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Cellulosic ethanol production via aqueous ammonia soaking pretreatment and simultaneous saccharification and fermentation

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Cellulosic ethanol production via aqueous ammonia soaking pretreatment and simultaneous saccharification and fermentation

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ABSTRACT

The most important energy challenges of the century are energy growth, energy security and climate protection. Cellulosic based fuels such as ethanol are poised to offer energy security, and economic and environmental benefits if the associated commercialization challenges are overcome. The main objective of this thesis is to evaluate a promising approach for cellulosic ethanol production and to present information to guide. This dissertation, completed in partial fulfillment of the requirements of the Ph.D. degree, is prepared in the journal paper format, and includes four papers that have been published in or are prepared for submission to a journal. The objective of the first research chapter was to evaluate the effectiveness of an aqueous-ammonia soaking pretreatment method on ethanol production from switchgrass at bench scale. We have determined that ammonia soaking pretreatment method can be an effective method for the pretreatment of switchgrass. It was observed that after ammonia soaking, lignin and hemicellulose were partially removed, while the cellulose content of the lignocellulosic feedstock was preserved. The results also show that there is a tradeoff between pretreatment intensity and enzyme loading. The objective of the second paper was to scale up the bench scale simultaneous saccharification and fermentation (SSF) procedure and to identify the key issues of cellulosic ethanol production at larger scales. Pilot scale experiments (50 and 350-L fermentations) showed promising results that were similar to bench scale experiments. Material handling of the feedstock and bacterial contamination were the biggest challenges of the pilot scale fermentations. The third paper presents the techno-economic feasibility analysis of a full scale aqueous-ammonia soaked switchgrass fermentation process. It was determined that even though the aqueous-ammonia soaking pretreatment method provides advantages such as operating at ambient

conditions, it is a capital-intensive process when implemented at commercial scale. Soaking time was the most important parameter that affected the total equipment cost. Feedstock and enzyme costs were identified to be the primary drivers of ethanol selling price. The objective of the last paper was to develop a rapid and easily adaptable SSF technique that offers the advantage of running a large number of samples at the same time using ammonia soaking as a pretreatment method. This research showed that ammonia soaking combined with SSF can be used as an easy and effective assay to determine ethanol yields of different feedstock. It was also observed that lignin concentration or near infrared reflectance spectroscopy can be used in directly to predict ethanol yields and can be used to guide biofuel feedstock selection in plant breeding research or in choosing feedstock for biofuel production.

CHAPTER 1. GENERAL INTRODUCTION

Increasing energy demand, rising fuel prices and environmental issues related to fossil fuel consumption have motivated governments, academia and entrepreneurs to focus on alternative energy sources. Our society and economy require secure, environmentally benign, economically feasible and sustainable energy supplies. Biorenewable resources and the fuels produced from them can reduce our dependence on petroleum from unstable countries, create new domestic job opportunities and improve environmental quality. One of the most widely promoted and promising alternative fuels is ethanol, which is currently being produced from corn in the U.S. The number of ethanol plants in the U.S. has increased dramatically from 50 in 1999 to 134 in 2007 with additional plants under construction (Renewable Fuel Association Statistics, 2008). However, the demand for corn as a fuel, food and feed has driven up corn prices and will eventually limit expansion of grain-based ethanol production. In addition, it has been shown corn grain ethanol can only satisfy a small portion (~10%) of the US energy demand (Perlack et al., 2005).

One approach to solving this problem is utilizing lignocellulosic materials for ethanol production (Lynd et al., 1991). Dedicated energy crops such as switchgrass, and agricultural and forestry residues are major sources of lignocellulose and have the potential to displace as much as 30% of U.S. petroleum consumption (Perlack et al., 2005). Lignocellulosic biomass is viewed as a renewable and sustainable ethanol feedstock, however the recalcitrant cellulosic structure, and hence the high cost of cellulosic ethanol production impedes its widespread commercialization. During the last two decades, a large literature has been generated by researchers addressing different issues of cellulosic ethanol production, including different pretreatment methods, enzymatic hydrolysis and saccharification of

cellulose, production of cost effective enzymes and development of new microorganisms and fermentation techniques. However, there is still a continuing debate about the best process designs and there is no single process that offers the most efficient way to produce ethanol from cellulosic biomass. This thesis does not claim to have the best process design for ethanol production and will not address all the issues of cellulosic ethanol, but rather attempts to evaluate one promising approach and to present information that can inform and guide the commercialization process.

Objectives

The objectives of the research for this thesis were:

- To evaluate the effectiveness of aqueous ammonia soaking on ethanol yield of switchgrass and to determine the effect pretreatment conditions on the composition of switchgrass
- To scale up the proposed bench scale SSF procedure and to identify the key issues of cellulosic ethanol production at larger scales.
- To analyze techno-economic feasibility of aqueous ammonia soaked switchgrass fermentation process in full scale
- To develop a rapid and easily adaptable SSF technique which offers the advantage of running a large number of samples at the same time

Thesis Organization

This thesis contains a general introduction, four research articles, a general conclusion as well as cited references and acknowledgments. The general introduction includes thesis objectives, thesis organization, the authors' role in each article and a brief

literature review. The first article entitled "*Aqueous Ammonia Soaking of Switchgrass Followed by Simultaneous Saccharification and Fermentation*" has been published in the journal of Applied Biochemistry and Biotechnology (Isci et al., 2008). This article shows that ammonia soaking at ambient conditions was an effective pretreatment method for ethanol production from switchgrass. The compositional changes that occurred during ammonia soaking are presented and different pretreatment conditions and enzyme loadings are compared in terms of ethanol production. The second research article entitled "*Pilot Scale Fermentation of Aqueous Ammonia Soaked Switchgrass*" has been accepted by the journal of Applied Biochemistry and Biotechnology. It reports on a series of scale up trials of the bench-scale experiments proposed in the previous article and identifies challenges in pilot scale fermentations such as material handling and bacterial contamination. The third article, entitled "*Techno-economic Analysis of Aqueous Ammonia Soaked Switchgrass Fermentation*", analyzes the economic feasibility of aqueous ammonia soaked switchgrass fermentation and identifies the processes and the parameters that have the largest impact on the capital cost and the ethanol selling price. The third article will be submitted to the journal of Bioresource Technology. The last research article, accepted by the journal of BioEnergy Research, is entitled "*A Rapid Simultaneous Saccharification and Fermentation (SSF) Technique to Determine Ethanol Yields*". This article demonstrates that the ammonia soaking pretreatment combined with SSF can be used for rapid feedstock screening. A rapid and easily adaptable SSF technique was developed which offers the advantage of running a large number of samples at the same time. The technique is demonstrated using a set of genetically diverse corn stover samples. It is also shown that ethanol yields of the stover samples could be predicted from simple compositional data and near infrared reflectance spectroscopy

(NIRS). Following the fourth article is a general conclusion and discussion of future research directions suggested for improving the ammonia-soaking pretreatment method and the feasibility of cellulosic biofuel production in general. References for the general introduction and each paper are included at the end of each chapter.

Authors' Role

All of the research articles presented in this thesis are written by the primary author with the guidance and assistance of co-authors. Unless otherwise stated in the research articles, all the methods described in this thesis were performed by the primary author.

In the first article (Chapter 2) Jennifer Himmelsbach (MS Student, Iowa State University) contributed to work by helping the primary author in the experiments. She has also helped with the design of bench scale ammonia washing system. Dr. Anthony L. Pometto (Professor, Department of Food Science & Human Nutrition, Iowa State University) contributed guidance and discussion and also helped with culture preparation and fermentation experiments. Dr. D. Raj Raman and Dr. Robert P. Anex (Associate Professors, Department of Agricultural & Biosystems Engineering, Iowa State University) provided guidance and assistance. The experiments were performed with the use of materials and equipment in Dr. Raman and Dr. Anex's laboratory (3242 NSRIC Building). Yeast cultures were prepared in Dr. Anthony Pometto's laboratory.

In the second article (Chapter 3) Jennifer Himmelsbach designed the pilot scale ammonia soaking steeping tanks and helped in every step of the fermentation experiments which were performed in the Iowa State University Fermentation Facility, Food Science Department under the supervision of Dr. Anthony Pometto. Dr. John K. Strohl (Assistant Scientist, Department of Food Science & Human Nutrition, Iowa State University) provided

guidance and assistance in using 50-L and 350-L fermentors. Dr. Anthony Pometto provided intellectual discussion and guidance on large scale fermentation experiments. Dr. Raj Raman and Dr. Robert Anex helped during ammonia soaking experiments and provided guidance and assistance.

In the third article (Chapter 4) Dr. Feroz Kabir (Visiting Scientist, Center for Sustainable and Environmental Technology, Iowa State University) helped with the mass balance and designing the flow charts. He also designed unit processes using ASPEN Plus software and provided guidance in cost calculations. Dr. Robert Anex contributed by providing new approaches and guidance.

In the fourth article (Chapter 5) Pat Murphy (PhD Candidate, Department of Agricultural & Biosystems Engineering) provided assistance throughout the experiments. Dr. Kenneth J. Moore (Professor, Department of Agronomy, Iowa State University) guided the primary author with his knowledge on Near Infrared Reflectance Spectroscopy (NIRS) and statistical experiment designs. The compositional data and NIRS calibrations were done in Dr. Moore's lab in the Department of Agronomy with the help of his laboratory members and Pat Murphy. Dr. Robert Anex provided the ideas and helped structuring the article.

Literature Review

The most important energy challenges of our century are growth in energy demand, energy security and climate protection. The dependency on petroleum and its products is increasing daily. The petroleum consumption of the United States is about 20.7 million barrels/day as of 2007 and the dependence on net petroleum imports is 60% (Energy Information Administration, 2007). Negative economic and social impacts are expected when our society faces disruptions in oil supplies.

Fuels derived from cellulosic biomass are expected to offer energy security, and economic and environmental benefits. However, there are still issues that impede their commercial production such as feedstock selection, pretreatment, cost, distribution and compatibility of the fuels, and the transition of the fossil fuel economy to a bio-economy. This chapter aims to give a brief overview of lignocellulosic ethanol production and to provide context for the concepts discussed in the later chapters of the thesis.

Lignocellulosic Biomass Structure

Lignocellulosic biomass refers to plant biomass which is composed of cellulose, hemicellulose and lignin. The plant cell wall of lignocellulosic biomass can be considered as a composite material consisting of cellulosic microfibrils embedded within a matrix of hemicellulose and lignin. In general, lignocellulosic biomass contains 35-50% cellulose, 20-35% hemicellulose, 10-15% lignin on dry basis (Wyman, 1994). The interactions among these cell components inside the plant cell hinder the hydrolysis of the carbohydrates into fermentable sugars.

Cellulose

Cellulose is polymer of glucose molecules which are covalently linked with β-1,4 glycosidic bonds. The β configuration orients the glucose molecules in such a way that each polymer forms hydrogen bonds with the adjacent ones and coalesces into very strong, long and straight microfibrils. The microfibrils usually contain 30-36 hydrogen bonds with diameters of ~3 nm. The lengths of the microfibrils are unknown but single glucans containing up to 14000 units have been identified (Somerville et al., 2004). Microfibrils are further aggregated into fibrils, which constitute cellulose fibers.

Due to hydrogen bonding within the cellulose structure, cellulose can form very tightly packed crystallites. This regularly ordered structure can sometimes be so tight that no enzyme or water can penetrate into it. However, cellulose fibers in nature are not purely crystalline and have amorphous regions, which make the fibers at least partially hydrated. This characteristic of the natural cellulose enables enzymes access to substrate during hydrolysis (Moore and Hatfield, 1991).

Hemicellulose

Hemicellulose is also a plant polysaccharide and comprises 20-35% of the biomass of most plants. Unlike cellulose, hemicellulose is not chemically homogeneous and composed of polymers of pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose), and sugar acids (Saha, 2003). Xylan and glucomannan are the main constituents of hardwood and softwood hemicelluloses, respectively (McMillan, 1993). In grasses like switchgrass, hemicellulose is mainly present as arabinoxylan. Xylose molecules bond together to from a xylan backbone structure and arabinose forms bonds with the free hydroxyl group of the xylose molecules. Due to their amorphous morphology, hemicelluloses can be hydrolyzed easily by chemical and enzymatic treatments (Moore and Hatfield, 1991).

Lignin

Lignin is phenylpropane based polymer derived from cinnamyl alcohols (coumaryl, sinapyl, coniferyl). Lignin is formed as a result of free radical reaction in which the monomeric precursors are condensed in a random arrangement. It gives strength and rigidity to plant cell and protects the plant from diseases and microorganisms (Moore and Hatfield, 1991).

Lignin in grasses can be classified as core and non-core lignin. The core lignin is primarily, etherified and methoxylated cinnamyl alcohol polymers. The non-core lignin includes esterified and etherified cinnamyl acids with varying degrees of methoxylation which are either bound to core lignin or to hemicellulose. It has been suggested that these chemical linkages between lignin, cell wall polysaccharides and proteins hinders enzymatic hydrolysis of polymeric sugars (Moore and Hatfield, 1991). The primary cinnamyl acids that play a role in limiting cell wall digestion are ferulic acid and *p*-coumaric acid. Ferulates and *p*-coumarates that are esterified to polysaccharides or lignin are extracted with alkali at room temperature. Extraction at high temperatures, however, is required to cleave both ester- and ether-linked hydroxycinnamic acids (Moore and Jung, 2001). These aromatic molecules are antimicrobial and needs to be removed prior to SSF.

Lignocellulosic Ethanol Production

The overall process of biochemical lignocellulosic ethanol production is summarized in Figure 1. The material handling process receives the biomass and prepares the feedstock for ethanol production. This step usually involves mechanical size reduction and washing. Subsequent to feedstock handling, biomass is pretreated in order to increase enzyme accessibility to fermentable sugars and remove possible fermentation inhibitors. Different pretreatment methods have been proposed and studied to overcome the recalcitrance of lignocellulosic hydrolysis. The main goals are to break up/alter the biomass structure and to reduce crystallinitiy of cellulose.

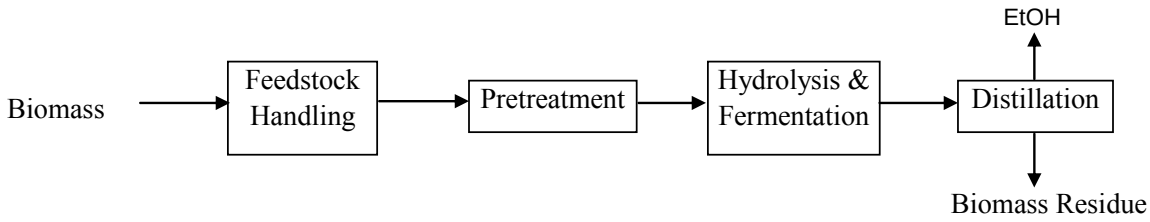

Figure 1. Block flow diagram of cellulosic ethanol production

Chemical and physical pretreatment methods have been extensively studied. Mosier et al. (2005a) have reviewed characteristics of the most studied pretreatment methods including steam explosion, liquid hot water, dilute acid, AFEX (ammonia fiber explosion), ARP (ammonia recycled percolation) and lime treatment.

In steam explosion, biomass is subjected to high pressure steam followed by an explosive decompression. Acetic acid and other acids released during the process help to hydrolyze the hemicellulose portion; hence the accessibility of cellulose by enzymes is improved (Schultz et al., 1983; Brownell and Saddler, 1984; Heitz et al., 1991). The hot water process uses compressed water in liquid form at high temperatures (~200 ºC) to pretreat the biomass (van Walsum et al., 1996; Allen et al., 1996; Mosier et al., 2005b). The hot water process cleaves hemiacetal linkages and liberates acids, which facilitates the breakage of ether linkages in biomass (Antal, 1996). Dilute acid treatment involves heating a biomass-acid mixture directly or indirectly, which hydrolyses the hemicellulose fraction (Grohmann et al., 1985; Torget et al., 1990, 1991, 1992). AFEX is similar to the steam explosion process, but ammonia is used instead of steam. AFEX removes some of the hemicellulose and lignin and disrupts the crystalline structure of cellulose (Dale, 1986; Dale and Moreira, 1982; Dale et al., 1996). The ARP process, on the other hand, utilizes aqueous

ammonia for pretreatment. Liquid ammonia at elevated temperature passes through a column reactor packed with biomass (Yoon et al., 1995; Iyer et al., 1996; Kim and Lee, 1996; Kim et al., 2002). Ammonia recycled percolation process causes swelling of cellulose and a phase change in the crystal structure. It is also reported that glucuronic cross-links are hydrolyzed by ammonolysis reactions further increasing the accessibility of carbohydrates (Lin et al., 1981). Lime pretreatment involves spraying a lime water mixture onto the biomass at relatively lower temperatures and pressures (Chang et al., 1997, 1998; Karr and Holtzapple, 1998, 2000). Pretreatment time can be reduced by increasing the temperature (Playne, 1984). Lime treatment removes lignin, acetyl groups and uronic acid substitutions in hemicellulose and improves hydrolysis of cellulose (Chang and Holtzapple, 2000).

After pretreatment, biomass is subjected to hydrolysis and fermentation. In most studies enzymatic hydrolysis and fermentation is performed in one step, which is also known as SSF (simultaneous saccharification and fermentation) (Takagi et al., 1977). Cellulase enzymes are widely used to hydrolyze cell-wall polysaccharides in the SSF process. A cellulosic enzyme (EC 3.2.1.4) system consists of three major components: endo-glucanase, exo-glucanase and β-glucosidase. Endo-glucanase randomly attacks internal glycosidic bonds in the cellulose chain and acts mainly on amorphous regions of cellulose. Exo-glucanase hydrolyzes from the chain ends and produces predominantly cellobiose. Cellobiose is cleaved to form two glucose molecules by β-glucosidase (Figure 2) (Eveleigh, 1987). In the SSF process, the released glucose is then consumed by the fermentative organism.

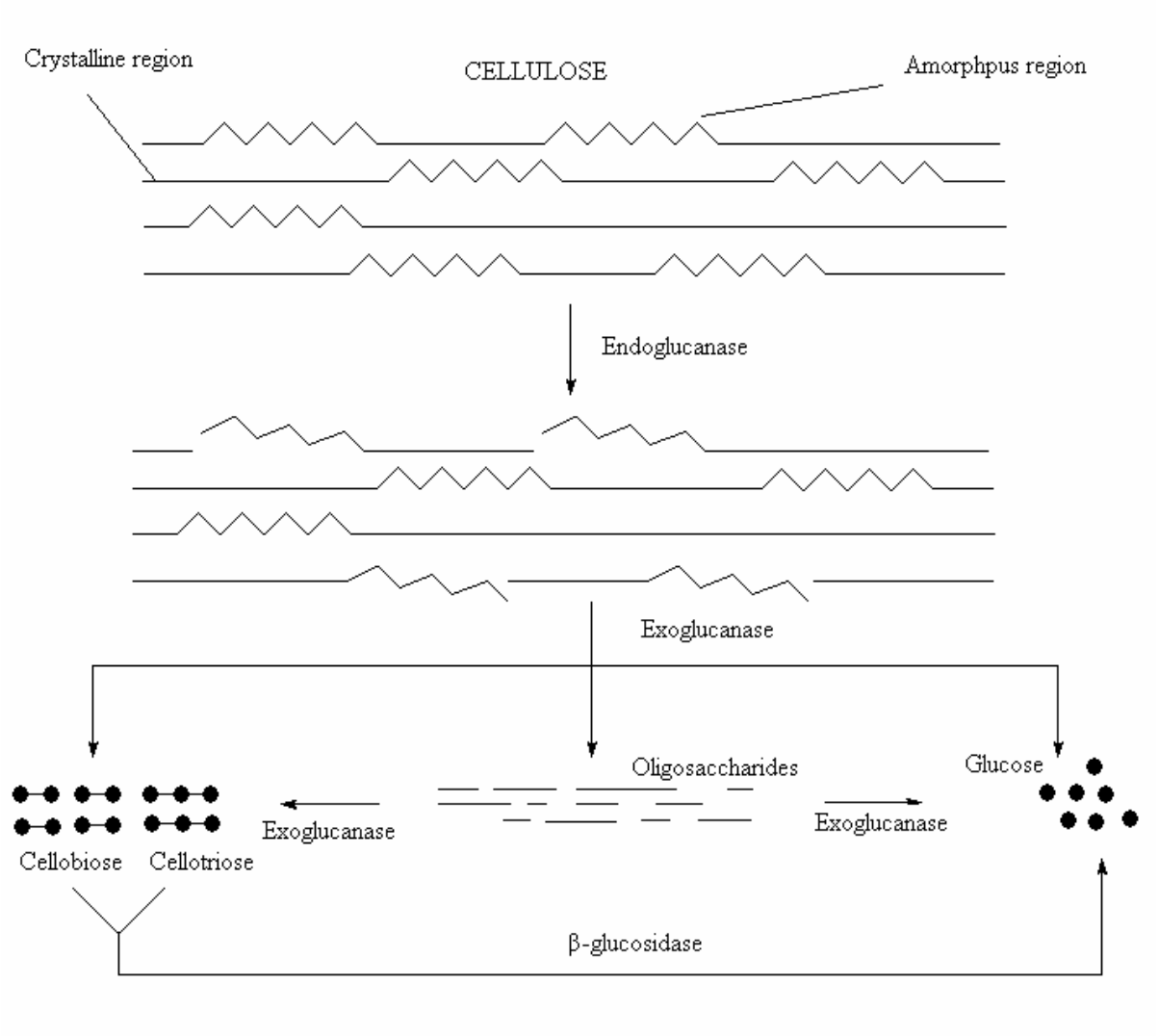

Figure 2. Enzymatic hydrolysis of cellulose (Eveleigh, 1987)

Fungi such as *Trichoderma ssp.* and *Aspergillus ssp.* are the main cellulase-producing microorganisms used and crude enzymes produced by these microorganisms are commercially available. Commercial cellulase enzymes are usually a mixture of enzymes that includes hemicellulases as well as cellulase (Isci et al., 2008).

Like many enzymes, cellulases are inhibited by their end products. Exo-glucanase activity is inhibited by cellobiose, whereas β-glucosidase activity is inhibited by glucose

production (Wright et al., 1988). Therefore, researchers have focused on SSF to overcome the end product inhibition during the hydrolysis step. As stated above, SSF is a process in which enzymatic hydrolysis and fermentation are carried out simultaneously in one reactor. Since glucose is rapidly consumed by the fermentative microorganism, end product inhibition of enzymes is greatly reduced. Takagi et al. (1977) were the first to describe SSF in the literature. Simultaneous saccharification and fermentations is preferred to SHF (separate hydrolysis and fermentation) not only due to reduction in end product inhibition but also due to reduced capital cost. In the SHF process, hydrolysis and fermentation take places in different reactors and solid liquid separation is required after hydrolysis. Using separate reactors for hydrolysis and fermentation also increases the chance of contamination (Wright et al., 1988; Grohmann, 1993). Ohgren et al. (2006) have also reported that higher ethanol concentrations can be achieved in SSF process which reduces the contamination levels. The most important disadvantage of SSF process is the limitation of process optimization for higher sugar and ethanol yields (Grohmann, 1993). Cellulolytic enzymes are usually more active at higher temperatures (~50ºC) relative to the optimum temperatures of common fermentative microorganism such as *Saccharomyces cerevisiae* (~35ºC). The SSF process usually takes place at the lower temperatures which are optimal for the fermentative organisms.

