CELLULOSE DIGESTIBILITY, ETHANOL YIELD, AND LIGNIN RECOVERY

FROM CORN STOVER FRACTIONATED BY A TWO-STAGE DILUTE-ACID

AND DILUTE-ALKALINE PROCESS

Except where reference is made to the work of others, the work described in this thesis is my own or was done in collaboration with my advisory committee. This thesis does not include proprietary or classified information.

_____

David B. Joiner

Certificate of Approval:

_____	_____
Ram B. Gupta Professor Chemical Engineering	Yoon Y. Lee, Chair Professor Chemical Engineering
_____	_____
Christopher B. Roberts Professor Chemical Engineering	Stephen L. McFarland Dean Graduate School

CELLULOSE DIGESTIBILITY, ETHANOL YIELD, AND LIGNIN RECOVERY FROM CORN STOVER FRACTIONATED BY A TWO-STAGE DILUTE-ACID AND DILUTE-ALKALINE PROCESS

David B. Joiner

A Thesis

Submitted to

the Graduate Faculty of

Auburn University

in Partial Fulfillment of the

Requirements for the

Degree of

Master of Science

Auburn, Alabama

December 16, 2005

CELLULOSE DIGESTIBILITY, ETHANOL YIELD, AND LIGNIN RECOVERY FROM CORN STOVER FRACTIONATED BY A TWO-STAGE DILUTE-ACID AND DILUTE-ALKALINE PROCESS

David B. Joiner

_____
Signature of Author

_____
Date of Graduation

THESIS ABSTRACT

CELLULOSE DIGESTIBILITY, ETHANOL YIELD, AND LIGNIN RECOVERY FROM CORN STOVER FRACTIONATED BY A TWO-STAGE DILUTE-ACID AND DILUTE-ALKALINE PROCESS

David B. Joiner

Master of Science, December 16, 2005
(B.S. Chem. Eng., Auburn University, 2003)

78 Typed Pages

Directed by Y. Y. Lee

Dilute-acid pretreated corn stover (PCS) from NREL has been delignified using dilute sodium hydroxide extraction to see how this treatment affects enzymatic hydrolysis and simultaneous saccharafication and fermentation (SSF) of the resulting cellulose-rich fibers. The process is intended to extract and recover the lignin from stover in a relatively pure form as a co-product of the bioethanol process, while also increasing ethanol yields.

The results from this treatment indicate that about 75% of the lignin can be extracted and recovered from the PCS using 0.4% NaOH at 50°C while maintaining high enzymatic hydrolysis rates. The results show that the highest SSF ethanol yield based on 9.5 FPU/g glucan cellulase loading and 6% total solids is seen with the hot-water insoluble PCS (HWI-PCS) treated with 0.4% NaOH at 50°C for 30 min. This sample has an overall ethanol yield of 77% and an ethanol concentration of 22 g/L after 120 hours.

ACKNOWLEDGMENTS

I would like to kindly thank my advisor, Dr. Yoon Young Lee, for his support and guidance while inspiring me in the field of bioethanol research and from whom I have learned so much. I would also like to extend thanks to my group members, Dr. Tae Hyun Kim, Dr. Yongming Zhu, Rajesh Gupta, Hattem Harraz, and Suma Peri, as well as Dr. Qian Xiang and Dr. Parag Garhyan for the many insightful discussions, pleasant work environment, and cooperation throughout this research.

My family deserves special recognition for their continual love, encouragement, and support which has helped me so much throughout my life. I would like to acknowledge my sisters Karen and Marianne, my brother Stephen, and my parents Harry and Suzanne Joiner.

Funding for this research was provided by Abengoa Bionergy R&D through the U.S. Department of Energy (DOE). I would especially like to thank Qian Xiang and Quang Nguyen for their support of this project.

Style manual or journal used Bioresource Technology

Computer software used Microsoft Office XP (Professional)

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. INTRODUCTION

Bioethanol is a clean, renewable energy source that has been identified as an important alternative to petroleum for a variety of environmental, economical, and strategic reasons (Wyman, 1996). Bioethanol is important to US energy security because it is produced from domestically grown lignocellulosic biomass feedstocks such as corn stover, wheat straw, and sugar cane bagasse, as compared with gasoline which is produced from petroleum that must be largely imported from politically unstable regions.

Bioethanol produced from lignocellulosic feedstocks is advantageous to the United States economy because of our vast farm land available and relatively small amount of natural oil reserves. It is essential for national energy security to have a domestically grown energy source to fuel the transportation sector, which consumes more than 25% of the total annual energy according to the 2005 Ethanol Industry Outlook (Renewable Fuels Association) and consumes 68% of the US oil supply. Berdetsky et al. (2005) have made a strong case for reducing US dependence on oil in order to protect America's energy security and have proposed increasing fuel efficiency of vehicles and developing lignocellulosic ethanol in the near-term while switching to an entirely biofuel-based economy in the long-term, among other suggestions.