Various fermentative microorganisms have been considered for improving ethanol production from lignocellulosics. As stated above, the most commonly used microorganism is *Saccharomyces cerevisiae*, which is also known as baker's yeast. Even though this microorganism is capable of producing relatively high ethanol concentrations, it can only metabolize glucose (a 6-carbon sugar). However, in order to improve process economics, it is

desirable to convert both pentoses and hexoses to ethanol (Lynd et al., 1999). Yeasts such as *Pichia stipitis*, *Candida shehatae*, and *Pachysolen tannophilus* are naturally capable of fermenting both glucose and xylose to ethanol (Lynd et al., 1999; Bothast et al., 1997; Schneider et al., 1981). However, it has been reported that these yeasts produce about one-fifth the ethanol of *S. cerevisiae* (Chandakant and Bisaria, 1998) which also negatively impacts process economics. These yeasts are also reported to be sensitive to possible by-products of pretreatment and hydrolysis (Hahn-Hagerdal et al., 2006).

One approach to overcome these issues is to genetically engineer a microorganism that can ferment both hexoses and pentoses, produce large amounts of ethanol, and tolerate both inhibitory compounds and high ethanol concentrations. Several microorganisms including *Escherichia coli, Klebsiella oxytoca* and *Saccharomyces cerevisiae* have been genetically modified to ferment both xylose and glucose into ethanol (Hahn-Hagerdal et al., 2006; Lynd et al., 1999), however, more research is needed to increase ethanol titers and to adapt these microorganism to industrial conditions. The ultimate low-cost configuration for cellulosic ethanol production is integrating cellulolytic enzyme generation and fermentation capability in one microorganism, an approach known as consolidated bioprocessing (Lynd et al., 2005).

The final step in cellulosic ethanol production is separation. Fractional distillation can concentrate ethanol to 95.6% (w/v). This mixture is an azeotrope with a boiling point of 78.1°C, and cannot be further purified by distillation, but can be further concentrated using molecular sieves, desiccation or extractive distillation. Other ethanol separation techniques such as gas stripping, vacuum, membrane processes, and liquid extraction have been also suggested in literature (Cardona and Sanchez, 2007).

Commercialization Challenges

A major barrier to the commercialization of cellulosic ethanol is its high cost of production relative to gasoline. The most important parameters that affect the costs of production are feedstock (collection, transportation and storage), pretreatment and enzyme (Aden and Ruth, 2001). Currently, pretreatment techniques are very capital intensive (Eggeman and Elander, 2005). Advancements in pretreatment methods will significantly reduce the cellulosic ethanol price. In addition, the engineered microorganisms that are capable of producing high ethanol yields will improve process economics.

Co-products are also important to decreasing the cost of cellulosic ethanol and to achieving an environmentally friendly process. Various by-product streams, mostly organic in nature are expected to be generated during the processing of lignocellulosic biomass. These organic by-products will have an important value either as a feedstock for fuel or as a source of other value added co-products. Some likely co-products are organic acids, organic alcohols, and aromatic chemical intermediates (Tsao et al., 1999; Iyer et al., 2000; Bordern et al., 2000; Altaras et al., 2001; Holtzapple et al., 1999). In addition, different integrated biorefinery scenarios are being considered to make the process more profitable and sustainable. One of the approaches is to utilize the unfermented portion of biomass for power and electricity generation by combustion (Aden and Ruth, 2001). Another option could be syngas production via gasification of the unfermented portion of the biomass. There will be more opportunities once the side streams are fully characterized.

There are currently 11 companies planning to produce cellulosic ethanol commercially, these are: Abengoa Bioenergy (30 MMgal/y in Kansas), Alico, Inc. (Florida), BlueFire Ethanol, Inc. (17 MMgal/y in California), Gulf Coast Energy (70MMgal/y in

Florida), Mascoma Corp., (40 MMgal/y in Michigan), Iogen (18 MMgal/y in Idoha), POET (25 MMgal/y in Iowa), Range Fuels (20 MMgal/y in Georgia), SunOpta (20 MMgal/y in Minnesota), Xethanol (8 MMGal/y in Florida) (Ethanol Producer Magazine, 2007). There are other demonstration plants under construction that should help guide the commercialization of cellulosic ethanol.

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CHAPTER 2. AQUEOUS AMMONIA SOAKING OF SWITCHGRASS FOLLOWED BY SIMULTANEOUS SACCHARIFICATION AND FERMENTATION

A paper published in the *Applied Biochemistry and Biotechnology* Journal

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Abstract

Simultaneous saccharification and fermentation (SSF) of switchgrass was performed following aqueous ammonia pretreatment. Switchgrass was soaked in aqueous ammonium hydroxide (30%) with different liquid-solid ratios (5 and 10 mL/g) for either 5 or 10 days. The pretreatment was carried out at atmospheric conditions without agitation. A 40-50% delignification (Klason lignin basis) was achieved, whereas cellulose content remained unchanged and hemicellulose content decreased by approximately 50%. The *Saccharomyces cerevisiae* (D_5A)-mediated SSF of ammonia-treated switchgrass was investigated at two glucan loadings (3 and 6%) and three enzyme loadings (26, 38.5 and 77 FPU/g cellulose), using Spezyme CP. The percentage of maximum theoretical ethanol yield achieved was 72. Liquid-solid ratio and steeping time affected lignin removal slightly, but did not cause a significant change in overall ethanol conversion yields at sufficiently high enzyme loadings. These results suggest that ammonia steeping may be an effective method of pretreatment for lignocellulosic feedstock.

Introduction

Switchgrass (*Panicum virgatum*) is a warm season, perennial grass which is resistant to harsh conditions, pests and diseases (1). It is also capable of producing high biomass yields at low fertilizer application rates (1). These attributes along with the environmental benefits associated with perennial vegetation make switchgrass a good candidate for a dedicated energy crop (2). However, lignin in lignocellulosic feedstocks is known to create obstacles such as inhibition of enzymatic hydrolysis and microbial activity in the ethanol fermentation. Besides the goal of reducing compounds that may inhibit fermentation of sugars to ethanol, pretreatment is also required to either partially remove or break up the lignin structure, so that enzymes can diffuse into the cellulose polymer and degrade it into monomeric fermentable sugars. While a variety of pretreatment methods have been developed and tested at lab-scale (3), pretreatment of biomass remains one of the most costly steps in lignocellulosic biofuels production and affects subsequent operations (4). For example, improvements in pretreatment can reduce the amount of enzymes (cellulases) used (4). Teymouri et al. (5) indicated effective enzymatic hydrolysis of AFEX-treated biomass at enzyme loadings as low as 7 FPU/g of glucan could be achieved by adjusting the pretreatment parameters. Kim et al. (6) reported the enzymatic digestibility of corn stover treated by the ammonia recycled percolation to be 90% with an enzyme loading of 10 FPU/ g-glucan. Although many biological, chemical, and physical methods have been attempted over the years, further development of pretreatment methods is needed to reduce overall costs of lignocellulosic bioconversion (7). Dilute acid treatment, water pretreatment with pH control, AFEX (ammonia fiber explosion), ARP (ammonia recycle percolation), and lime pretreatment are among the most promising and most studied technologies (3). Many of these

pretreatment methods, however, require high temperature and/or high pressure. The extreme conditions used to increase the digestibility of the biomass decrease the reaction time required for the pretreatment, but they increase capital and operating costs. Extreme conditions may also cause the formation of compounds that are inhibitory to the fermentative organisms, and they may cause degradation of some fraction of the fermentation substrate. For these reasons, ambient temperature and pressure pretreatments are of interest.

Removing lignin with alkaline chemicals to improve cellulose digestibility and ammonia steeping/soaking at room temperature has been previously studied on several types of biomass (8, 9, 10). The steeping method is a simple method that does not require high pressures and high temperatures. Ammonia soaking of corn stover at room temperature can remove as much as 74% of the lignin, but retain nearly 100% of the glucan and 85% of the xylan (8).

Currently, simultaneous saccharification and fermentation (SSF) is one of the most commonly used processes for ethanol production (5, 6, 8, 11-15). This process combines two steps in the same vessel to generate ethanol: enzymatic break down of the complex sugars into glucose, and fermentation of the glucose into ethanol by yeast. This process has been widely adopted due to reduction of glucose inhibition during enzymatic hydrolysis. In addition, the risk of bacterial contamination and capital investments are lower, since both the hydrolysis and the fermentation steps take place in the same reactor. Although SSF of switchgrass has been studied following numerous types of pretreatment (11-13, 16), the effect of aqueous ammonia soaking at room temperature and atmospheric pressure on ethanol yield of switchgrass has not been reported. The objectives of this study were to determine the

effect of soaking time and liquid-solid ratios on the composition of ammonia-steeped switchgrass, and on ethanol production from SSF of ammonia-steeped switchgrass.

Materials and Methods

Switchgrass samples were collected from mature stands of the Cave-in-Rock cultivar while dormant (early spring) in Chariton, IA. Dry switchgrass was ground to a size of 5-6 mm by the Biomass Energy Conversion Center (BECON), Nevada, IA. The composition of the switchgrass except for Klason lignin was determined by Iowa State University, Department of Agronomy using the ANKOM method (ANKOM Technol. Corp., Fairport, NY) as described by Vogel et al. (17). Untreated switchgrass contained 42% cellulose, 31% hemicellulose, 6% acid detergent lignin, 22% Klason lignin and 0.7% ash.

Cellulase enzyme (Spezyme CP, lot no: 301-05021-011) was provided by Genencor International (Palo Alto, CA) and had an activity of 77 filter paper units (FPU)/mL, measured using standard procedures (18). The yeast (*Sacccharomyces cerevisiae* D_5A) was supplied by National Renewable Energy Laboratory and preserved at 4°C after freeze drying with 20% skim milk.

Forty grams of dry switchgrass was soaked in reagent-grade 29.5 wt% aqueous ammonium hydroxide (Fisher Scientific Inc.) in 1L high density polyethylene bottles at room temperature without any agitation. Two different aqueous ammonia loading rates (5 and 10 mL/g) were applied for both 5 and 10 d. Each treatment was performed in duplicates. At the end of the pretreatment, the biomass was washed in the same bottles with 20 L DI water using a customized fluidized bed-biomass washing system (Figure 1). For treated fiber washing, deionized water was supplied from the bottom of the bottle which was placed on top of a magnetic stir plate and the rinsate was collected from the top of the bottle. A metal

screen and glass wool was used to keep the biomass inside the bottle during washing. This system allowed homogeneous continuous rinsing of the biomass *in situ* to minimize reactor handling.

The pretreated samples were then analyzed for cellulose, hemicellulose (as described above) and for Klason lignin. The biomass was dried completely for Klason lignin analysis which was performed following Crawford and Pometto (19) with a slight modification, namely that glass fiber filters (Fisherbrand, G6, 1.6 μm) were used for capturing lignin residues instead of Whatman #1 filter papers. Employing the glass fiber filters avoided errors due to the rapid adsorption of atmospheric humidity onto the dry filter papers.

The wet biomass obtained from the washing system was used for simultaneous saccharification and fermentation experiments following established procedures (20). Specifically, 250-mL Erlenmeyer flasks were used for fermentation with 100-mL working volume. Two cellulose loadings (3 and 6%), and three enzyme loadings (26, 38, and 77 FPU/g cellulose) were evaluated on ammonia-treated switchgrass. The switchgrass after the pretreatment contained 56.6% cellulose with 80% wet basis moisture content. Example cellulose loading calculation for one flask (20):

$$26.6\,g\ wet\ pretreated\ biomass \times 20\%\ solid\ content \times 56.6\%\ cellulose\ content$$
$$= 3.01\,g\ cellulose\ in\ 100\,mL\ working\ volume\ fermentation\ flask$$

The fermentation media contained 1 % w/v yeast extract, 2 % w/v peptone and 0.05 M citrate buffer (pH 4.8). Yeast-free saccharification flasks were run alongside each fermentation flasks to monitor sugar production in the absence of fermentative organisms. Samples were analyzed for sugars (cellobiose, glucose, and xylose) and ethanol by HPLC (Varian ProStar 210, MetaCarb 87P column with mobile phase of water, flow rate of 0.4

ml/min, column temperature of 80^{o}C, and injection volume of 20 µl) with a refractive index detector. Total sugars and reducing sugars in both saccharification and fermentation flasks were determined by using phenol-sulfuric (21) and DNS methods (22), respectively. Water soaked switchgrass and α-cellulose were fermented using the same procedure and reported as control and reference, respectively. All of the experiments are performed in duplicate (n=2).

Theoretical ethanol yields were calculated as follows considering the maximum (51%) conversion of glucose into ethanol by yeast (8).

$$Theoretical\ ethanol\ \ yield\ (\%) = \frac{Ethanol\ \ produced\ (g)\ in\ reactor}{Initial\ sugar\ (g)\ in\ reactor \times 0.511} \times 100$$

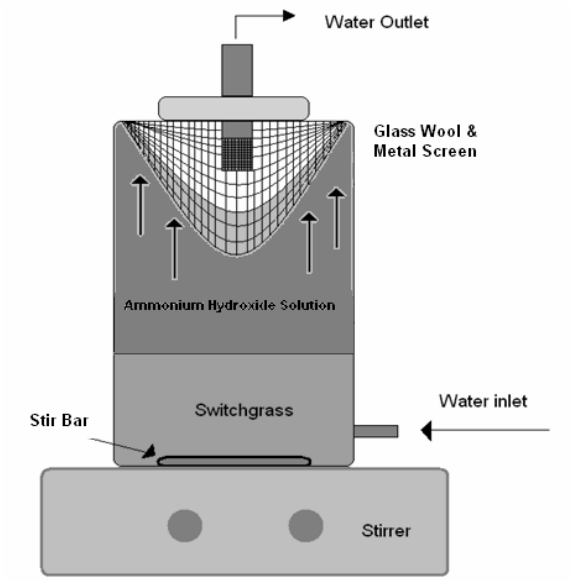

Figure 1. Fluidized bed biomass washing set-up.

Results and Discussion

Ammonia steeping proved to be an effective method for removing Klason lignin, preserving the cellulose fraction, and enhancing the subsequent SSF of switchgrass. Figure 2 illustrates the effect of different treatments on the recovery of sugars and total dry biomass. Almost all the cellulose was retained, while nearly half of the hemicellulose was removed with ammonia treatment. More than 75% of the original dry biomass was collected after soaking. Removing hemicellulose has sometimes been considered a desired characteristic of a biomass pretreatment because this reduces inhibitory compounds such as furfural generated from hemicellulose degradation via dilute acid treatment at high temperature and pressures (23) and some process designs have included fermentation of hexose and pentose sugars in separate reactors. However, the development of genetically modified microorganism capable of fermenting both pentose and hexose sugars offers the advantage of greater ethanol yields (24, 25) and lower capital cost. Therefore, the feasibility of capturing the rinsate pentoses should be determined in future studies. In contrast to the 50% removal of hemicellulose that we observed, Kim and Lee (8) observed around 15% xylan reduction after 10 d of aqueous ammonia soaking of corn stover with 12 mL/g loading (they did not report hemicellulose reductions specifically). The higher reduction in pentose polymers in our study could be due to the thorough washing of treated biomass employed to ensure neutral pH before fermentation; the degree of washing performed by Kim and Lee was not reported. However, the difference might also reflect fundamental cell wall differences between corn stover and switchgrass.

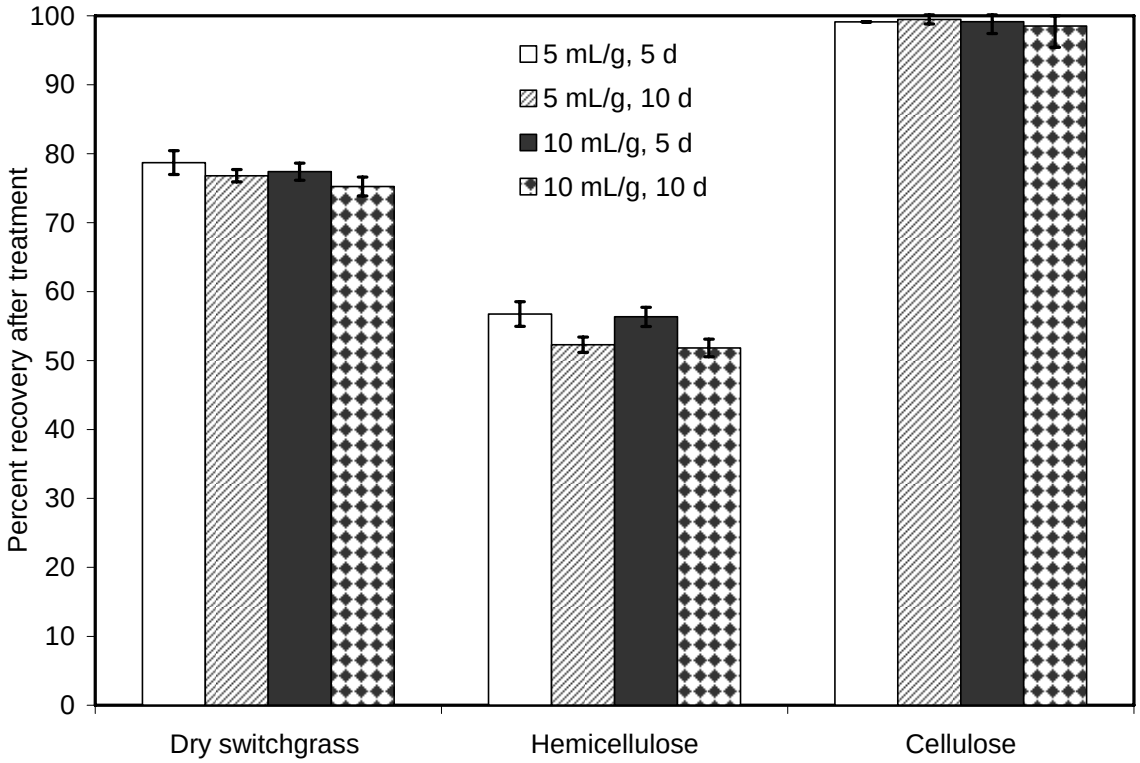

Figure 2. Effect of different aqueous ammonium hydroxide loadings (ml/g of ground switchgrass) and soaking time on the recovery of dry switchgrass, hemicellulose, and cellulose (n=2).

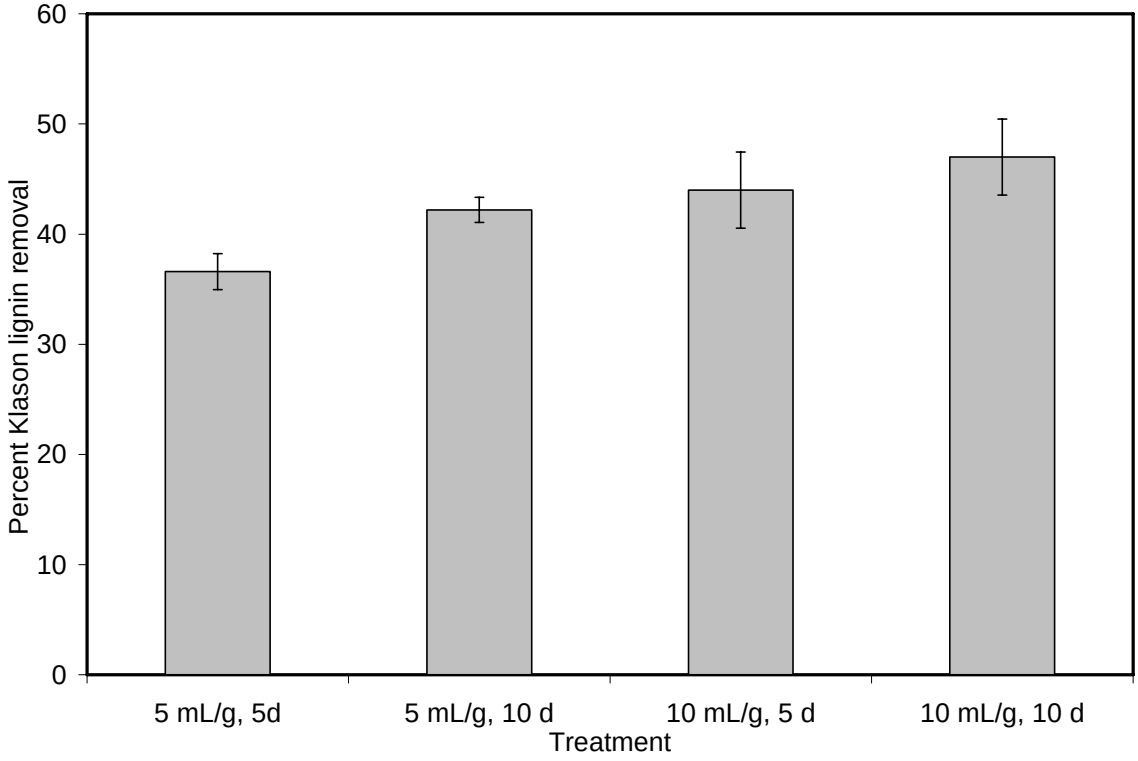

Figure 3. Percent klason lignin removal with different aqueous ammonium hydroxide loadings (ml/g ground switchgrass) and soaking time (n=2).

Estimates of lignin content can vary greatly between different procedures (26). For example, although acid detergent lignin (ADL) and Klason lignin are both common for determination of forage lignin content, Klason lignin values are generally two to four times greater than ADL estimates for grasses (26). This study used Klason lignin values to be consistent with previous studies (8, 10, 14, 15).

The influence of different treatment conditions on Klason lignin is presented in Figure 3. As anticipated, more lignin was removed with higher aqueous ammonia loadings and longer treatment. The highest delignification (47%) was achieved with ammonium hydroxide soaking for 10 d at 10 mL/g biomass. These results are consistent with Kim and

Lee (8) who report approximately 50% lignin removal from corn stover in 4 d with loading of 12 mL/g ground corn-stover. Cao et al. (10) report that between 80-90% lignin can be removed from corn cobs in 24 h with an ammonium steeping ratio of 5 mL/g at 26ºC. Higher delignification with lower pretreatment duration in that case could be due to structural differences between corn cob and corn stover. Chang et al. (14) reported approximately 30% lignin solubilization after lime treatment of switchgrass. Kim and Lee (15) have also reported that the ARP (ammonia recycle percolation) process can remove up to 85% of lignin from corn stover.

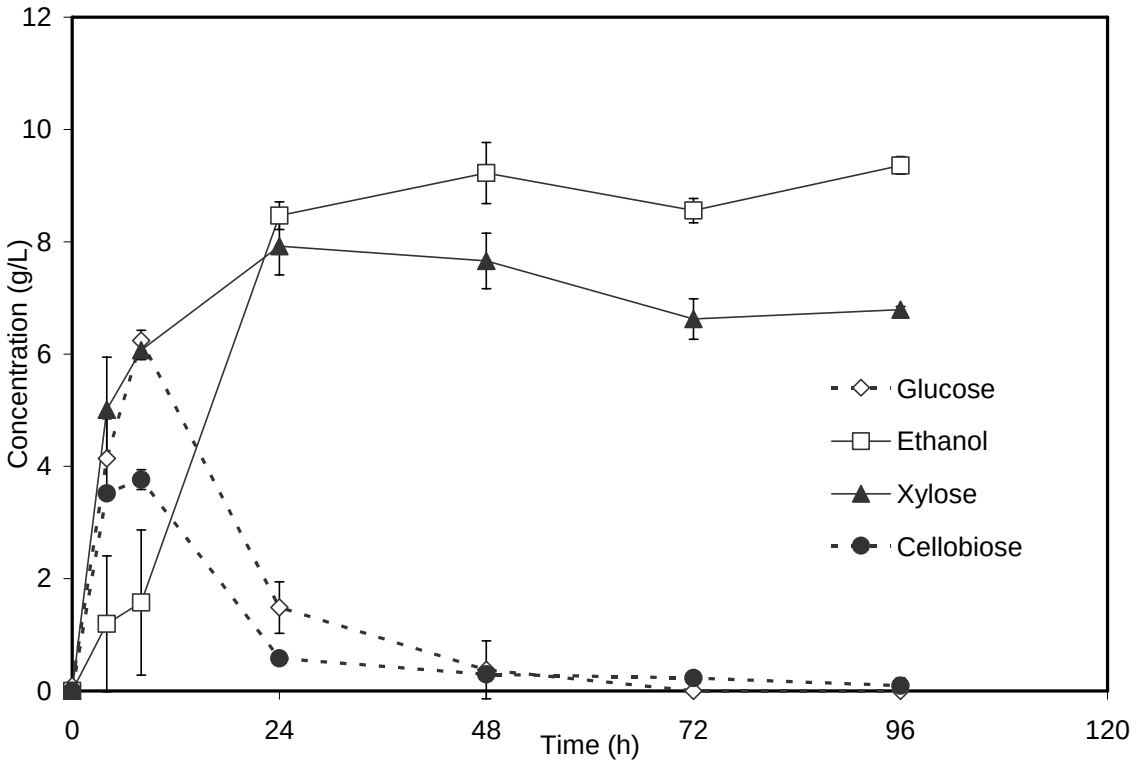

Figure 4. Time courses of sugars and ethanol concentrations for SSF of aqueous ammonium hydroxide steeped switchgrass (5 mL/g, 5 d, 3% cellulose, 77 FPU/g cellulose) (n=2).

The SSF of aqueous ammonia pretreated switchgrass followed a classical SSF production curve in which ethanol concentration increased with time while glucose and cellobiose concentrations increased initially then decreased once the yeast began consuming sugars at higher rates (Figure 4). The accumulation of xylose indicated the presence of hemicellulases in our enzyme complex, and could potentially inhibit ethanol fermentation (8). The glucose concentrations were generally higher than cellobiose concentrations throughout the fermentations indicating the effective conversion of cellobiose into glucose by β-glucosidase in the enzyme solution. The ethanol concentration remained relatively constant after 24 h, but to ensure fermentation was completed, the 96 h data are presented in subsequent figures.

Two extreme ammonium pretreatment conditions were selected (5 d with 5 mL/g, and 10 d with 10 mL/g switchgrass) to explore the effect of steeping time and loading rate on sugar release and ethanol production (Figure 5). The lower residual sugar concentrations observed in the fermentation flasks (compared to saccharification flasks) suggested the enzymes and yeast were metabolizing sugars. As expected, higher aqueous ammonium hydroxide loadings and longer soaking times led to greater sugar release in subsequent saccharification tests. This implied higher ethanol concentrations could be achieved by these more aggressive conditions, and this is borne out in the data presented in Figure 6, which illustrates the final ethanol concentrations achieved through SSF of pretreated switchgrass. The highest ethanol concentration (22 g/L) was observed in flasks fermenting switchgrass treated for 10 d at 10 mL/g ground switchgrass, using a glucan loading rate of 6 % and an enzyme loading rate of 38.5 FPU/g cellulose. This also corresponded to the treatment showing the highest sugar release (Figure 5, T4). Pretreatment loading rates and durations

had no significant effect on ethanol production at high enzyme loadings (Figure 6, T1 and T2). However, at medium and low enzyme loadings (38.5 and 26 FPU/g cellulose, respectively), final ethanol concentrations were sensitive to pretreatment conditions in the range studied. Specifically, final ethanol concentrations increased approximately up to 40% when the pretreatment went from 5 d with 5 mL/g loading to 10 d with 10 mL/g aqueous ammonia loading (Figure 6, T3 vs. T4 and T5 vs. T6). Doubling the cellulose loading, while halving the enzyme loadings, resulted in a doubling of ethanol concentrations at 96 h (Figure 6, T1 vs. T3 and T2 vs. T4). This suggests effective conversions at higher cellulose concentrations with medium enzyme loadings. The trade-offs among pretreatment intensity, enzyme loadings, and cellulose loadings need to be addressed in detail prior to scale-up of the aqueous ammonia steeping procedure.

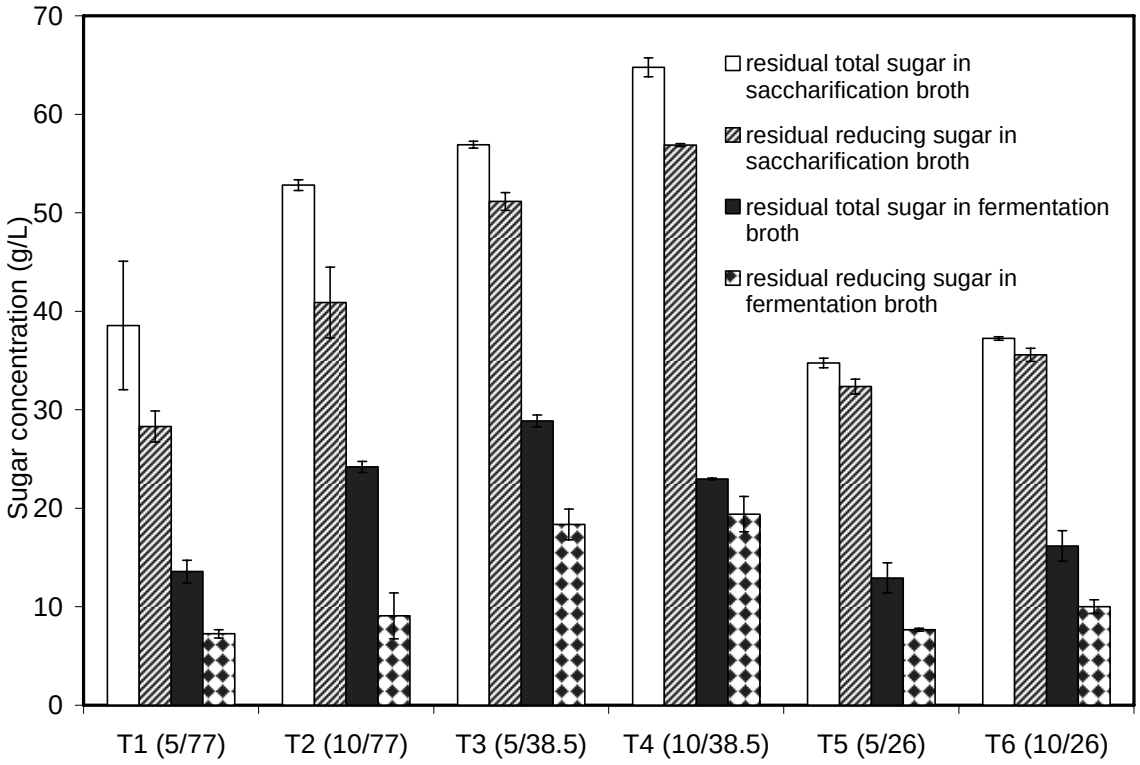

Figure 5. Effect of aqueous ammonium hydroxide loading and soaking time on sugar release at 96 h. Saccharification and fermentation flasks had two cellulose loadings (3 and 6 %) and three enzyme loadings (26, 38.5 and 77 FPU/ g cellulose). T1 (5/77): Treatment 1, 5 mL/g, 5d, 3% cellulose, 77 FPU/g cellulose; T2 (10/77): Treatment 2, 10 mL/g, 10d, 3% cellulose, 77 FPU/g cellulose; T3 (5/38.5): Treatment 3, 5 mL/g, 5d, 6% cellulose, 38.5 FPU/g cellulose; T4 (10/38.5): Treatment 4, 10 mL/g, 10d, 6% cellulose, 38.5 FPU/g cellulose; T5 (5/26): Treatment 5, 5 mL/g, 5d, 3% cellulose, 26 FPU/g cellulose; T6 (10/26): Treatment 6, 10 mL/g, 10d, 3% cellulose, 26 FPU/g cellulose.

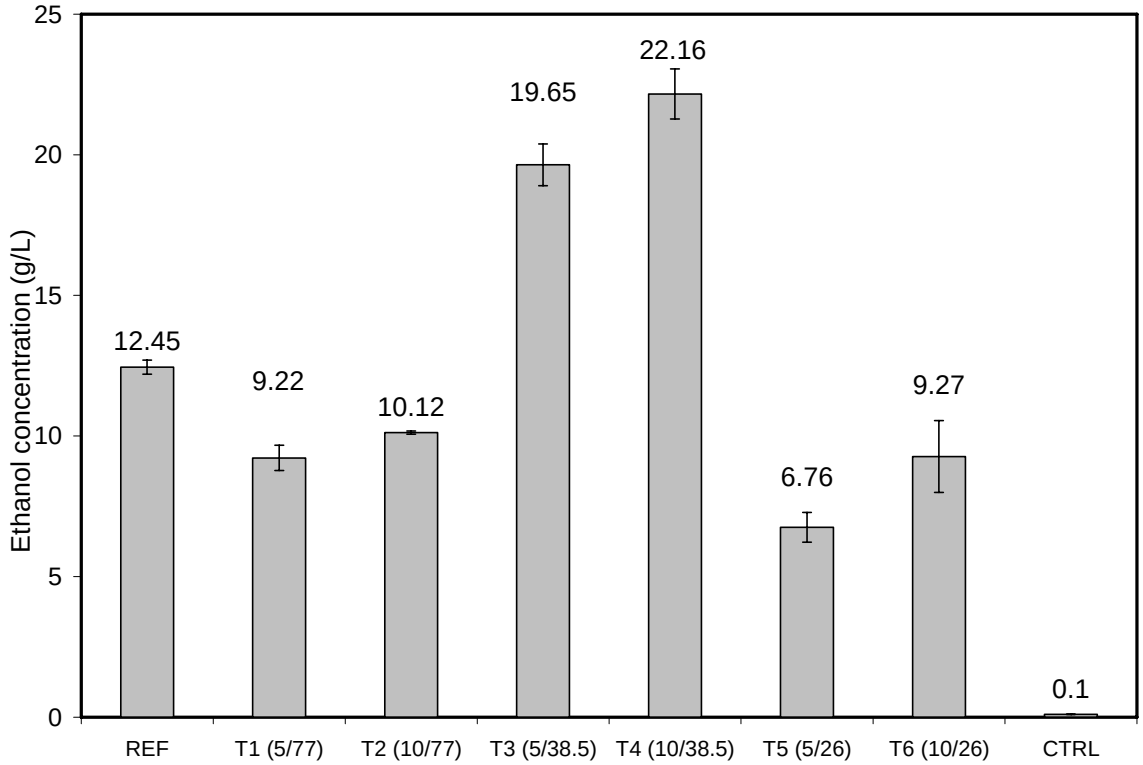

Figure 6. Ethanol concentrations in different fermentation flasks at 96 h. REF: reference, 3% α-cellulose fermentation, 77 FPU/g cellulose; T1 (5/77): Treatment 1, 5 mL/g, 5d, 3% cellulose, 77 FPU/g cellulose; T2 (10/77): Treatment 2, 10 mL/g, 10d, 3% cellulose, 77 FPU/g cellulose; T3 (5/38.5): Treatment 3, 5 mL/g, 5d, 6% cellulose, 38.5 FPU/g cellulose; T4 (10/38.5): Treatment 4, 10 mL/g, 10d, 6% cellulose, 38.5 FPU/g cellulose; T5 (5/26): Treatment 5, 5 mL/g, 5d, 3% cellulose, 26 FPU/g cellulose; T6 (10/26): Treatment 6, 10 mL/g, 10d, 3% cellulose, 26 FPU/g cellulose; CTRL: control, water soaked switchgrass fermentation, 5mL/g, 5d, 3% cellulose, 77 FPU/g cellulose.

Three percent α-cellulose fermentation with high enzyme loading (77 FPU/g cellulose) was performed as a reference. At equal glucan loadings, α-cellulose yielded approximately 25% more ethanol compared to aqueous ammonia pretreated switchgrass (Figure 6, REF vs T1 & T2). The lower ethanol productions from switchgrass fermentation were likely due to accumulation of inhibitory compounds such as xylose (8). Fermentation of water-soaked switchgrass was attempted as a negative control and no ethanol production was

observed, illustrating the critical role of aqueous ammonia soaking in overcoming the recalcitrance of switchgrass.

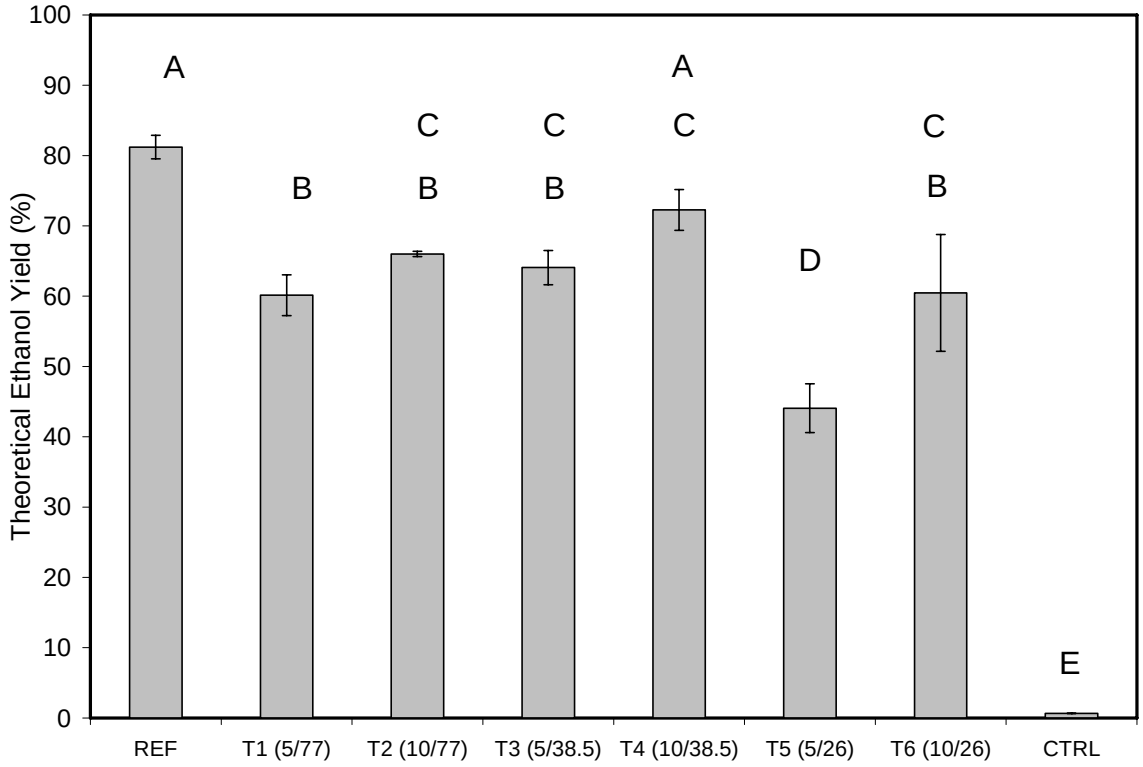

Figure 7. Theoretical ethanol yields of different fermentations. Letters on top of the columns indicate significant differences (Tukey test, $\alpha = 0.05$). REF: reference, 3% α-cellulose fermentation, 77 FPU/g cellulose; T1 (5/77): Treatment 1, 5 mL/g, 5d, 3% cellulose, 77 FPU/g cellulose; T2 (10/77): Treatment 2, 10 mL/g, 10d, 3% cellulose, 77 FPU/g cellulose; T3 (5/38.5): Treatment 3, 5 mL/g, 5d, 6% cellulose, 38.5 FPU/g cellulose; T4 (10/38.5): Treatment 4, 10 mL/g, 10d, 6% cellulose, 38.5 FPU/g cellulose; T5 (5/26): Treatment 5, 5 mL/g, 5d, 3% cellulose, 26 FPU/g cellulose; T6 (10/26): Treatment 6, 10 mL/g, 10d, 3% cellulose, 26 FPU/g cellulose; CTRL: control, water soaked switchgrass fermentation, 5mL/g, 5d, 3% cellulose, 77 FPU/g cellulose.

The percent of maximum theoretical ethanol yield achieved was computed for each of the SSF treatments using the conversion rate of 51 g ethanol per 100 g glucose. Results ranged from 72% for 10 d with the 10 mL/g treatments (Figure 7, T4), to 44% for 5 d with 5

mL/g and low enzyme case (Figure 7, T5). The average percent of maximum theoretical ethanol yield achieved was 60. Kim and Lee (8) reported 73% of maximum theoretical ethanol yield achieved after fermenting corn stover pretreated in aqueous ammonium hydroxide at 8 mL/g for 10 d. No statistically significant differences in percent of maximum theoretical ethanol yield achieved were observed between low, medium, and high enzyme loadings at high liquid-solid ratios and soaking times (Figure 7, T2, T4, and T6). In contrast, 5 d with 5 mL/g with the lowest enzyme loading had a statistically significantly lower ethanol yield (Figure 7, T5). This reinforces the interrelationship between enzyme requirements and pretreatment intensity discussed earlier.

Conclusion

Aqueous ammonia soaking at room temperature and atmospheric pressure is an effective pretreatment method for switchgrass prior to simultaneous saccharification and fermentation for ethanol production. The percent of maximum theoretical ethanol yield achieved by this method was as high as 72%, and this result reflected minimal optimization of the process. At high enzyme loadings, ethanol production was not greatly influenced by soaking time and liquid-solid ratio. However, at low enzyme loadings, significant increases in ethanol production were observed for the samples pretreated with higher intensity. The interrelationship between pretreatment conditions and enzyme requirements should be an area of further study and optimization.

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CHAPTER 3. PILOT SCALE FERMENTATION OF AQUEOUS AMMONIA SOAKED SWITCHGRASS

A paper accepted by the *Applied Biochemistry and Biotechnology* Journal

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Abstract

Aqueous ammonia steeped switchgrass was subject to simultaneous saccharification and fermentation (SSF) in two pilot-scale bioreactors (50 and 350 L working volume). Switchgrass was pretreated by soaking in ammonium hydroxide (30%) with solid to liquid ratio of 5 L ammonium hydroxide/kg dry switchgrass for 5 days in 75-L steeping vessels without agitation at ambient temperatures (15 to 33 ºC). SSF of the pretreated biomass was carried out using *Saccharomyces cerevisiae* (D_5A) at approximately 2% glucan and 77 filter paper units (FPU)/g cellulose enzyme loading (Spezyme CP). The 50-L fermentation was carried out aseptically, whereas the 350-L fermentation was semi-aseptic. The percentage of maximum theoretical ethanol yields achieved was 73% in the 50-L reactor and 52-74% in the 350-L reactor due to the difference in asepsis. The 350-L fermentation was contaminated by acid producing bacteria (lactic and acetic acid concentrations approaching 10 g/L), and this resulted in lower ethanol production. Despite this problem, the pilot-scale SSF of aqueous ammonia pretreated switchgrass has shown promising results similar to laboratory scale experiments. This work demonstrates challenges in pilot scale fermentations with material

handling, aseptic conditions and bacterial contamination for cellulosic fermentations to biofuels.

Introduction

Recovery of nutrients from biorefineries and recycling them back to crop fields can significantly improve the sustainability of biofuel production and can improve the overall energy balance of cellulosic ethanol systems (Anex et al., 2007). Advanced biorefinery designs usually integrate biological and thermochemical processes in which the unfermented portion of the biomass is thermally converted to produce additional fuels as well as heat and energy to drive the conversion processes. In theory, nutrient cycles can be closed by capturing the plant nutrients that are concentrated in ash and as gaseous ammonia produced during thermal conversion of the fermentation residue and by recycling them to crop production fields where feedstock are grown (Anex et al., 2007). To test this theory, quantities of fermentation residue sufficient to feed a pilot-scale gasifier were required. The target gasifier located at Iowa State University requires approximately 10 kg of dry fermentation residue to achieve the steady state operation required for consistent data generation (Do et al., 2007). Thus, one of the objectives of this study was to develop pilot-scale fermentation protocols that would generate a sufficient amount of fermentation residue for future gasification studies.

Pilot-scale fermentation experiments using steam exploded aspen and corn fiber have been reported previously in the literature (De Bari et al., 2002; Schell et al., 2004; Schell et al., 2007). De Bari et al. (2002) reported achieving 79% of theoretical ethanol yields from steam exploded aspen in helical stirred 10 and 50-L pilot-scale bioreactors. Schell et al. (2004) described an ethanol plant design which can continuously process a lignocellulosic

feedstock at a rate of 900 kg/day (dry weight) and they evaluated the equipment operation in the ethanol plant and generated performance data using dilute acid treated corn fiber. The authors have also discussed significant operational problems such as settling of solids during fermentation, difficulty in mixing, and bacterial contamination. In their second study, the authors (Schell et al., 2007) presented information on subsequent fermentation experiments and identified the primary source of contaminating microorganisms as *Lactobacillus* bacteria in the main fermentors. These papers provided valuable information on pilot-scale ethanol production from lignocellulosics; however, to our knowledge, there are no articles in the literature on pilot scale ethanol production from dedicated energy crops such as switchgrass or pilot-scale fermentation studies using aqueous ammonia pretreatment.

Ethanol production from switchgrass has been the focus of different studies (Isci et al., 2008; Alizadeh et al., 2005; Chang et al., 2001; Kurakake et al., 2001; Iyer et al., 1996); however all of these studies were performed at the bench scale. Therefore, the second objective of the study was to show that our previously proposed bench scale simultaneous saccharification and fermentation procedure (Isci et al., 2008) can be successfully scaled up.

Materials and Methods

Switchgrass samples were collected from mature stands of the Cave-in-Rock cultivar while dormant (early spring) in Chariton, IA. Dry switchgrass was ground to an average size of 5-6 mm in a tub grinder by the Biomass Energy Conversion Center (BECON), Nevada, IA. The compositions of the switchgrass (before and after pretreatment) were determined by Iowa State University, Department of Agronomy using the ANKOM method (ANKOM Technol. Corp., Fairport, NY) as described by Vogel et al. (Vogel et al., 1999). Untreated switchgrass (starting material) contained 32% cellulose, 31% hemicellulose, and 4.4% acid

detergent lignin, 27% Klason lignin and 0.7% ash. Klason lignin values were determined as explained by Isci et al. (Isci et al., 2008).

Cellulase enzyme (Spezyme CP, lot no:301-05330-206) was provided by Genencor International (Palo Alto, CA) and had an activity of 60 filter paper units (FPU)/mL, measured using standard procedures (NREL, Procedure no: 06, 1996). The yeast (*Sacccharomyces cerevisiae* D_5A) was supplied by National Renewable Energy Laboratory and preserved at 4°C after freeze drying with 20% nonfat dry milk.

Soaking in ammonium hydroxide, which was first studied by Kim and Lee (2005) as a biomass pretreatment method, was performed to enhance subsequent enzymatic hydrolysis of switchgrass. It has been shown at the lab scale (Isci et al., 2008) that the process partially removes lignin and hemicellulose, while preserving cellulose fraction of the biomass. To test the performance of the pilot scale pretreatment and fermentation, 4 kg of dry switchgrass was soaked in reagent-grade (29.5 wt%) ammonium hydroxide (Fisher Scientific Inc., Hanoverpark, IL) for 5 days in 75-L vessels at the Iowa State University Livestock Environment Building and Research Center (LEBRC) near Boone, IA, during the summer of 2007 (Himmelsbach et al., 2008). The vessels were operated without any agitation at ambient temperatures (15 to 33ºC). The ammonia soaked switchgrass from this first trial was used in the 50-L fermentation immediately after the soaking period. The design and performance of the pretreatment vessels have been described in detail elsewhere (Himmelsbach et al., 2008). From the subsequent soakings, approximately 80 kg of wet aqueous ammonia soaked switchgrass was generated and stored at -20ºC for the 350-L fermentation and thawed during the three days before the fermentation. Pilot scale simultaneous saccharification and

fermentation (SSF) was performed following established procedures (NREL, Procedure no: 08, 1996).

Fermentation Design

Extrapolating bench scale experiments suggested that approximately 12-20 kg of ammonia pretreated switchgrass (60-100 kg wet switchgrass) would need to be fermented to produce the 6-10 kg of residual. One steeping vessel run generated sufficient pretreated switchgrass for the 50-L fermentor, which allowed to directly scale-up to the 50-L fermentation. Performing a 50-L fermentation 7-times with pretreated fresh switchgrass was considered, however in order to produce a homogeneous residue, gain experience at larger scales and save time we chose to work at 350 L. The size of pretreatment vessel used required that pretreated switchgrass be stored by freezing until sufficient material could be generated to perform the 350-L fermentation.

The 50-L Fermentation

Approximately, 13.3 kg of wet switchgrass (80% moisture content) was generated from an initial trial of pilot-scale ammonia soaking, which contained 48% cellulose, 23% hemicellulose and 22% Klason Lignin. The average solid content of the wet switchgrass was determined by drying 6 switchgrass samples (20 gram each) taken from different locations of the pretreatment vessel at 60ºC for 3 days.

A 50-L steam-jacketed fermentor (Figure 1, Biostat U-50, B. Braun Biotech (Sartorius), Allentown, PA) was loaded with 13.3 kg wet switchgrass (which corresponds to 2.4% [w/v] cellulose concentration), 1% (w/v) yeast extract (Ardamine Z, Indianapolis, IN), 2% (w/v) peptone (Difco Laboratory, Detroit, MI), 0.05 M citrate buffer (pH 4.8), and

deionized water added to make a working volume of 50-L then sterilized at 121°C for 20 min. The 50 L fermentor was equipped with 3 Rushton type impellors, which operated at 130 rpm during fermentation. Once the fermentation media cooled down to 35ºC, the inoculum and enzyme (77 FPU/g cellulose) was added aseptically. The 1-L *S. cerevisiae* D_5A inoculum was prepared in 2-L shake flasks with 1% (w/v) yeast extract (Ardamine Z), 2% (w/v) peptone (Difco Laboratory) and 5% (w/v) dextrose (Fisher Scientific Inc.) at 35°C with shaking at 170 rpm for 24 h, and was inoculated with one freeze dried culture vial ($2 * 10^9$ cells/ mL).

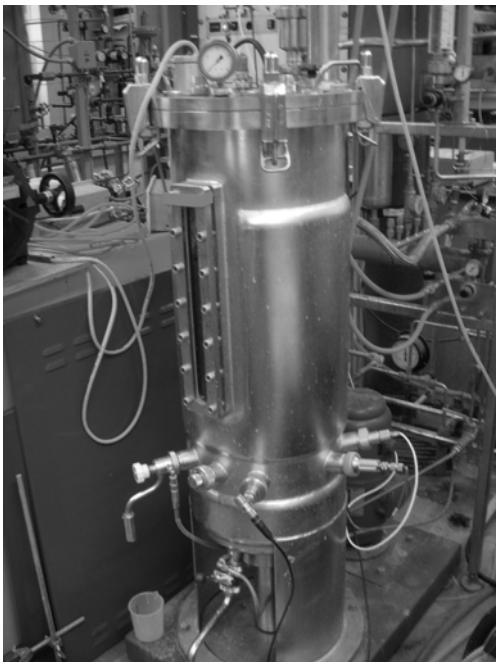

Figure 1. Biostat U-50, 50L fermentor.

The 350-L Fermentation

Approximately, 80 kg of wet switchgrass (80% moisture content) was generated by ammonia steeping in 75-L vessels by steeping for 5 days at a liquid to solid ratio of 5 L

aqueous ammonia/kg dry switchgrass (Himmelsbach et al., 2008). The pretreated switchgrass contained 45% cellulose, 23% hemicellulose and 23.5% Klason lignin. The solid content and fiber content of the pretreated material were determined as explained above.

A 350-L fermentor (Figure 2, Model PTT, Walker Stainless Equipment Co., New Lisbon, WI) was loaded with approximately 80 kg of thawed wet switchgrass with a moisture content of 80% (~2% w/v cellulose), 1% (w/v) yeast extract, 2% (w/v) peptone and 0.05 M citrate buffer (pH 4.8). A semi-aseptic method was used, such that first yeast extract, peptone, water and buffer were sterilized in the tank using steam jackets, and then unsterilized switchgrass was added incrementally over 24 h. Specifically, approximately one third of the switchgrass (~27 kg) was added at times 0, 5, and 24 h, which allowed substrate thinning via the cellulase (77 FPU/g cellulose). Incremental addition of wet switchgrass was done because the material was dense and clumpy, and we risked damaging the reactor impeller if all biomass were added at once. The single 30-cm diameter, 3-blade axial flow impeller was operated at 200 rpm throughout the SSF process.

The 10-L *S. cerevisiae* D_5A inoculum was prepared in 20 L fermentor (Bioflo 500, New Brunswick Scientific, Edison, NJ) with 1% (w/v) yeast extract (Ardamine Z), 2% (w/v) peptone (Difco Laboratories) and 5% (w/v) dextrose (Fisher Scientific Inc.) at 35^{o}C for 24 h with 250 rpm agitation and 5-10 L/min air flow. The inoculum was aseptically transferred into 350 L fermentor at time 0. Enzyme (77 FPU/ g cellulose) was added at the same time from the top of the fermentor using sterile containers based on the final concentration of treated switchgrass.

Figure 2. 350-L fermentor, Model PTT.

Theoretical ethanol yields were calculated as follows based on the maximum (51%) conversion of glucose into ethanol by yeast (Isci et al., 2008).

$$Theoretical\ ethanol\ yield\ (\%) = \frac{Ethanol\ produced\ (g)\ in\ reactor}{Initial\ sugar\ (g)\ in\ reactor \times 0.511} \times 100$$

At the end of each fermentation, the biomass fermentation residue remaining in the fermentors was pumped into containers and screened through 2 mm fiberglass mesh (charcoal fiberglass, New York Wire) and the captured solids were dried at 60ºC for 3 days.

Analytical Procedure

Samples were taken at time 0, 4, 8, 24, 48 and 72 h from 50 L fermentation and at time 0, 5, 24, 28, 48, 72, 96, 120 h from 350 L fermentation. The samples were analyzed for sugars (cellobiose, glucose, and xylose), ethanol and organic acids by HPLC (Varian ProStar 210) with a refractive index detector (Varian 355 RI). A MetaCarb 87P column (water as a mobile phase, flow rate of 0.4 ml/min, column temperature of 80^{o}C, and injection volume of 20 µl) was used for sugar analysis, while a Bio-Rad 87H column (0.01 N sulfuric acid as a mobile phase, flow rate 0.6 ml/min, column temperature 65^{o}C, and injection volume of 20 µl) was used for determination of ethanol and organic acid concentrations.

Results and Discussion

In our previous study (Isci et al., 2008), we showed that ammonia soaking at room temperature is an effective method for removing Klason lignin while conserving the cellulose fraction and enhancing the subsequent SSF of switchgrass at lab scale. Therefore, ammonia soaking at room temperature was also selected as the pretreatment method for these pilot-scale fermentation experiments. Approximately 30-35% weight loss was observed from the initial pilot scale ammonia soaking of switchgrass. The details of the compositional changes of switchgrass have been reported elsewhere (Himmelsbach et al., 2008). The focus of the current paper was to generate large amounts of biomass fermentation residue sufficient for gasification and to demonstrate that SSF of ammonia treated switchgrass is feasible at pilot scale under non-aseptic conditions.

It was also reported earlier that at higher enzyme loadings ethanol production was not greatly influenced by pretreatment intensity (Isci et al., 2008). Therefore, the experiments

reported in this paper used relatively high enzyme loading (77 FPU/ g cellulose). Figure 3 shows sugar and ethanol concentrations over time for the 50-L fermentation. A set of standard SSF trajectories similar to those previously obtained at lab scale (Isci et al., 2008) were observed. The ethanol concentration increased slowly during the first 24 h of fermentation, with a more rapid increase between 24 and 48 h. A rapid decrease in glucose concentration was simultaneously observed, indicating that yeast cells began to utilize glucose and convert it into ethanol effectively after 24 h. In a lab scale fermentation performed under similar conditions (Isci et al., 2008), the ethanol concentration reached its peak at 24 h when sugar was depleted, and remained constant thereafter. The slower response observed at pilot scale could be due to scale-up induced changes in mixing, shear forces, mass transfer and/or it might reflect the lower inoculum concentrations used in the 50-L fermentation. In bench scale, freeze dried inoculum was used and each fermentation flask (100 mL working volume) contained approximately $2*10^7$ cells. On the other hand, the inoculum of the 50 L fermentation had an absorbance at 620 nm of 0.5, which corresponds to a cell concentration of 0.23 g/L (dry wt) (Demirci and Pometto, 1999). It might have taken longer for the number of cells to reach a level where glucose consumption equaled or exceeded the rate of enzymatic hydrolysis. De Bari et al. (2002) have reported that doubling inoculum yeast concentration from 3 to 6 g/L produced nearly identical ethanol concentrations after 48 h in pilot scale fermentations.

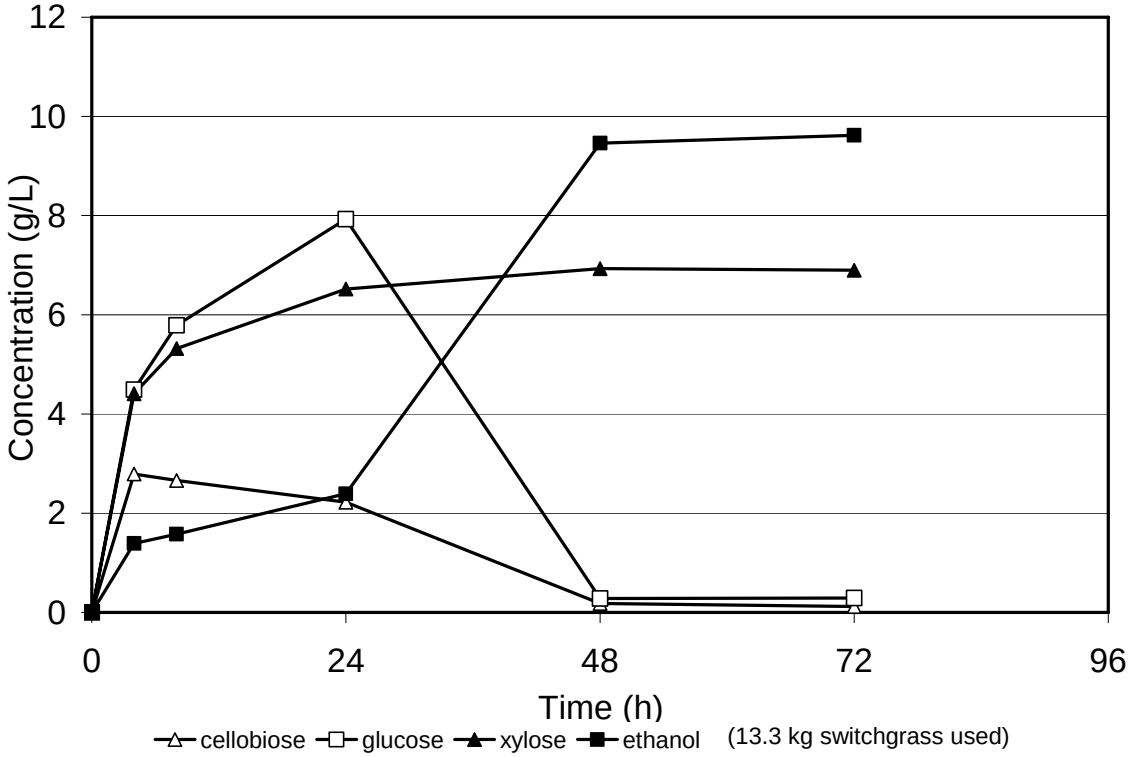

Figure 3. Time courses of sugars and ethanol concentrations for 50-L SSF of ammonium hydroxide steeped switchgrass (5 L/kg ammonia soaking for 5 days, 2.4% (w/v) cellulose, and 77 FPU/kg cellulose).

The theoretical ethanol yield of the 50-L fermentation was 73%, which was similar to the results (60-72%) obtained from lab scale fermentations (Isci et al., 2008). No lactic acid and acetic acid production was observed throughout the 50-L fermentation which indicates a successful sterilization was achieved.

Xylose concentrations (7 g/L) observed in the 50-L fermentation are similar to those observed in lab scale fermentations (6.79 g/L in 100 mL fermentation) as well, and reflected hemicellulase activity in the cellulase enzyme used. Xylose concentrations at this level could possibly inhibit ethanol production (Kim and Lee, 2005).

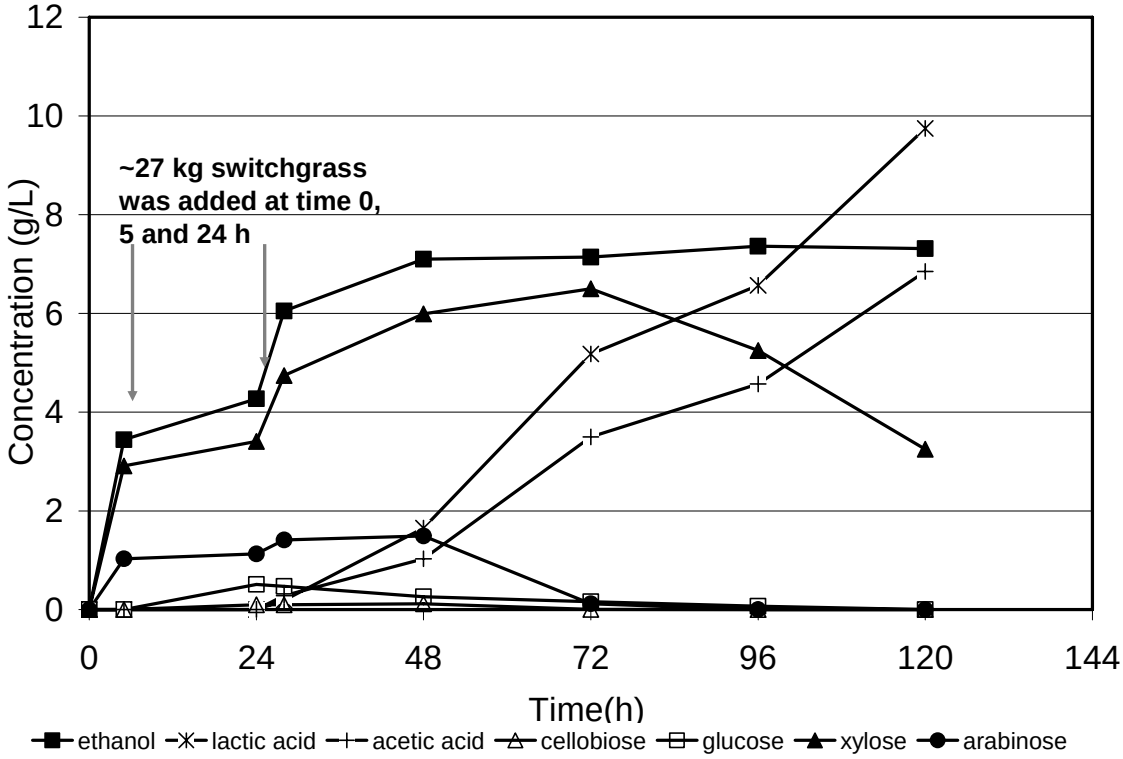

Figure 4. Time courses of sugars, ethanol and acid concentrations for the 350-L SSF of ammonium hydroxide steeped switchgrass. The total amount of wet switchgrass loaded was approximately 80 kg. (5 L/kg ammonia soaking for 5 days, ~2% (w/v) cellulose, and 77 FPU/kg cellulose).

Sugar, ethanol and organic acid concentrations over time in the 350-L fermentation are presented in Figure 4. The rate of ethanol production was observed to fluctuate significantly over the first 48 h, which corresponded to the incremental addition of pretreated switchgrass into the reactor. The ethanol concentration at 72 h was approximately 25% lower than that of the 50-L fermentation. This was likely due to bacterial contamination by heterofermentative lactic acid bacteria producing lactic acid and acetic acid, as evidenced by a continuous increase in both acid concentrations beginning at 24 h. The lactic acid and acetic acid concentrations reached to 9.75 and 6.85 g/L, respectively, at 120 h; these

concentrations are reportedly inhibitory to yeast growth and ethanol production (Maiorella et al., 1983). Schell et al. (2004 and 2007) also reported decrease in ethanol production due to inhibitory effect of the relatively high organic acid concentrations on yeast performance.

Unlike 50 L fermentation, cellobiose and glucose concentrations never exceeded 0.5 g/L throughout the fermentation, which signified that as soon as cellulose was hydrolyzed into glucose it was being consumed by yeast and/or contaminating bacteria. Arabinose was completely utilized by bacteria after 48 hours. Schell et al. (2007) have also identified the contaminating microorganisms as different stains of *Lactobacillus* in their fermentation broth which consumed arabinose readily. After the depletion of arabinose, xylose concentrations started to decrease as the organic acid concentrations continued to increase which proves that the contaminating bacteria was able to consume different sugars.

A gradual decrease in pH from 5.0 to 4.3 was observed between 48 and 120 h of fermentation due to organic acid production which also was an indication of contamination (no pH control was employed). The corn dry-grind ethanol industry usually observes 2 to 3 g/L lactic acid production in 60 h of fermentation which also indicates lactic acid bacterial growth (Dr. Anthony L. Pometto, personal communication, November 27, 2007). The lactic acid concentration in our experiment was approximately 3 g/L at 60 h, which shows that a similar pattern was being followed. Since it would be extremely difficult to produce lignocellulosic ethanol aseptically at industrial scale, ways to keep contamination levels at minimum must be determined in detail before scaling up. In industrial fermentations, the most common method to control contaminations is based on the antibiotics virginiamycin and penicillin (Connoly, 1997 and Lushia & Heist, 2005). Schell et al. (2007) have also showed that the lactic acid bacteria contaminations can be controlled by antibiotic

virginiamycin in industrial scale lignocellulosic fermentations. In addition, modified microorganisms that can ferment all of the available sugars (xylose, arabinose etc.) into ethanol could be used to compete with the contaminating microorganisms.

The pretreated switchgrass from the first pilot scale steeping trial was used for the 50-L fermentation which generated approximately 0.8 kg dry switchgrass residue at the end of fermentation. Based on this data, it was decided to repeat the pilot scale soaking 8 times which was expected to generate 6.4 kg fermentation residue. However, because of the problems encountered during this first pilot-scale pretreatment experiment, a design change was made (Himmelsbach et al., 2008). The redesigned steeping system, however, generated only 4.5-kg dry fermented switchgrass residue after fermentation in the 350-L fermentor. The difference between the amount of residue recovered and the predicted amount of fermentation residue was most likely due to the loss of fine particles during washing of pretreated switchgrass in the redesigned steeping system. It could also be attributed to a technical problem encountered on the first trial of the 350-L fermentation. As a consequence of mixing problems, the pretreated biomass was transferred back to containers from the fermentor before inoculation, which resulted in some loss of switchgrass. It was unknown exactly how much biomass was lost during the transfer. The agitation problem was overcome in the second 350-L fermentation trial by loading the fermentor with water initially, and then gradually adding the switchgrass. Schell et al. (2004) have also reported mixing difficulties in the first run of a 9000-L fermentor due to settling of solids. For their second run, the authors started the fermentor with sterile water which thinned the broth enough to allow adequate mixing.

Table 1. Mass flow of the process including theoretical ethanol yield estimations (all of the values are presented on dry basis)

		conservative case	medium case	best case
	50L	**350L**	**350L**	**350L**
Switchgrass Before Pretreatment (kg)	4[a]	32[a]	32[a]	32[a]
Switchgrass After Pretreatment (kg)	2.7[a]	21[c]	16[c]	15[c]
Cellulose in pretreated switchgrass (kg)[b]	1.3	9.6	7.2	6.8
Ethanol yield (%)[b]	73	52	70	74
Switchgrass After Fermentation (kg)	0.8[a]	4.5[a]	4.5[a]	4.5[a]

[a] Measured values
[b] Calculated values (percent ethanol yields are calculated based on the total cellulose in the fermentor using the formula presented in methods section)
[c] Estimated values

Due to uncertain loss of biomass in the 350-L fermentation as mentioned above, estimates have been made of the possible theoretical ethanol yields achieved (Table 1). The lower limit (conservative case) was calculated assuming same amount of biomass was recovered from the redesigned pilot-scale soaking vessels as in the first soaking design trial and there were no biomass loss from the 350-L fermentor. In that case 21 kg pretreated biomass (2.6 kg dry pretreated switchgrass recovered from first steeping vessel and totally eight 75-L aqueous ammonia pretreatment was performed) should be available for ethanol conversion. Based on this assumption, the theoretical ethanol yield calculated as 52% for the most conservative case. However, it was clear from lower amount of fermentation residue generation (~4.5 kg) that a significant amount of biomass was lost, which meant a better conversion rate was achieved in reality. Based on the biomass residue recovered from both the 50-L and 350-L fermentations, it could be estimated that originally there were 15 kg dry pretreated switchgrass for 350-L fermentation, which corresponds to a theoretical ethanol

yield of 74%. If the total biomass loss from the process (including both the pretreatment and fermentation) was assumed to be the half of the initial switchgrass, the theoretical ethanol yield would have been 70%. The last two estimates are more likely to be close to real values, which are also similar to lab scale ethanol yields (Isci et al., 2008).

Conclusion

Simultaneous saccharification and fermentation of ammonia soaked switchgrass was scaled up successfully with minor contamination and mixing problems. Better techniques to control the bacterial contamination and to improve mixing of wet biomass in the fermentor need to be studied for further scaling up trials. The theoretical ethanol yields achieved were between 52-74% which were similar to laboratory scale results. Due to loss of biomass during the washing step of the pretreatment and the first trial of the 350-L fermentation, a lower amount of fermentation residue was generated than expected.

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CHAPTER 4. TECHNO-ECONOMIC ANALYSIS OF AQUEOUS AMMONIA SOAKED SWITCHGRASS FERMENTATION

A paper to be submitted to the *Bioresource Technology* Journal

Asli Isci, Feroz K. Kazi, and Robert P. Anex

Abstract

A techno-economic model is used to analyze the economic feasibility of biochemical conversion of switchgrass to ethanol using an aqueous-ammonia soaking pretreatment process. The modeled biorefinery includes an aqueous-ammonia soaking pretreatment section along with two-stage ammonia recovery units, simultaneous saccharification and co-fermentation, distillation, waste water treatment and combustion units. Ammonia recovery units are designed using ASPEN Engineering Software which includes two steam stripping units. A spreadsheet model was developed by adapting the National Renewable Energy Laboratory's (NREL) lignocellulosic ethanol production process model (Aden and Ruth, 2001). Ammonia soaking pretreatment and ammonia recovery sections designed in this study replace NREL's dilute acid pretreatment section. The model assumes a 2000 metric ton (dry)/day switchgrass feed rate. In the base case scenario, the ammonium hydroxide (30%) to solids ratio is assumed to be 5L/kg and the soaking time is taken as 5 days. The results show that at full-scale aqueous-ammonia soaked switchgrass fermentation is a capital intensive process. Even though the pretreatment reactor unit cost is low, the cost of the pretreatment section is still high due to the large number of reactors and filters needed. The pretreatment

section was found to be the second most expensive after the combustion section and it contributes to 24% of the total equipment cost in the base case design. The ammonia recovery units were not large contributors to equipment cost. The baseline scenario resulted in an ethanol selling price of $2.99/gal. Sensitivity results showed that feedstock cost have the largest impact on ethanol selling price, whereas soaking time was the most important parameter affecting the total equipment cost. Ethanol yields were also significant economic drivers of the process. Under the range of conditions and configurations examined, aqueous ammonia soaking was found not to be an economically viable pretreatment method.

Introduction

Aqueous ammonia soaking pretreatment of biomass for ethanol production has been performed recently at bench and larger scales (Kim and Lee, 2005; Isci et al., 2008a; Isci et al., 2008b). Aqueous ammonia soaking is an attractive pretreatment method because it does not require high temperatures or pressures and preserves the cellulose fraction of biomass while partially removing lignin and hemicellulose (Isci et al., 2008a). Even though the ammonia soaking method does not require extreme pretreatment conditions, capital and operations costs can still be high due to water and ammonia usage and the need for extensive ammonia recovery. In this paper a process model is used to assess the techno-economic performance of an integrated biorefinery that utilizes the aqueous ammonia soaking pretreatment process.

Process modeling and economic analysis of lignocellulosic ethanol production have been the focus of several biorefinery evaluation studies (Perez et al., 1981; Nagle et al., 1999; Aden and Ruth, 2001; Eggeman and Elander, 2005). The process simulation package, ASPEN Plus has been used in several of these studies to perform mass and energy balances

and to identify the variables that have major impact on the process performance and economics. These analyses have found that pretreatment, hydrolysis and fermentation unit performance are the largest contributors to production cost of cellulosic ethanol. The Biomass Refining Consortium for Applied Fundamentals and Innovation (CAFI) project has compared different types of pretreatment in terms of economic performance (CAFI PROJECT, Eggeman and Elander, 2005). The CAFI studies found that all of the designs were capital intensive and there was little differentiation in process economics among the pretreatment options. Although the economic performances of several different types of pretreatment have been compared, there are no articles in the literature that report on the economics of aqueous-ammonia soaking pretreatment. This paper focuses on identifying the process economic impacts of the aqueous-ammonia steeping pretreatment method.

Materials and Methods

A detailed flow diagram (Figure 1) and an Excel®-based process model have been developed based on aqueous-ammonia soaking pretreatment data obtained from bench and pilot-scale experiments (Isci et al., 2008a, 2008b). The model is divided into 10 sections (known as "areas"), including feedstock handling (Area-100), pretreatment (Area-200), ammonia recovery system 1 (Area-300), ammonia recovery system 2 (Area-400), hydrolysis and fermentation (Area-500), distillation-evaporation-dehydration (Area-600), storage (Area-700), combustor (Area-800), waste water treatment (Area-900) and utilities (Area1000). The feedstock in this analysis is switchgrass with the composition shown in Table 1. The composition of switchgrass was taken from a previous laboratory analysis that was also the source of estimated process parameters (Isci et al., 2008a).

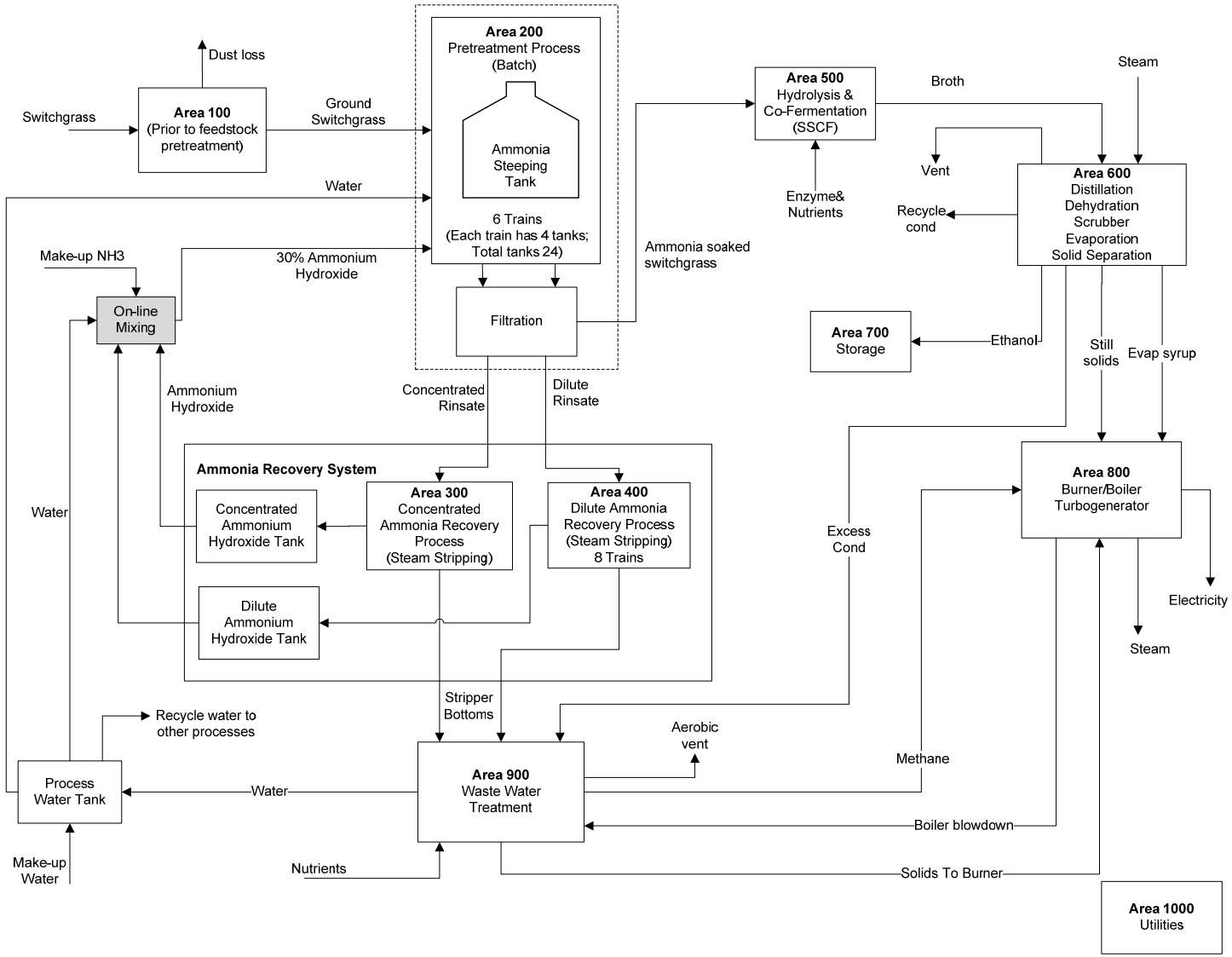

Figure 1. Process flow diagram of aqueous ammonia soaked switchgrass fermentation

The cost estimates are based on the assumption that this is the "nth" plant as described in NREL's technical report (Aden and Ruth, 2001). The plant is assumed to be operational 24 hours per day, 350 days per year. In order to be consistent with and allow comparison with other studies the base scenario plant size is defined as 2000 dry ton/day of feedstock (15% moisture content) into the pretreatment unit. Other assumptions of the base scenario are presented in Table 2.

Design and Cost Calculations

Feedstock Handling (Area 100)

The feedstock handling section (Area 100) is assumed to be similar to NREL's design. The feedstock washing step is eliminated in our model because unlike dilute acid pretreatment, ammonia soaking process does not require wet biomass. In this area the feedstock is received and ground and then sent to the pretreatment unit. Equipment sizes and costing are adapted from the NREL's design and adjusted for differing flow rates.

Table 1. Composition of switchgrass

Composition of Switchgrass (% dry basis)	
Cellulose	42
Hemicellulose	31
Klason Lignin	22
Acid Detergent Lignin	5.9

Table 2. Base case scenario assumptions

Parameter	Assumptions
Ammonia Consumption By Reactions	1.5% of total ammonia
Ammonium Hydroxide to Solids Ratio	5 L/kg
Wash Water to Solids Ratio	50 L/kg
Soaking Time	5 days
Cellulose to Ethanol Conversion	95%
Xylose to Ethanol Conversion	85%
Switchgrass Cost	$30/dry US ton
Enzyme Price	$5.52/lb
Pretreatment Reactor Price	$165,000/unit

Pretreatment (Area 200)

The major equipment in the pretreatment section are the ammonia soaking tanks, pneumapress filters, and screw and belt conveyers. The base scenario residence time is taken to be 5 days. Vessel size is calculated based on the bulk density of ground switchgrass with a 20% headspace volume. One train of soaking unit consists of 4 tanks and each tank has a volume of 1.1 million gal. The number of trains required is equal to the soaking time in days (plus one additional train to accommodate downtime). For example, in the base case scenario the residence time is 5 days, therefore the number of trains required is 6 and the total

number of tanks needed is 24. The cost of pretreatment tanks is estimated based on the cost of the ethanol storage tank reported in NREL's report with modifications for material handling. These modifications increase the pretreatment tank cost 40% relative to the simple storage tank.

The ammonium hydroxide (30% w/w) to solids ratio in the pretreatment reactors is 5 L/kg in the base scenario. Different soaking ratios are also studied during sensitivity analysis. After switchgrass is soaked in 30% ammonium hydroxide for 5 days, it is sent to the pneumapress filters by screw conveyers. A single pneumapress filter is used for each pretreatment train.

Filtration is performed in 2 sequential steps. In the first step, the tank is drained and switchgrass is pressed to extract concentrated ammonium-hydroxide solution (concentrated rinsate). The concentrated rinsate is sent to ammonia recovery system 1 (Figure 2). In the second filtration step, switchgrass is washed with water and pressed. The resulting dilute ammonium hydroxide solution (dilute rinsate) is sent to ammonia recovery system 2 (Figure 3). For this analysis, it is assumed that 1.5% of the total ammonia is consumed in reactions (e.g., ammonolysis) during pretreatment. The pretreated and washed solids are transferred to the fermentation unit with belt conveyers.

Ammonia Recovery Systems (Area 300-400)

A two-step ammonia recovery system is designed using ASPEN Plus simulator 2004.1 (Aspen Technology, INC, Cambridge, MA). Equipment costs are estimated using engineering design and cost estimation data from Peters et al. (2003). The concentrated ammonia recovery system (Figure 2) consists of a feed pump, steam stripper, reboiler, flash drum, water scrubber, scrubber reflux condenser and two additional condensers. The

recovered ammonium hydroxide is collected from the flash drum, scrubber and condenser and stored in the concentrated ammonia tank. The stripper bottom is sent to the waste water treatment (WWT) unit. The system is designed such that stripper bottom would contain only 5 ppm ammonia (The ammonia loss through concentrated ammonia recovery-system was calculated as 0.001% of the total ammonia feed).

The dilute-ammonium hydroxide stream generated after washing pretreated switchgrass is sent through a separate recovery train (Figure 3). This system consists of a feed pump, steam stripper, stripper reboiler, reflux condenser and an ammonia condenser. The flow rate of the dilute ammonium hydroxide stream in the base case scenario is too large to be passed through a single stripper system and considering the maximum practical size of a stripper it is determined that eight dilute ammonia recovery trains are needed. The recovered ammonium hydroxide solution is collected from condenser and stored in the dilute ammonium hydroxide tank. Ammonia concentration in the stripper bottom is 5 ppm (accounting for 0.02% of total ammonia in the feed) and is sent to WWT. Total ammonia loss from the plant is calculated and included in the pretreatment unit as make-up ammonia. In the base case scenario, total ammonia loss is calculated as 1.69 % of the original ammonia that is used in the pretreatment area. The concentration of ammonia recovered from area 300 and 400 is assumed to be adjusted back to 30% using concentrated ammonia before being recycled back to the pretreatment unit.

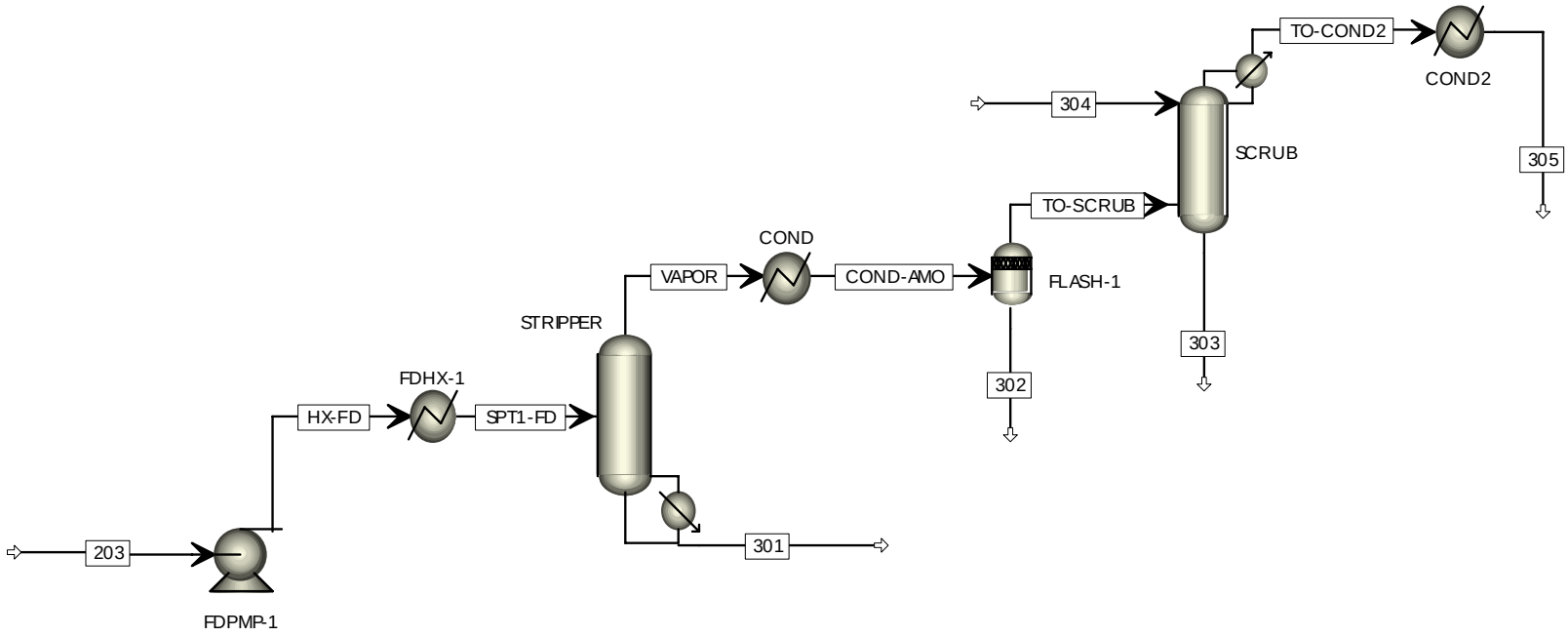

Figure 2. Flow Diagram of Area 300 (Concentrated Ammonia Recovery Process) (203: concentrated rinsate, 301: stripper bottoms to WWT, 302-303-305: ammonium hydroxide to concentrated ammonia tank, 304: water for scrubbing)

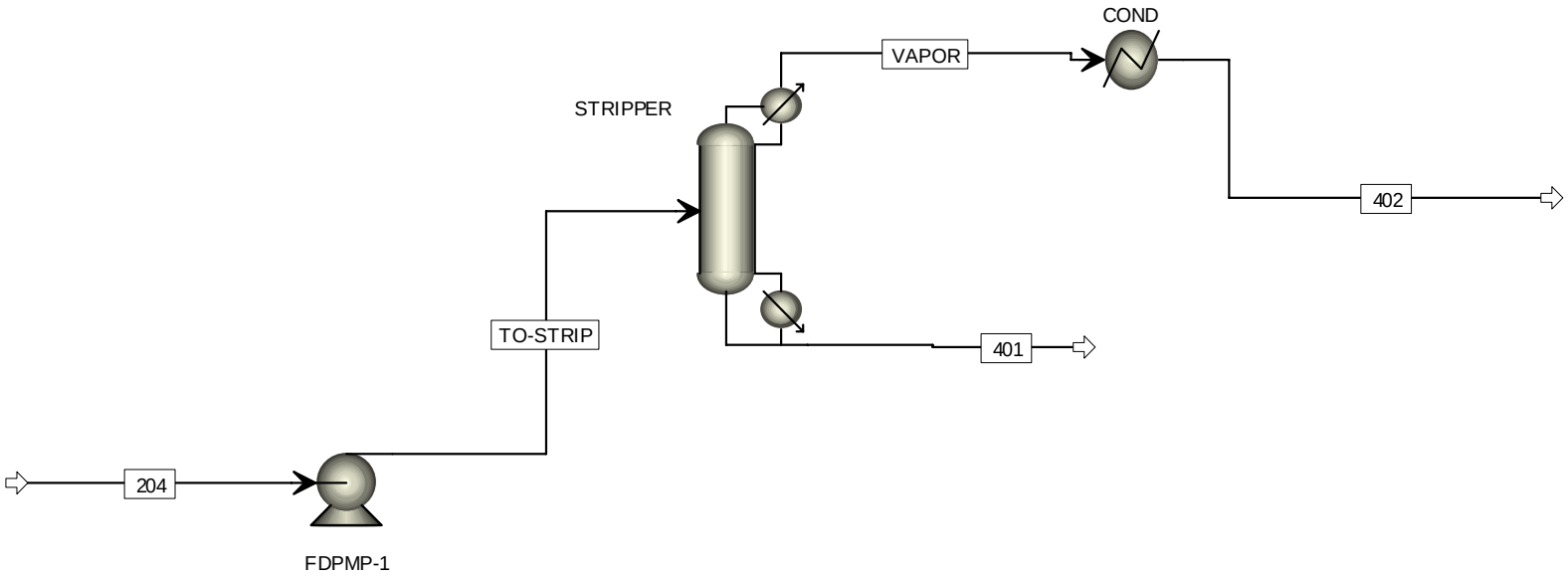

Figure 3. Flow Diagram of Area 400 (Dilute Ammonia Recovery Process) (204: dilute rinsate, 401: stripper bottom to WWT, 402: ammonium hydroxide to dilute ammonium hydroxide tank

Hydrolysis and Fermentation (Area 500)

The pretreated lignocellulosic material is sent to the fermentation unit. The fermentation conditions are taken to be the same as assumed in NREL's technical report (Aden and Ruth, 2001) (Table 3). Since a simultaneous saccharification and co-fermentation (SSCF) process is assumed in this area, no separate saccharification vessels are included in the design. To facilitate comparison with results of the NREL analysis, in the base case scenario we also assumed that a microorganism will be commercially available that can ferment glucose and xylose with 95 and 85% ethanol yields, respectively. Cellulase loading (30 FPU/ g cellulose) is selected based on the results of our previous study (Isci et al., 2008a). It was reported in the same study that at this level of enzyme loading, ethanol yields were not affected greatly by pretreatment intensity.

Table 3. Condition of SSCF in base scenario

Temperature	(ºC)	41
Initial fermentation solids	(%)	10
Residence time	(d)	1.5
Size of vessel	(gal)	1,365,000
Number of vessels	-	5
Inoculum Level	(%)	10
Corn Steep Liquor Level	(%)	0.25
Diammonium phosphate (DAP) Level	(g/L fermentation broth)	0.33
Cellulase loading*	FPU/g cellulose	30

*Assuming total enzyme activity as 60FPU/ml enzyme

Fermentor capital cost is scaled based on the total volume of the fermentation broth. An additional 20% increase in the costs of fermentors and agitators is included to account for

modifications to the vessels to accommodate the SSCF process. Other equipment in this area is assumed to be 40% of the total area cost.

Distillation, Dehydration, Evaporation, Solid-Liquid Separation (Area 600)

This area includes the beer column, rectification column, evaporation system, molecular sieve and pneumapress filter. As described in NREL's report, distillation is performed in two steps. In the first beer column most of the water is removed and in the second rectification column, ethanol concentration is increased to 95%. The rest of the water is removed by vapor phase molecular sieve adsorption. The bottoms from the beer column are filtered and sent to the combustor. The liquid from the filter is concentrated in a multiple effect evaporator. The concentrated syrup from the evaporator is mixed with the solids being sent to the combustor, and the evaporated condensate is used as recycle water (Aden and Ruth, 2001). Equipment is sized according to modeled flow rates and the costs are adjusted accordingly.

Storage (Area 700)

In Area 700, all tank designs and costs are taken from the NREL's design report. The tanks that are not required in the ammonia steeping process, such as the sulfuric acid tank, are eliminated. All tank and pump costs are corrected based on design flow rates.

Combustor (Area 800)

This area is designed to generate steam and electricity by burning unconverted portions of lignocellulosics. Burning these by-product streams allows the plant to be self sufficient in energy and reduces solid waste disposal cost and generates additional revenue through sales of excess electricity (Aden and Ruth, 2001). This design philosophy increases

the plant capital cost compared to a plant design that utilizes fossil fuels to generate heat and power. We have followed this same "minimum fossil fuel" design approach to allow more direct comparison of plant performance. The design of this section is also adapted from the NREL design. The size and the cost of equipment in this area are scaled from the NREL design according to the predicted total solids coming into the burner from the WWT unit and Area-600.

Wastewater Treatment (Area 900)

The wastewater treatment section treats process waste water for reuse to reduce the plant makeup water requirement. NREL's wastewater unit is adapted by updating the cost based on the solid fraction of the waste streams coming from the pretreatment area. As in the NREL design report, we also assumed that WWT produces waste water sufficiently clean that it can be recycled in the process and no water is sent to municipal treatment facilities.

Process Economics

Equipment costs are taken either from NREL's report (Aden and Ruth, 2001) or from Peters et al. (2003) and adjusted to 2007 dollars (Bureau of Labor Statistics, 2007). The installation factors are also taken from NREL's report. Total project investment includes the cost of several items in addition to the installed equipment as summarized below in Table 4.

Table 4. Additional costs for determining total project investment (Aden and Ruth, 2001)

Item	Amount
Warehouse	1.5 % of total installed equipment cost
Site Development	9% of the installed process equipment cost
Prorateable Costs	10% of total installed cost
Field Expenses	10% of total installed cost
Home Office and Construction	25% of total installed cost
Project Contingency	3% of total installed cost
Other cost (start-up, permission etc.)	10% of total capital investment

All components of the variable operating costs (Table 5) including raw materials, waste handling charges and by-product credits are taken from NREL's design report and adjusted to our process flow rates. Fixed operating costs include labor and various overhead items which are also taken from NREL's design report. General overhead is assumed to be 60% of applied total salaries and covers items such as safety, general engineering, general plant maintenance, payroll overhead (including the benefits), plant security, janitorial and similar services, phone, light, heat, and plant communications (Aden and Ruth, 2001). Annual maintenance and repairs are estimated as 2% of the total installed equipment cost. Insurance is estimated 0.7% of the total installed cost. These estimates are based on a representative Midwest US location.

Table 5. Variable operating costs

Variable Operating Costs	Flow (kg/h)	Cost (2000$/lb)	(2007$)/y
Biomass Feedstock	99,020	0.015	$27,510,618
Make up- anhydrous ammonia	1,907	530[i]	$8,488,856
Corn Steep liquor	1,650	0.0804	$2,956,327
Cellulase	17,629	5.52	$24,095,819
Diammonium Phosphate	1,712	0.0706	$2,693,232
Propane	20	0.0022	$981
Make up-Water	303,001	0.0001	$675,236
Boiler Feed Water Chemicals	1	1.3497	$30,078
Cooling Water Chemicals	1.9	1.0204	$43,205
WWT Chemicals	225.81	0.1579	$794,580
WWT Polymers	0.761	2.551	$43,255
Ash Disposal	4802	0.0094	$1,005,861
Electricity Credit	20040[ii]	0.041[iii]	$8,305,229
Extra electricity for not using steam in pretreatment unit			$1,031,894

i: in $/ton anhydrous ammonia (Carolan et al., 2007)
ii: units in kW
iii: units in $/kWh

Discounted Cash Flow Analysis

A discounted cash flow analysis method is used to determine the minimum selling price per gallon of ethanol produced. The discounted cash flow analysis fixes the internal rate of return and then iterates on the selling cost of ethanol until the net present value of the project is zero.

Table 6. Discounted cash flow parameters

Plant life	20 years
Start up period	1 year
Salvage value	10% of capital investment
Annual depreciation method	Straight line method
Income Tax Rate	30 %
Internal Rate of Return	15 %

Sensitivity Analysis

The base case scenario includes several uncertain assumptions that may have a large impact on equipment cost and ethanol selling price. Some of these assumptions are selected for sensitivity analysis in order to determine and compare the impact of these assumptions on ethanol selling price and equipment cost. The Crystal Ball (Denver, CO) software program is used in combination with the Excel® model to calculate the probability distributions and to determine model sensitivity in terms of how much the variation in each parameter contributes to variation in the ethanol selling price and total equipment cost. The selected parameters and their distributions are shown in Table 7. Some of the assumptions (cellulose conversion yield, xylose conversion yield and feedstock cost) in the base case scenario were taken from NREL's design report (Aden and Ruth, 2001). As can be seen in table 7, these assumptions are modified from the base case to reflect current realistic values.

The values of some of the parameters selected for sensitivity analysis (Table 7) are correlated and this will impact the likelihood of model outcomes. There may be other correlated parameters in the model that are not recognized in this analysis. Parameter correlations can affect the sensitivities of model outputs to parameter values significantly; however it is difficult to identify and quantify all correlations. The Crystal Ball program allows parameter values to be correlated either with a positive or negative correlation coefficient. The correlations assumed in this analysis are presented in Table 8. In sensitivity analysis 10,000 iterations were run using the probability distributions shown in Table 7.

Table 7. Input parameters of sensitivity analysis

Parameter	Distribution Function	Most Probable	Standard Deviation	Minimum	Maximum
Ammonia Consumption By Reactions	Triangular	1.5% of total ammonia	-----	1% of total ammonia	2% of total ammonia
Ammonium Hydroxide to Solids Ratio	Triangular	5 L/kg	-----	3 L/kg	10 L/kg
Wash Water to Solids Ratio	Triangular	50 L/kg	-----	25 L/kg	75 L/kg
Soaking Time	Triangular	5 days	-----	2 days	10 days
Cellulose to Ethanol Conversion	Triangular	85%	-----	70%	96%
Xylose to Ethanol Conversion	Triangular	70%	-----	40%	90%
Switchgrass Cost	Triangular	$50/dry US ton	-----	$25/dry US ton	$100/dry US ton
Enzyme Price	Triangular	$5.52/lb	-----	$3.86/lb	$11.04/lb
Pretreatment Reactor Price	Lognormal	$165,000/unit	$33,160	$0	Infinity

Table 8. Correlations between assumptions

	Ammonium Hydroxide to Solids Ratio	**Wash Water to Solids Ratio**	**Soaking Time**	**Cellulose to Ethanol Conversion**	**Xylose to Ethanol Conversion**
Ammonium Hydroxide to Solids Ratio		+	NC	+	+
Wash Water to Solids Ratio			NC	NC	NC
Soaking Time				+	+
Cellulose to Ethanol Conversion					NC
Xylose to Ethanol Conversion					

+, shows positive correlation between two assumptions (correlation coefficient assumed to be 0.85 for all of them)
NC: no correlation

Results and Discussion

Aqueous ammonia soaking is an attractive pretreatment method for ethanol production from lignocellulosic biomass, since the process operates at atmospheric pressure and ambient temperatures. However, this study shows that aqueous ammonia soaked switchgrass fermentation is a capital intensive process. The total project investment (TPI) for the base case scenario was determined as $277.5 million and breakdown of the TPI is presented in Figure 4.

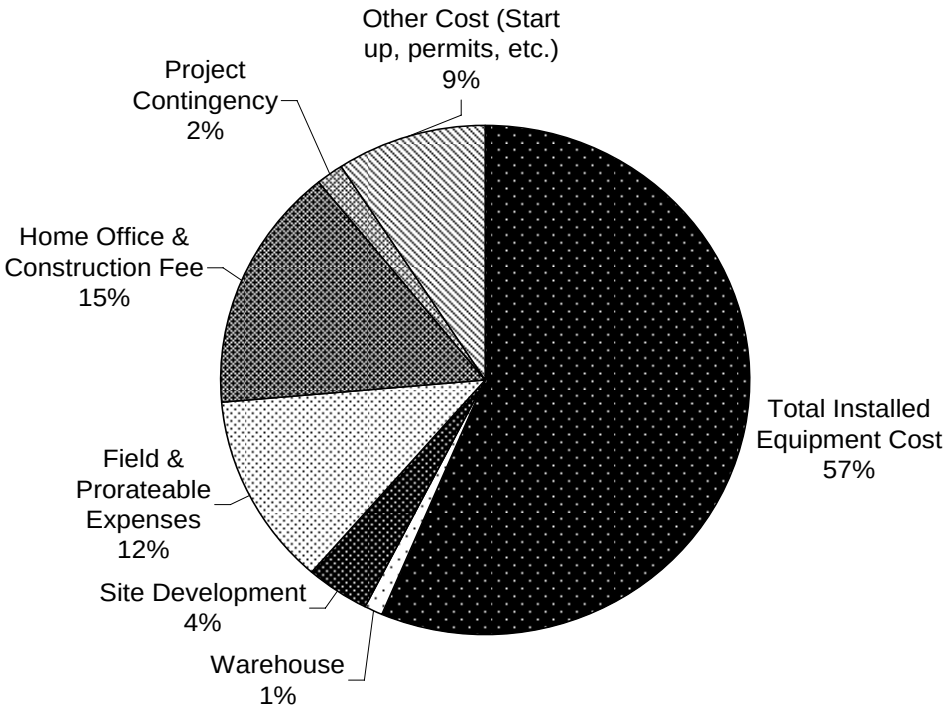

Figure 4. Breakdown of total project investment

Total installed equipment cost was approximately $157 million (2007 dollars) in the base case scenario. The other costs (Table 4) are computed from the total installed equipment cost; therefore, it is important to examine the total installed equipment cost in detail.

The breakdown of total equipment cost is shown in Figure 5. As can be seen, the most expensive section is the combustion area. This section is integrated to generate electricity and steam and to reduce the dependence of the plant on fossil fuels. However, including such a complex system in this process not only increases the capital cost but will also raise the cost of installation and operation. The second most expensive area is pretreatment. Even though unit cost of a soaking vessel is low, the large number of required vessels increases the total pretreatment capital cost. We find that soaking vessel and pneumapress filter costs account

for 73% of the pretreatment area cost. Overall the pretreatment area including the two ammonia recovery systems accounts for one-third of the total equipment cost. Unexpectedly, the ammonia recovery systems do not represent a large fraction of the capital cost. The steam and power generation system accounts for the other one-third of the total equipment cost. These percentages show a distribution of capital costs similar to a plant based on dilute acid pretreatment (Eggeman and Elander, 2005). However, total project investment in our base case is almost $40 million higher than NREL's dilute acid process, which is directly reflected in the minimum ethanol selling price.

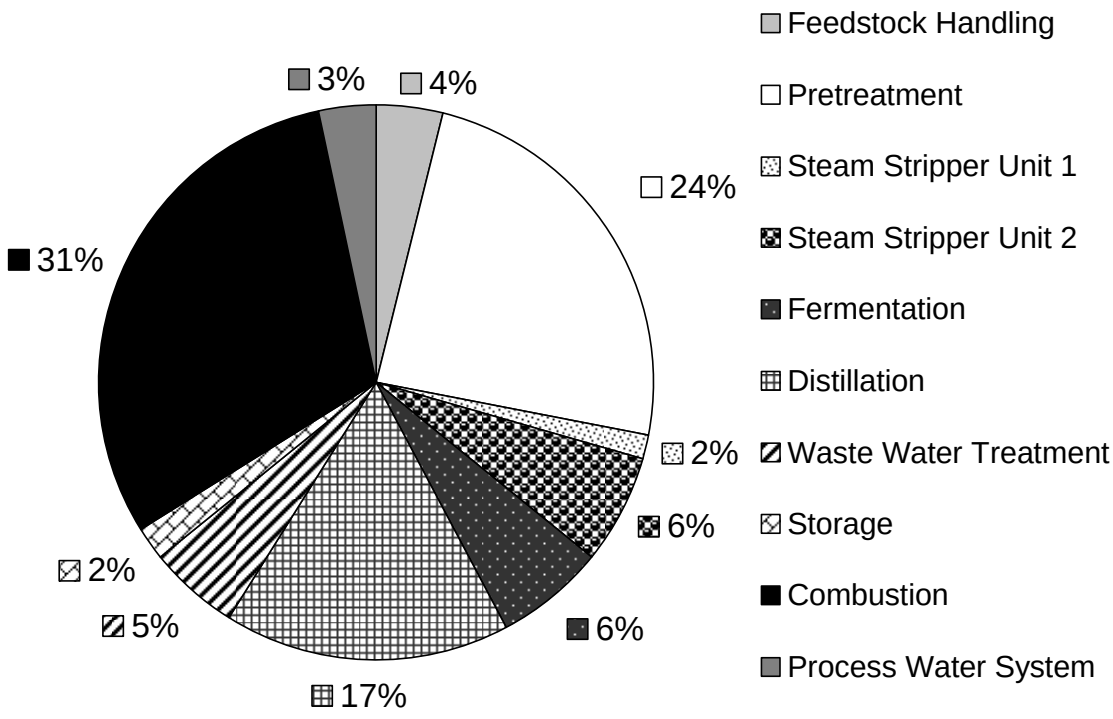

Figure 5. Cost contribution of each area to total equipment cost

In the base case scenario it is assumed that with the more active enzymes that will be available in the future, better conversion rates will be achieved (Aden and Ruth, 2001). The theoretical ethanol yields of glucose and xylose are taken to be 95 and 85%, respectively. Total annual ethanol production is determined to be 50.5 MMgal/y. Even with these optimistic assumptions, the capital investment requirement is found to be $5.50/gal ethanol produced. In comparison, biorefineries based on other pretreatment methods have a capital investment requirement ranging from $2.82/gal ethanol to$5.14/gal ethanol produced (the results are adjusted to 2007 dollars) (Eggeman and Elander, 2005). The aqueous ammonia soaking method is close to the upper end of this range. Compared to corn-dry mill ethanol technology that requires capital investment of $1.12-1.69/gal ethanol (adjusted to 2007 dollars) (BBI International, 2003), cellulosic biorefineries require very large capital investment.

Eggeman and Elander (2005) suggest that an ideal cellulosic ethanol process should have a minimum ethanol selling price (MESP) of $1.12/gal (adjusted to 2007 dollars). In this ideal case, pretreatment related capital and operating costs are assumed to be zero and sugar yields (glucose and xylose) after enzymatic hydrolysis are assumed to be 100%. In our base case scenario, MESP is determined as $ 2.99/gal which is about $1.90/gal more than the ideal case. As mentioned above, the base case scenario included optimistic assumptions (conversion yields and feedstock cost in Table 2) related to future conditions and therefore the MESP of the base case scenario is not the most likely near term outcome. When more likely parameter assumptions (the most probable numbers in Table 7) are integrated into the model, MESP increases to $3.90/gal ethanol. This increase shows how sensitive the MESP is

to conversion yields and feedstock cost which are the most significantly modified parameters.

The assumptions in Table 7 are used to generate probability distributions for MESP and total equipment cost. As mentioned, feedstock cost, cellulose and xylose conversion yields were adjusted to more probable values to determine the likely near future outcome. Figure 6 is the histogram of the minimum ethanol selling price and shows that the expected value of MESP is $4.45/gal. This reflects a more likely outcome than the base case, accounting for updated expectations of parameters such as feedstock price and conversion efficiency. The explanation of the shift in expected value of MESP from $3.90/gal to $4.45/gal is the skewness of the distributions assumed in Table 7 reflecting parameter uncertainty. Similarly, Figure 7 shows that the expected cost of the installed equipment is approximately $169 million, which is almost $10 million more than the base case scenario.

Sensitivity analyses are performed on the parameters shown in Table 7. Figure 8 shows the contribution of the variation in each sensitivity parameter to the variation in MESP. As can be seen, MESP is most sensitive to feedstock and enzyme costs which accounted for 63 and 10% of the variation in MESP, respectively. This shows that of the parameters examined, feedstock cost will play the most important role in determining the ethanol selling price. It also shows the importance of reduction in enzyme consumption. In fact, if the enzyme loading is decreased by half in the base case scenario the MESP reduces 24 cents/gal.

Ethanol yields from glucose and xylose, soaking time and ammonium hydroxide to solids ratio have similar effects on MESP. It is also important to note that these parameters

affect the MESP negatively, such that when there is an increase in cellulose conversion, ethanol price decreases.

Soaking time is the most influential parameter on total equipment cost (Figure 9). This was an anticipated result, since the soaking time greatly influences the pretreatment area cost. Ethanol yields also have an important impact on equipment cost since they directly affect the fermentation area cost. Ammonium hydroxide to switchgrass (solids) ratio and water consumed during pretreatment washing step accounted for 15 and 11% of the variation in MESP, respectively. These two parameters have a large influence on the pretreatment and ammonia recovery unit costs.

It is also important to note that the correlations between the selected parameters greatly affect the sensitivity results. One of the important correlations that has not been modeled is the correspondence between the amount of enzyme consumed and the intensity of the pretreatment. As we have previously reported (Isci et al., 2008a), when ammonium hydroxide to solids ratio and the soaking time increased, the amount of enzyme required decreases. This correlation could significantly influence the ethanol selling price, since enzyme cost is 34% of the direct operating cost.

The sensitivity results showed that even though the proposed biorefinery system is capital intensive under the current assumptions; the economics of the process can be improved with improved performance in targeted sections. Reduction in soaking time and enzyme consumption, better conversion yields, and reduced feedstock cost are the key improvements that would reduce the ethanol selling price and capital cost. It is also worth noting that increasing the maximum allowable solids concentration not only in pretreatment section but also in fermentors will save a significant amount of money. In this study, it was

assumed that the fermentors will contain only 10% solids limited by mixing requirements in the fermentor. Low solids concentrations result in low end product concentrations which increase distillation costs. Improvements that allow increased solids concentrations in the fermentor will significantly improve process economics.

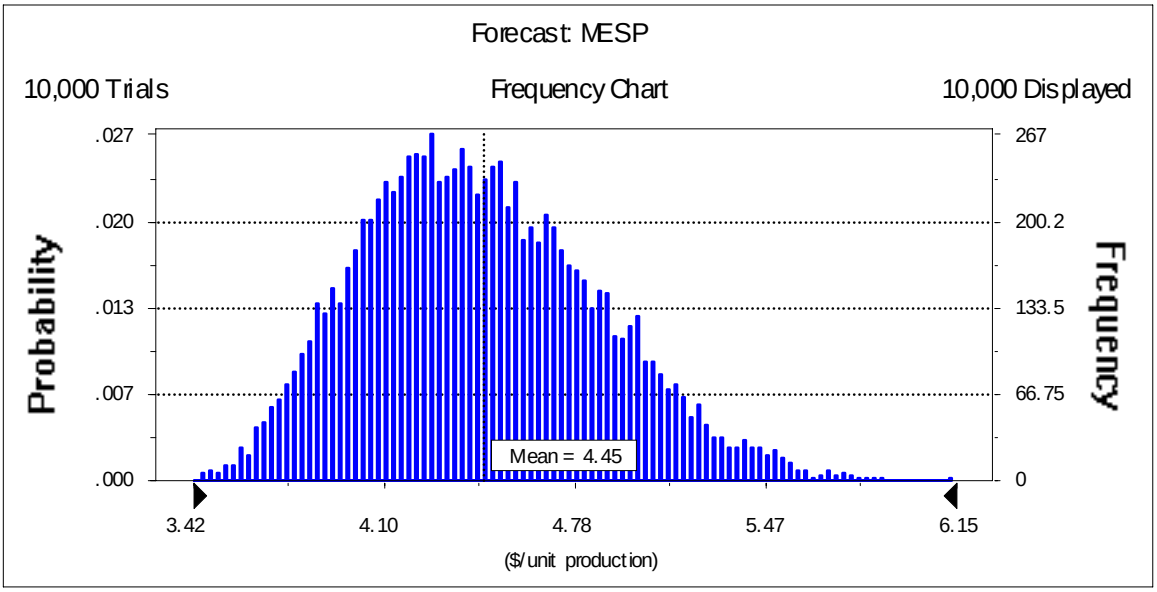

Figure 6. Histogram of minimum ethanol selling price

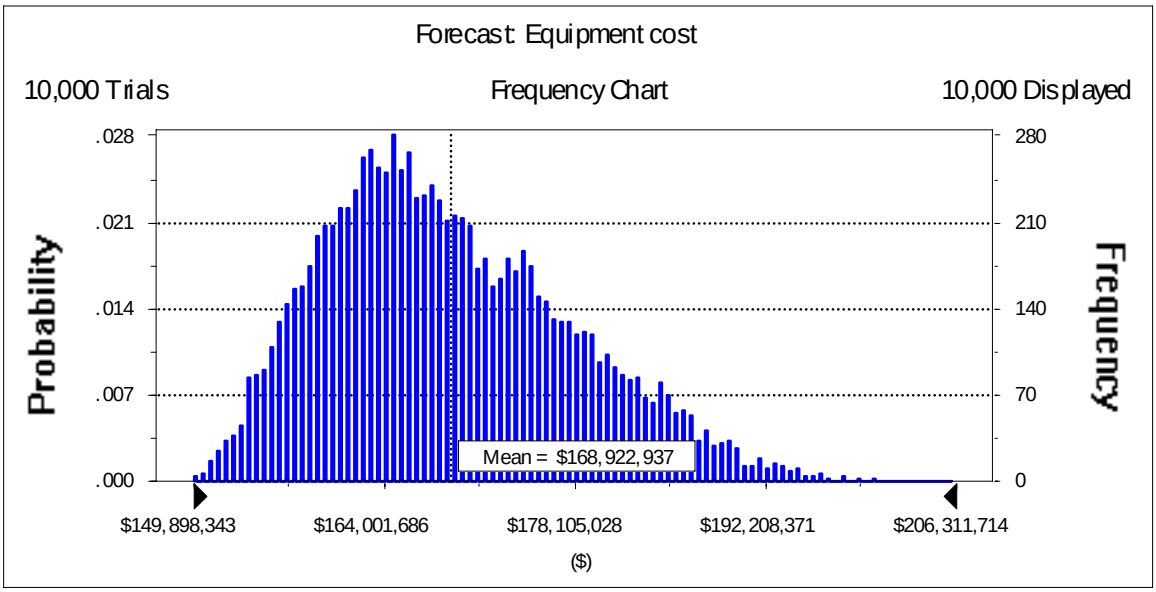

Figure 7. Histogram of total installed equipment cost

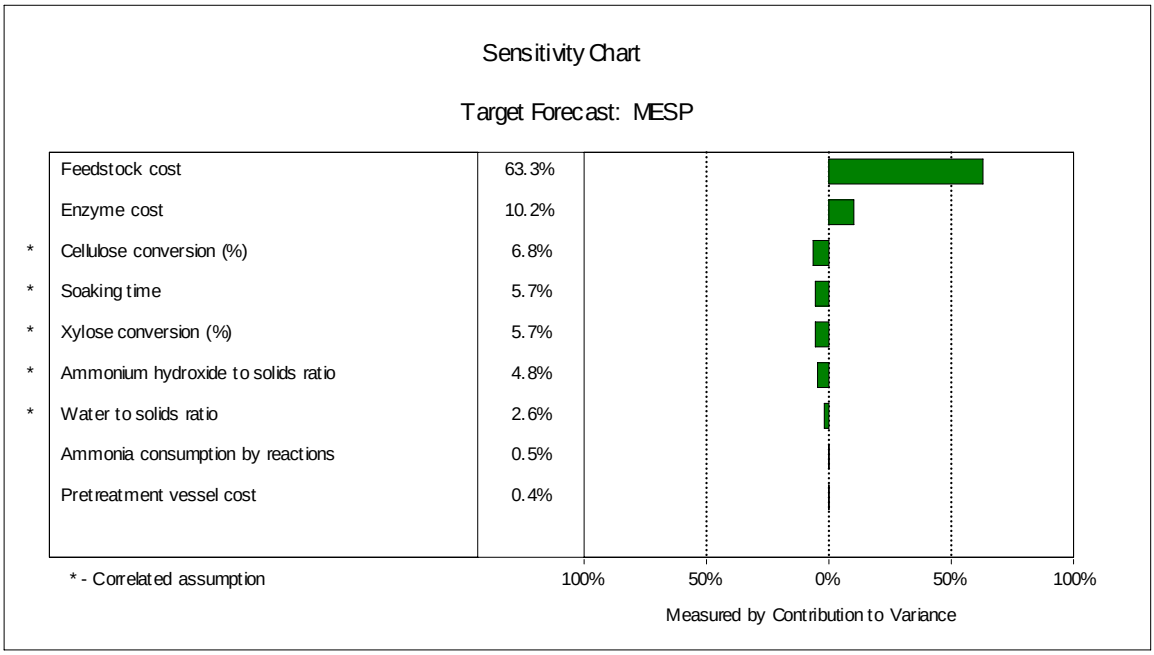

Figure 8. Sensitivity chart of minimum ethanol selling price

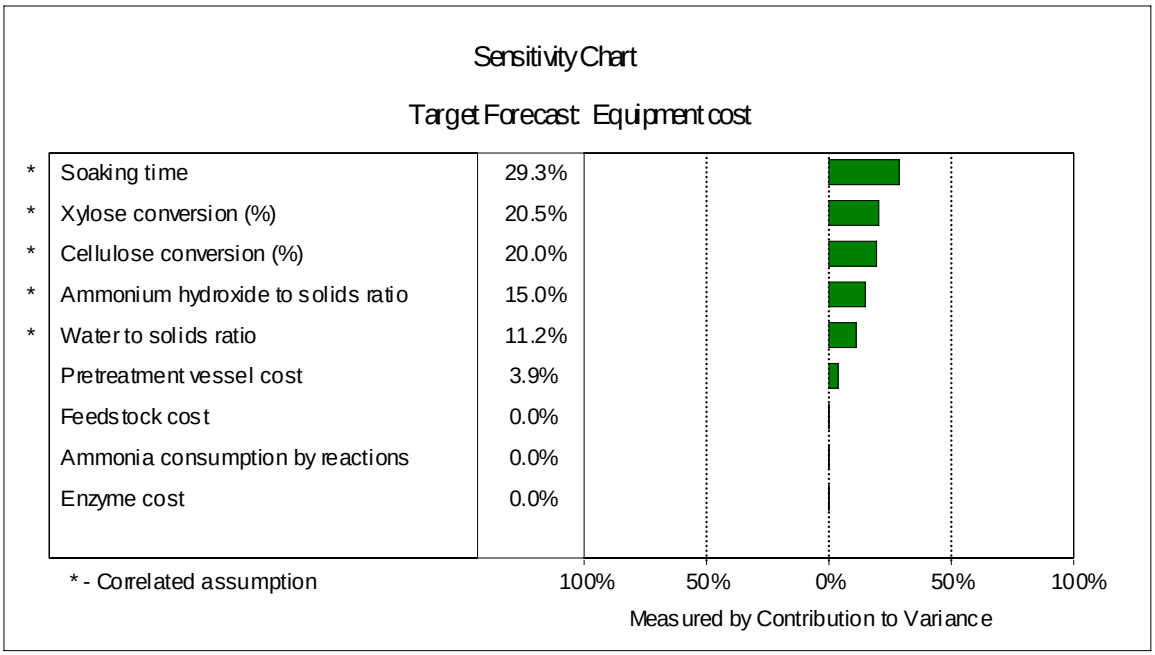

Figure 9. Sensitivity chart of total installed equipment cost

Conclusion

The techno-economic feasibility of ethanol production through aqueous-ammonia soaking and simultaneous saccharification and co-fermentation process was analyzed. The results show that ammonia soaking is a capital intensive process compared to NREL's dilute acid treatment-based biorefinery and is much more capital intensive than corn dry mill ethanol technology. Pretreatment and combustion are the most expensive areas in the overall process. The base case scenario which is based on an optimistic view of future technology development has an ethanol selling price of $ 2.99/gal. However, less optimistic assumptions show that the ethanol selling price may be as high as $4.45/gal. These results suggest that ammonia soaking pretreatment is not a cost competitive pretreatment method at commercial scale. However, sensitivity analysis revealed that performance may be better if improvements

allow shorter soaking time, increased solids to liquid ratio, lower enzyme loading, and better conversion yields.

Acknowledgements

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CHAPTER 5. A RAPID SIMULTANEOUS SACCHARIFICATION AND FERMENTATION TECHNIQUE TO DETERMINE ETHANOL YIELDS

A paper accepted by the *BioEnergy Research* Journal

Asli Isci, Patrick T. Murphy, Robert P. Anex, and Kenneth J. Moore

Abstract

We have developed a relatively simple simultaneous saccharification and fermentation (SSF) technique to determine the ethanol production potential for large sets of biomass samples. The technique is based on soaking approximately 0.5 grams of a biomass sample in aqueous ammonia at room temperature and at atmospheric pressure for 24 hours, then fermenting with *Saccharomyces cerevisiae* D_5A for 24 hours using Spezyme CP, for enzymatic hydrolysis of structural polysaccharides. We have tested the technique on a set of corn stover samples representing much of the genetic variability in the commercial corn hybrid population. The samples were weighed into modified Ankom filter bags (F57) before soaking to avoid biomass loss during the process. Fermentation samples were analyzed for ethanol after 24h by HPLC. Theoretical ethanol yields of the samples ranged between 44.9 and 73%. We observed that theoretical ethanol yields were highly correlated ($r^2 = 0.90$) with acid detergent lignin concentration while a low correlation was observed between cellulose concentration and ethanol yield. Near infrared spectra of corn stover samples were also examined. The coefficient of determination (r^2) from regression of predicted versus measured percent theoretical ethanol yield was 0.96. This result suggests that using NIRS is a

promising method for predicting ethanol yield, but larger calibration sets are necessary for obtaining improved accuracy for larger sample populations. We conclude that the developed SSF technique could be applied to large numbers of biomass samples to rapidly estimate ethanol yields and to compare different biomass samples in terms of theoretical ethanol yields.

Introduction

Simultaneous saccharification and fermentation (SSF) is currently the most common fermentation method used for producing ethanol from lignocellulosic feedstock. It integrates an enzymatic hydrolysis of cellulose into glucose and fermentation of glucose into ethanol within the same vessel. The method reduces the likelihood of end product inhibition by glucose and bacterial contamination. The current standard SSF method (NREL, LAP 08, 1996) to determine ethanol yield is time-consuming and not suitable for large sets of samples. Large sample sets may be required in industry and for agronomic plant breeding research when comparing and checking feedstock quality in terms of ethanol production.

Two fermentation assays are reported in the literature for predicting the fermentability of cellulosic biomass to ethanol. Weimer et al. (2005) proposed using an *in vitro* ruminal (IVR) digestibility assay as an indirect predictor of ethanol yields. They compared three different forage samples (eastern gamagrass, big bluestem and switchgrass) by subjecting both to SSF and IVR fermentations and determined the correlation of gas accumulations in both fermentations. Even though the IVR method offers the advantage of a non-aseptic operation and high sensitivity due to more gas generation than the SSF system, the correlations appears to be dependent on sample type. For example, lower correlations were observed when they used switchgrass as a feedstock. The authors claimed SSF of

switchgrass possibly contained inhibitory compounds which resulted in unfermented sugar accumulation, thus the lower correlations (r^2=0.2). Hoskinson et al. (2007) have also reported an SSF technique, which is based on the method described by Weimer et al. (2005) to estimate the ethanol potential of corn stover samples with different harvesting scenarios. Their samples were subjected to dilute acid treatment prior to fermentation. In this technique, the fermentations were carried out in air tight serum vials. This can cause solubilization of the carbon dioxide generated during fermentation which can decrease the pH of the solution and inhibit yeast fermentation. Both techniques require about one gram of pretreated material which in some cases may not be available.

The main objective of our study was to develop a rapid and easily adaptable SSF technique which offers the advantage of running a large number of samples at the same time. We also aimed to predict the ethanol yields of samples with compositional (cellulose, hemicellulose, lignin) data and NIRS calibrations.

Materials and Methods

Corn stover samples were obtained from a set of different hybrids, populations, and population crosses genetically diverse for compositional properties, which were part of a larger collaborative field experiment between the University of Wisconsin-Madison and Iowa State University. Samples obtained from the University of Wisconsin-Madison were grown at Madison and Arlington, WI in 2005 and 2006. Samples obtained from Iowa State University were grown at Ames and Ankeny, IA, in 2005 and at Ames and Belmond, IA in 2006. All corn entries were grown in triplicate at each location and in each year. The Wisconsin samples were comprised of 11 hybrids and one breeding population and the Iowa samples were comprised of 10 hybrids, one breeding population, and one population cross.

After the manual grain harvest of corn plots occurred, sub-samples of the corn stover were taken and dried at 38°C for four days. Samples were ground with a Wiley mill (Thomas Scientific, Inc., Swedesboro, NJ) to pass a 1-mm screen. Composite samples of the dried, ground material were made by combining 2 g of each of the six samples from each location (Wisconsin and Iowa) within each year (2005 and 2006).

Composition of the corn stover samples were determined using the ANKOM method (ANKOM Technol. Corp., Fairport, NY) as described by Vogel et al (Vogel et al., 1999). Table 1 presents the samples and their compositions. Hemicellulose concentration was calculated as the difference between neutral detergent fiber and acid detergent fiber and cellulose concentration was calculated as the difference between acid detergent fiber and acid detergent lignin. Dry matter of the samples was determined by drying 0.5-g subsamples at 103°C for 72 h in a forced-air oven to correct of the moisture contained in the samples.

Cellulase enzyme (Spezyme CP, lot no: 301-05021-011) was provided by Genencor International (Palo Alto, CA) and had an activity of 60 filter paper units (FPU)/mL, measured using standard procedures (NREL, LAP 06, 1996). The yeast (*Sacccharomyces cerevisiae* D_5A) was supplied by National Renewable Energy Laboratory. The yeast culture was grown in 1% (w/v) yeast extract, 2% (w/v) peptone and 5% (w/v) dextrose for 24 hours at 35ºC rotating at 170 rpm and freeze dried in 2-mL serum vials with 20% (w/v) nonfat dry milk. The vials were preserved at 4°C until the day of fermentation. The number of yeast cells per vial was determined to be 2.4 X 10^9 cells/mL according to hemocytometer counts.

Table 1. Compositions and theoretical ethanol yields of corn stover samples (dry basis)

Entry	Year	%NDF[a]	%ADF[b]	%Cellulose[c]	%Hemicellulose[d]	%ADL[e]	% TEY[f]
W64A X A619	2005	67.95±0.13	36.44±0.02	33.54±0.03	31.51±0.15	2.05±0.01	56.0±0.95
W64A X A619 bm3	2005	60.22±0.74	31.09±0.48	29.75±0.41	29.13±0.26	0.98±0.04	73.0±3.33
WQS C3 Syn2	2005	64.85±0.26	33.87±0.25	31.71±0.11	30.98±0.06	1.67±0.05	65.5±1.42
WQS C3 X HC33	2005	69.64±0.62	36.88±0.01	34.66±0.09	32.77±0.63	1.83±0.03	58.4±1.76
W601S X LH244	2005	71.39±0.21	40.02±0.12	37.39±0.17	31.38±0.08	1.94±0.12	58.7±0.77
W602S X LH198	2005	72.49±0.00	39.44±0.12	36.95±0.04	33.05±0.12	1.96±0.10	56.5±3.42
W603S X LH227	2005	75.87±0.71	41.36±0.54	38.56±0.51	34.50±0.17	2.40±0.07	53.4±0.84
W604S X TR7245	2005	71.45±0.1	39.09±0.15	36.54±0.13	32.37±0.05	2.07±0.04	55.1±2.72
W605S X HC33	2005	74.06±0.65	40.20±0.50	37.23±0.38	33.86±0.15	2.41±0.01	55.8±1.70
LH227 X LH279	2005	75.73±0.25	41.36±0.37	38.35±0.33	34.37±0.13	2.52±0.07	52.7±0.23
DK5143	2005	73.33±0.25	39.99±0.29	37.39±0.36	33.34±0.54	2.05±0.10	54.5±0.54
Mycogen F697 bm3	2005	64.46±2.00	35.08±0.74	33.46±0.56	29.38±1.26	1.23±0.04	68.9±1.55
W64A X A619	2006	57.40±1.42	31.15±0.93	28.60±0.84	26.25±0.48	1.95±0.11	58.7±2.89
W64A X A619 bm3	2006	61.13±0.46	32.47±0.48	30.93±0.52	28.66±0.94	1.10±0.07	72.4±1.54
WQS C3 Syn2	2006	60.51±0.18	33.12±0.79	30.80±0.55	27.39±0.62	1.80±0.09	63.7±1.45
WQS C3 X HC33	2006	63.63±0.43	34.67±0.58	32.20±0.53	28.96±1.00	2.06±0.06	53.4±2.08
W601S X LH244	2006	69.62±0.79	39.62±0.48	36.67±0.29	30.00±0.30	2.32±0.11	53.9±2.01
W602S X LH198	2006	69.06±0.66	39.26±0.46	36.40±0.45	29.81±0.20	2.40±0.06	53.9±1.54
W603S X LH227	2006	74.68±0.09	42.20±1.40	38.78±1.09	32.48±1.49	2.86±0.26	44.9±2.24
W604S X TR7245	2006	65.22±0.93	36.74±0.28	33.83±0.42	28.48±1.21	2.36±0.11	56.9±1.72
W605S X HC33	2006	62.96±0.56	34.58±0.23	31.77±0.21	28.38±0.79	2.31±0.09	53.6±2.37
LH227 X LH279	2006	71.42±0.1	40.88±0.73	37.44±0.53	30.54±0.63	3.12±0.25	48.9±0.66
DK5143	2006	71.75±1.57	41.03±0.33	38.08±0.32	30.72±1.24	2.47±0.02	50.4±1.41
Mycogen F697 (bm3)	2006	61.21±0.76	34.14±0.10	32.46±0.01	27.07±0.66	1.23±0.03	68.0±3.83

[a]NDF: neutral detergent fiber, [b]ADF : acid detergent fiber, [c] Cellulose: ADF-ADL(adjusted for ash), [d] Hemicellulose: NDF-ADF
[e]ADL: acid detergent lignin, [f]TEY: theoretical ethanol yield

Pretreatment

Approximately 1.6 mm from the top of each F57 Ankom filter bag (made from polyester) was cut to make the bag small enough to fit in a 25-mL fermentation flask. Each filter bag received 0.5 g of corn stover sample, and was sealed at the top using a heat sealer. Filter bags were placed in 1-L plastic bottles (every bottle contained 6 filter bags) and soaked in 100 mL of reagent grade 30% aqueous ammonium hydroxide for 24 hours. An average of 30% dry matter loss was observed after 24 hours of aqueous ammonia soaking, indicating sufficient pretreatment based on previous lab scale experiments (Isci et al., 2008).

The bags were washed with deionized water until ammonia odor was eliminated (Isci et al., 2008). To decrease washing time, the bags were also squeezed by hand and washed several times under running deionized water. Each corn stover sample were pretreated in triplicates.

Fermentation

Simultaneous saccharification and fermentation was performed aseptically following established procedures (NREL, LAP 08, 1996) with modifications. Washed filter bags with corn stover samples inside them were loaded into 25 mL Bellco DeLong flasks (Bellco Glass Inc, Vineland, NJ) along with 1% (w/v) yeast extract, 2% (w/v) peptone and 0.05 M citrate buffer (pH 4.8). The total working volume was 10 mL.

The filter bags were cut before placement into flasks as shown in Figure 1 to accelerate mass transfer during fermentation. Uncut bags were observed to expand during autoclaving and trapping all the liquid inside and preventing enzyme and yeast contact with

the substrate. The cut bags were submerged totally in the fermentation media with a help of spatula before autoclaving.

All flasks were capped with stainless steel closures (Bellco Glass, Inc., Vineland, NJ) and covered with aluminum foil. The flasks were allowed to cool to room temperature following sterilization at 121ºC for 20 min by autoclaving. Afterwards, sterile water was added aseptically to correct for the amount vaporized during autoclaving. Finally, 0.5 mL enzyme and 0.2 mL of rehydrated freeze dried yeast were transferred into each flask. Each flask contained approximately 2 X 10^7 cells and was incubated at 35ºC for 24 hours rotating at 170 rpm.

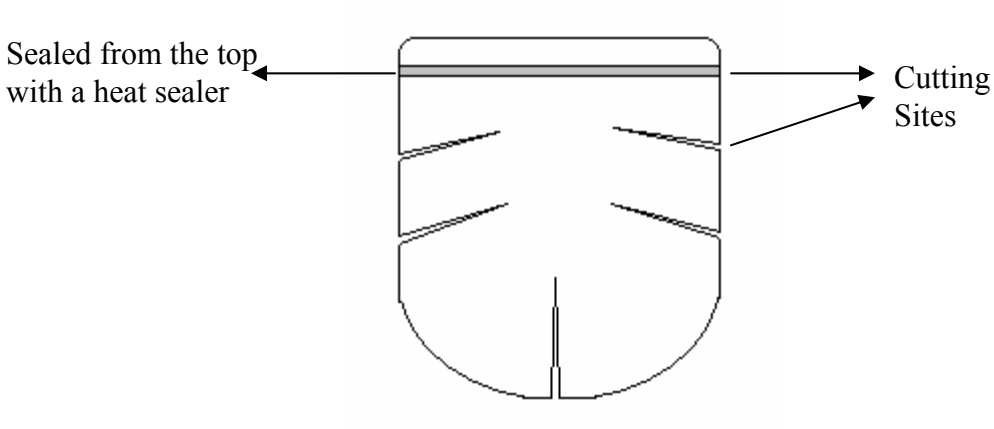

Figure 1. Schematic representation of a cut filter bag

The 24-h fermentation period was selected based on preliminary experiments done using a corn stover sample (W64A X A619 bm3). A total working volume of 10 mL was too small to allow for sampling over time to determine ethanol concentration without affecting the fermentation experiment. Therefore, 8 filter bags were prepared for sampling in parallel, using the same corn stover sample, and fermented to determine the change of ethanol

concentration over time. Two flasks were taken out of the incubator at 7, 24, 48 and 72 h and analyzed for ethanol concentrations. Ethanol concentration peaked at 24 h (Figure 2), then started to decrease. For this reason, we have selected 24 h as the completion point for fermentation. The decrease in ethanol concentration may be due to consumption of ethanol by yeast after depletion of glucose (Piskur et al., 2006) or due to evaporation of ethanol.

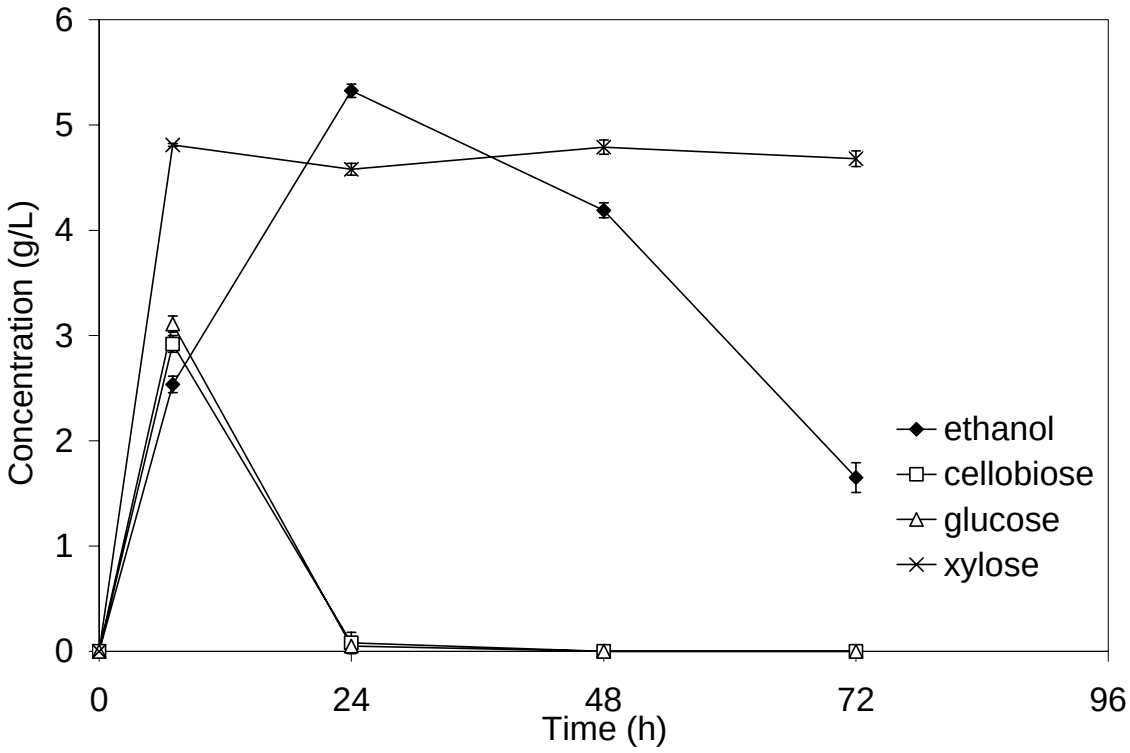

Figure 2. Change of ethanol concentrations in flasks over time

As a control, ammonia soaked empty filter bags were fermented using the same procedure along with the corn stover samples and no ethanol production was observed.

Analysis

Samples were analyzed for sugars (cellobiose, glucose, and xylose) and ethanol by HPLC (Varian ProStar 210) with a refractive index detector (Varian 355 RI). A MetaCarb 87P column (water as a mobile phase, flow rate of 0.4 ml/min, column temperature of 80^{o}C, and injection volume of 20 µl) was used for sugar analysis, while a Bio-Rad 87H column (0.01 N sulfuric acid as a mobile phase, flow rate 0.6 ml/min, column temperature 65^{o}C, and injection volume of 20 µl) was used to determine ethanol concentration.

Theoretical ethanol yields were calculated based on a theoretical ethanol yield of 51 g per 100 g of glucose for yeast (Isci et al, 2008; Kim and Lee, 2005).

$$Theoretical\ ethanol\ yield\ (\%) = \frac{Ethanol\ produced\ (g)\ in\ reactor}{Initial\ sugar\ (g)\ in\ reactor \times 0.511} \times 100$$

Fermentations were preformed in triplicate, in three batches. A randomized complete block design was used to perform the experiments in which the batches were considered to be the blocking factor. Linear correlations between compositional data and ethanol yields as well as multiple regression equations were determined by JMP (SAS Institute, Cary, NC) using a stepwise multiple regression approach.

NIRS

The samples were scanned in triplicate using a Foss NIRSystems 6500 spectrophotometer (NIRSystems, Silver Springs, MD). The NIR instrument records the mean spectrum of 3 scans of each sample over the wavelength region of 400–2500 nm at 2 nm intervals. Equations were developed using modified partial least squares (MPLS) using

reference chemistry for percent theoretical ethanol yield. Calibration equations were evaluated and validated using 6 sample subsets in a cross validation scheme. The performance of calibrations was assessed by evaluating the standard error of validation (SEV), coefficient of determination (R^2), and bias (Westerhaus et al., 2004).

Results and Discussion

Previously, it was reported that the cellulose concentration does not change with aqueous ammonia soaking at room temperature and atmospheric pressure (Isci et al., 2008; Kim and Lee, 2005). Based on this fact, theoretical ethanol yields can be calculated easily, since the weight of cellulose inside one filter bag is known before the process. The theoretical ethanol yields of different corn stover samples from Wisconsin can be seen in Table 1. The highest and lowest ethanol yields achieved were 73 and 45% respectively, belonging to variety W64A X A619 bm3 (year 2005) and W603S X LH227 (year 2006). These results were consistent with the bench scale (100 mL total working volume) SSF data (Isci et al., 2008) in which 72% theoretical ethanol yield was the maximum observed from switchgrass fermentation. Even though W64A X A619 bm3 (year 2005) had a lower cellulose concentration than W603S X LH227 (year 2006), the former generated more ethanol. This result can be attributable to the samples' lignin concentration. Figure 3 shows the relationship between lignin concentration of different corn stover samples and their percent theoretical ethanol yields. We observed that acid detergent lignin concentration of the samples were highly correlated (r^2=0.90) with ethanol yields. As the lignin concentration in the biomass decreased the ethanol generation capability of a corn stover sample increased.

This result was anticipated, since lignin has been known to create obstacles to ethanol fermentation by limiting the accessibility of hydrolytic enzymes to fermentable plant cell-

wall components in the fermentation vessel. On the other hand, we observed a lower correlation (r^2=0.50) between the cellulose concentration of stover samples and percent theoretical ethanol yields (Figure 4). The stover sample with the highest cellulose and lignin concentration (W603S X LH227, year 2006) generated the lowest ethanol yield. This suggests that lignin concentration of stover samples may be a more important feature than cellulose concentration when determining or estimating ethanol generating capabilities.

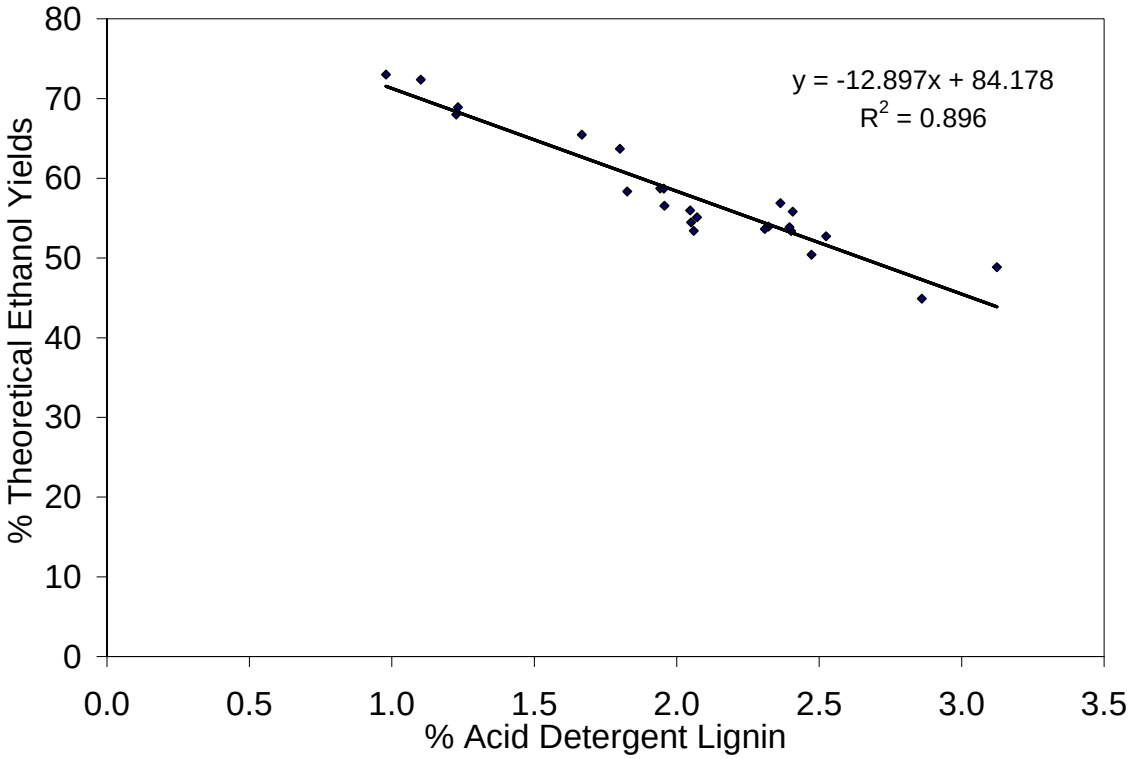

Figure 3. The correlation between acid detergent lignin concentration of corn stover samples and percent theoretical ethanol yields. (Theoretical ethanol yields are average of three replicates)

Acid detergent lignin (ADL) concentration can be determined easily using the ANKOM method described in the methods section. The high correlation between ADL

concentration and percent ethanol yield can be used to guide biofuel feedstock selection and/or plant breeding research.

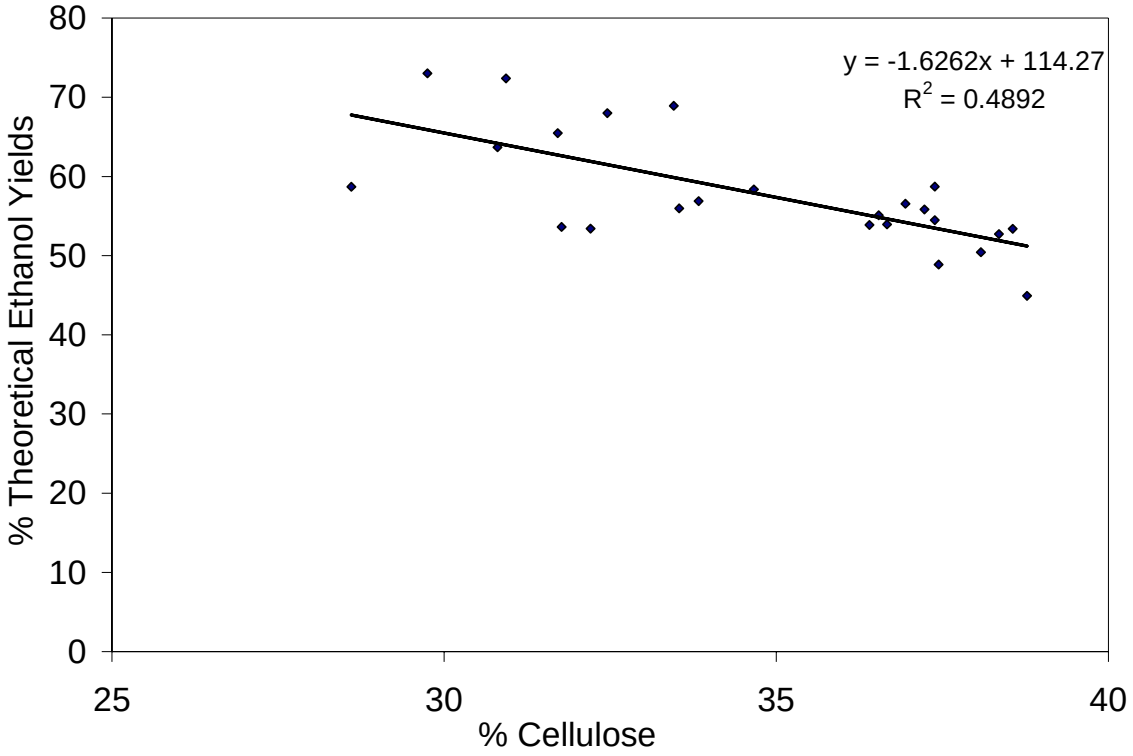

Figure 4. The correlation between cellulose concentration of corn stover samples and percent theoretical ethanol yields. (Theoretical ethanol yields are average of three replicates)

We observed a low correlation (r^2=0.21) between ethanol yield and percent hemicellulose (Figure 5). Because *Saccharomyces cerevisiae* is not capable of utilizing five-carbon sugars; the low correlation was an expected result. In addition to these correlations, multiple regression equations were developed and analyzed (Table 2). We determined that when cellulose and hemicellulose were included in the ethanol yield prediction model, the root mean square error (a measure of the differences between values predicted by a model

and the values actually observed) and r^2 did not improve significantly. Consequently, acid detergent lignin concentration can solely be used for estimation of ethanol yields for similar corn stover samples.

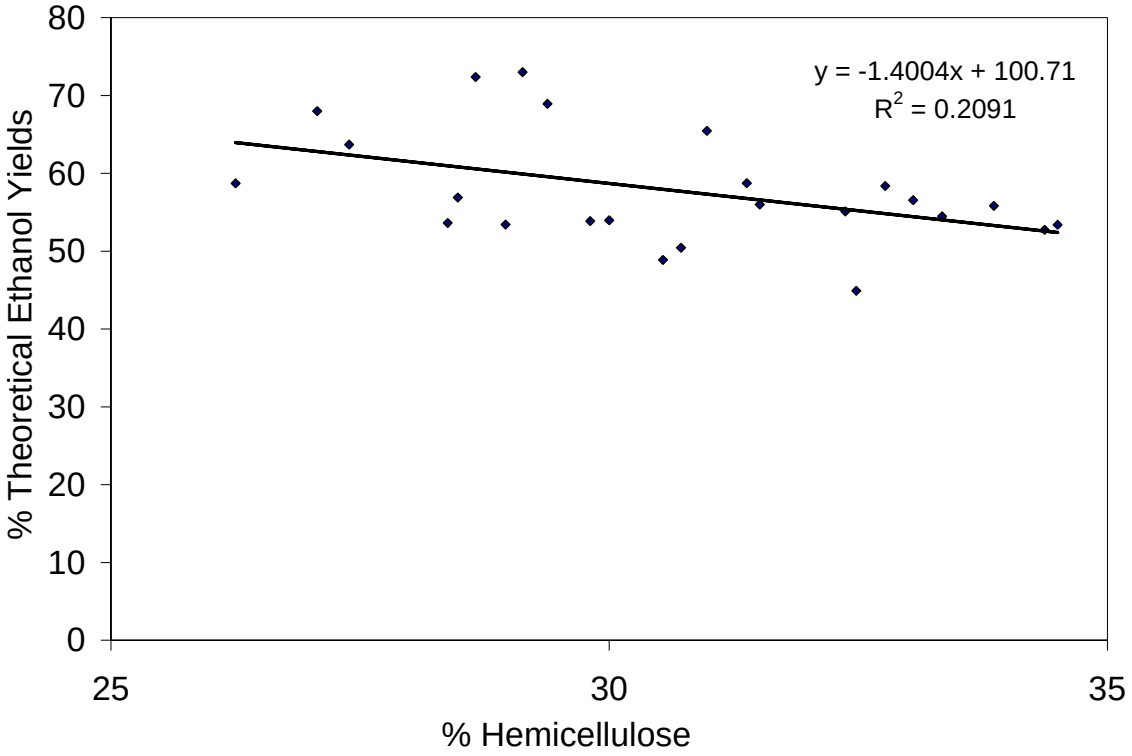

Figure 5. The correlation between hemicellulose concentration of corn stover samples and percent theoretical ethanol yields. (Theoretical ethanol yields are average of three replicates)

Table 2. Multiple regression results

MODEL number	MODEL	r^2	RMSE
1	% cellulose,%hemicellulose,% ADL	0.90	2.41
2	% cellulose,% ADL	0.90	2.36
3	%hemicellulose,% ADL	0.90	2.36
4	% cellulose,%hemicellulose	0.51	5.29
5	% ADL	0.90	2.38
6	% cellulose	0.49	5.30
7	%hemicellulose	0.21	6.59

Cellulose and hemicellulose concentrations were calculated from neutral detergent fiber (NDF) and acid detergent fiber (ADF) concentrations as described in materials and methods section (Cellulose=ADF-ADL, Hemicellulose=NDF-ADF). Since these are the primary measurement rather than calculations, the correlations between NDF, ADF and theoretical ethanol yields were of interest as well. Figure 6 shows that both NDF and ADF have low correlations (r2=0.47 for NDF and r2=0.60 for ADF). ADF had a slightly better correlation than NDF which may be explained by the removal of hemicellulose content on the ADF step. As stated above, our fermentative microorganism is not capable of fermenting pentose sugars. Therefore, when hemicellulose was removed during ADF step, there is a possibility of increasing the correlation.

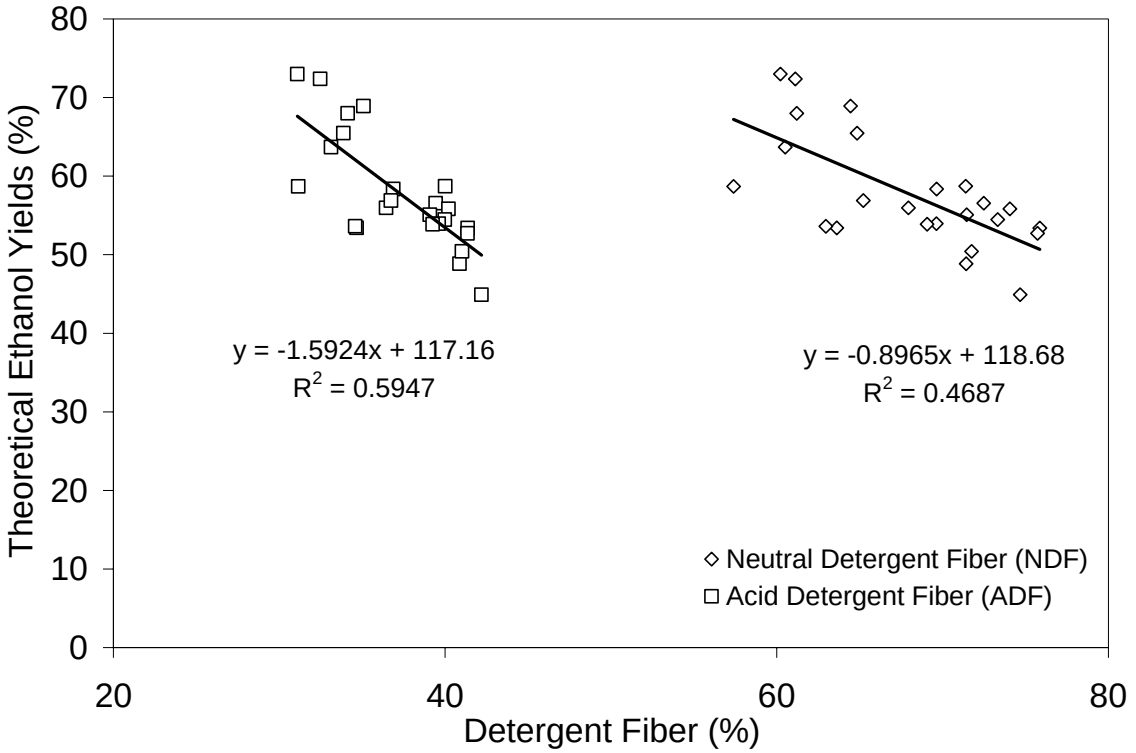

Figure 6. The correlation between NDF, ADF and percent theoretical ethanol yields (Theoretical ethanol yields are average of three replicates)

Table. 3 NIR Results

Constituent	% Theoretical Ethanol Yields
Math treatment	1,4,4,1
Mean	57.369
Range	44.90-73.00
Stdev	6.928
SEL	2.215
SEC	1.329
SECV	2.036
R^2	0.963
F value	15.12
1-VR	0.920

Stdev: Standard Deviation
SEL: Standard Error of Laboratory Results,
SEC: Standard Error of Calibration,
SECV: Standard Error Cross-Validation,
R^2: Coefficient of determination,
VR: The ratio of unexplained variance to total variance

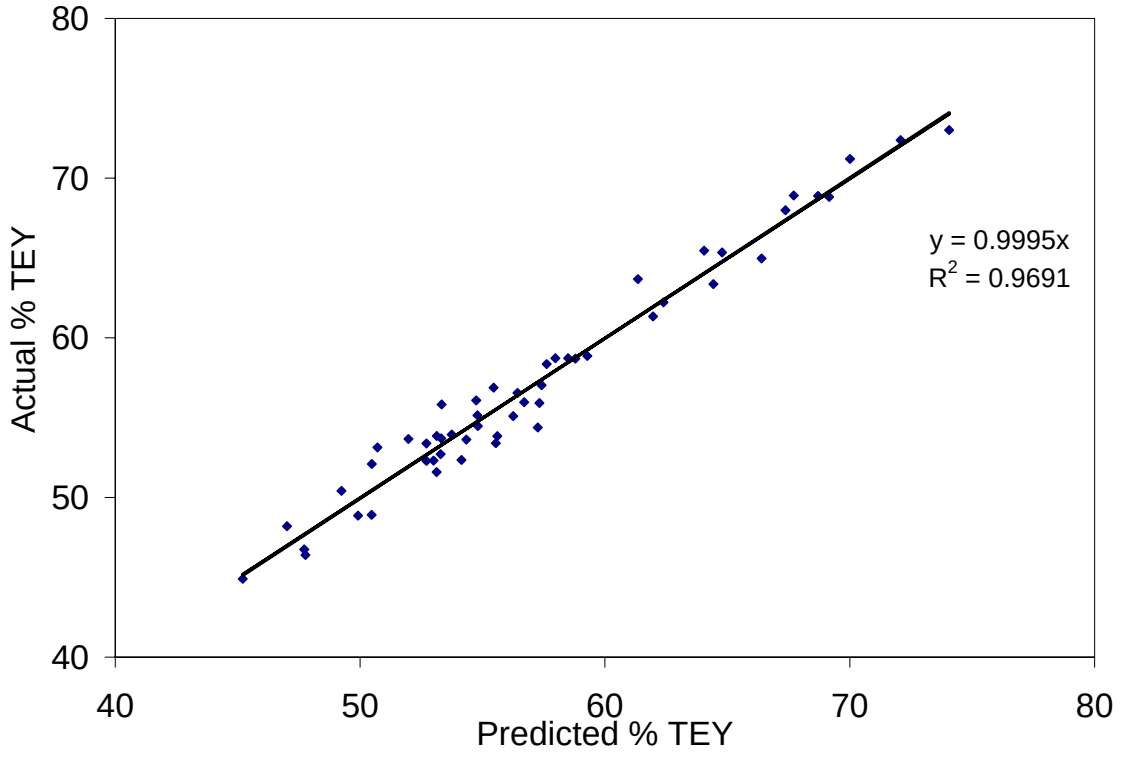

Figure 7. Correlation between actual ethanol yields obtained from fermentation experiments and predicted ethanol yields using NIRS calibration equations

Although laboratory analysis using spectrophotometry to estimate forage quality is common, little work has been done to estimate the ethanol production potential of biomass using near infrared spectroscopy. We determined that NIRS predictions have high correlation with actual percent theoretical ethanol yield (Table 3 and Figure 7). The results suggest that the method can be used to estimate percent theoretical ethanol yields of corn stover with high accuracy. However, more samples from different locations and years are needed to improve the calibration.

Conclusion

We have developed a rapid and relatively easy SSF technique to determine theoretical ethanol yields of a large number of corn stover samples. Theoretical ethanol yields between 45-73% were observed without optimizing our SSF technique. We observed a high correlation between percent acid detergent lignin and theoretical ethanol yields which can be used as a prediction method of ethanol yields for corn stover samples in further studies. NIRS also showed promising results with high coefficient of determination, and also could be used for ethanol yield estimation after addition of more stover samples into the calibration data set.

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CHAPTER 6. GENERAL CONCLUSION

General Discussion

Cellulosic ethanol promises to provide not only a renewable transportation fuel but also reduced dependence on foreign oil supplies. In this thesis, a cellulosic ethanol production method using an aqueous-ammonia (30%) soaking pretreatment and simultaneous saccharification and fermentation (SSF) is presented at several scales and in several contexts. Several important conclusions are drawn from this work. In Chapter 2, "*Aqueous Ammonia Soaking of Switchgrass Followed by Simultaneous Saccharification and Fermentation*", the effectiveness of ammonia soaking was examined in a laboratory setting. It was determined that soaking switchgrass in ammonium hydroxide alters the structure of the material and facilitates enzymatic hydrolysis and ethanol production. Ethanol yields as high as 73% were achieved. It was also observed that enzyme consumption can be reduced as the intensity of the pretreatment is increased. In Chapter 3, "*Pilot Scale Fermentation of Aqueous Ammonia Soaked Switchgrass*", previously developed bench-scale SSF experiments were scaled up to 50 and 350L. The results showed that materials handling of semi-solid feedstock slurries can be problematic at larger scales. Obtaining a homogeneous mixing in the fermentors and bacterial contamination during fermentation were the important challenges at pilot scale. The first two projects have showed us that aqueous ammonia soaking is an effective pretreatment process but scale-up may be difficult. However, a pretreatment method needs to be economically feasible as well technically feasible in order to be commercially viable. Therefore, in Chapter 4, "*Techno-economic Analysis of Aqueous Ammonia Soaked Switchgrass*", we have analyzed the economically viability of the process as part of a

commercial-scale integrated biorefinery. It was found that ethanol production using ammonia soaking of switchgrass is likely to be a highly capital intensive process. Long residence time during pretreatment increases pretreatment cost, the total fixed capital cost and, in turn, the ethanol selling price. Sensitivity analysis showed that enzyme and feedstock cost had the largest impact on mean ethanol selling price. Although, ammonia soaking may not be a cost competitive pretreatment method, it is still an attractive method at bench scale due to its simplicity. In Chapter 5, entitled "*A Rapid Simultaneous Saccharification and Fermentation Technique to Determine Ethanol Yields*", we have developed a relatively simple SSF technique to rapidly assess the ethanol production potential for large sets of biomass samples. This method was based on soaking feedstock in aqueous ammonia for 24 hours and then fermenting it for 24 hours. The technique was successfully demonstrated on a large set of corn stover samples. Through this investigation it was found that acid detergent lignin (ADL) concentration and theoretical ethanol yields were highly correlated which suggests that ADL can be used as a rapid prediction method for ethanol yields for corn stover. We have also shown that NIRS calibration curves can be developed that predict ethanol yields of lignocellulosic biomass such as corn stover. Such NIRS methods may be even more useful in screening biomass ethanol potential because the calibrations reflect more compositional information than the simple ADL measurement.

Future Work

As described in Chapter 4, soaking time is an important parameter that affects the process cost aqueous ammonia soaking. Therefore, it is extremely important to optimize pretreatment conditions further. Different soaking times and soaking ratios can be examined in the search for increased ethanol production. In addition, it is important to search for

improved methods of ammonia recovery that avoid washing the pretreated feedstock. This would not only reduce water consumption but also decrease the waste water treatment cost. One approach could be vacuum recovery of ammonia after pretreatment. However, the presence of inhibitory compounds generated during pretreatment might become an issue if the pretreated material is not washed. The trade offs need to be explored more fully before ammonia soaking is deemed to be feasible or infeasible.

One of the biggest problems encountered at pilot scale was bacterial contamination. It is not feasible to sterilize the feedstock at larger scales using standard methods. Therefore other methods need to be analyzed to reduce contamination. It is known that ammonia at high concentrations has a sterilization effect and if the pretreated material is protected from contacting non-sterile environments, the contamination levels can be reduced. One approach could be soaking the feedstock in a sterile reactor, and after vacuum recovery of ammonia, sterilized fermentation media could be added from the side of the reactor with sterile pipes and fermentation can be done in the same reactor. This approach could also decrease the capital cost as well, since both pretreatment and fermentation could be done in the same reactor.

There are many possibilities for improving the aqueous ammonia soaking pretreatment method. There are still many unknowns in the cellulosic ethanol production process and commercially viable processes may still be years away. However, cellulosic ethanol will continue to be a very appealing way of reducing foreign energy dependency, combating global warming, and reducing the environmental impacts of fuel production.

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Finally, I wish to thank to my family, Nursel & Osman Isci, for their continuous love and encouragement and for always believing in me. This thesis was simply impossible without them.

This thesis is dedicated to my family and to my beloved mother, Gulay Isci (1951-1993). She has been my inspiration and my light in every day of this journey.