Because biomass is naturally resistive to hydrolysis due to its recalcitrance, pretreatment is needed in order to increase the hydrolysis rates to levels suitable for

commercial scale ethanol production. Various pretreatment methods have been studied including steam explosion, acid, and alkaline among others (Sun and Cheng, 2002). Generally, acid treatment solubilizes the hemicellulose while alkaline treatment solubilizes the lignin.

This paper focuses on a dilute-alkaline process to extract and recover the lignin from NREL dilute-acid steam-pretreated corn stover (PCS), and the effect of delignification on enzymatic hydrolysis and simultaneous saccharification and fermentation is investigated.

NREL dilute-sulfuric acid pretreated corn stover (PCS)

Schell et al. (2003) have described the dilute-acid pretreatment for corn stover using a pilot-scale reactor at the National Renewable Energy Laboratory (NREL) ethanol process development unit (PDU) in Golden, CO, and Schell et al. describe the pilot plant and data acquisition and control system in more detail in 2004. For the NREL PCS used in this paper, corn stover has been treated with 1.4% H_2SO_4 and heated to 190ºC by direct steam injection for 10 min followed by explosive decompression at the NREL PDU. This treatment solubilizes a large portion of the hemicellulose and swells the cellulose fibers.

Dilute-alkaline treated NREL PCS

Alkaline treatment is the dominant method for pulp production from both wood and non-wood resources and is a preferred method of delignification of agricultural residues because of the good solubility of extractives such as silica (Glasser, 2000). Little sugar degradation occurs during alkaline treatment. In 1991, Elshafei et al. reported that

4% NaOH treatment of corn stover at 25°C for 1 hr gives 95% cellulose conversion after 24 hours enzymatic hydrolysis. Varga et al. (2002) have recently described acidic and alkaline treatment of corn stover and reported that dilute-alkaline treatment is more suitable than a more concentrated base considering economic, performance, and environmental reasons.

Varga et al. also reported in 2002 that a significant portion of the cellulose solubilizes using 1% NaOH treatment at 120°C for 1 hr under pressure, and up to 53% cellulose solubilization occurred with 10% NaOH. However, McDonald et al. (1983) show that mainly lignin and hemicellulose components solubilize during dilute caustic treatment, while cellulose remains relatively untouched.

In this paper, NREL PCS is first washed with hot water to remove the hemicellulose-rich hydrolyzate which can be fermented separately or added back into the fermentation reactor. The hot-water insoluble PCS (HWI-PCS) is then treated with dilute sodium hydroxide in order to extract the lignin fraction. The enzymatic hydrolysis of the delignified HWI-PCS samples is very good at cellulase loadings of 5 and 7 FPU/g glucan and cellulose digestibilities of 90-100% are attained within 36 hours.

For the HWI-PCS sample treated with 0.4% NaOH at 50°C for 30 minutes, the simultaneous saccharification and fermentation (SSF) result is promising with up to 77% ethanol yield using *Saccharomyces cerevisiae* and cellulase loading of 9.5 FPU/g glucan. Fractionating corn stover by dilute-acid followed by dilute-alkaline treatment appears to give adequate ethanol yields and concentrations while providing a lignin co-product.

Objectives

The objective of this research is to improve the SSF ethanol yield and enzymatic hydrolysis of fractionated NREL standard pretreated corn stover (PCS) while recovering the lignin fraction in relatively pure form. Three main experiments were conducted for the delignified PCS:

- Cellulose digestibility
- Simultaneous saccharification and fermentation (SSF)
- Lignin recovery

II. LITERATURE REVIEW

Fuel ethanol background

The idea of using ethanol as a fuel dates back to the Samuel Morey's prototype internal combustion engine in 1826 (Ethanol Timeline, 2005), and during the 1890's ethanol fueled trains, farm machinery, and automobiles (Bernton et al., 1982). In 1908, Henry Ford used a blend of alcohol and gasoline to power the Model T, and around that time people began to debate whether gasoline or ethanol would become the fuel of choice. Due to WWI fuel demand, 50-60 million gallons of ethanol were produced per year in 1917-1918. Ethanol was later banned when Prohibition started in 1919, and it could only be sold when blended with petroleum during that time (Ethanol Timeline, 2005).

Prohibition ended in 1933, and gasohol (6% to 12% ethanol blend at the time) gained a market in the Midwest; however, the accessibility of cheap western oil and a propaganda campaign by the American Petroleum Institute quelled ethanol demand during the 1930's. Ethanol production was stimulated briefly by WWII, and then virtually no fuel ethanol was produced between 1945 and 1978 due to cheap gasoline (Bernton et al., 1982).

Data from the 2004 US DOE Energy Information Administration Annual Energy Review (Fig. II-1) show the ethanol consumption for the transportation sector has been

increasing over the past 20 years, especially over the past 5 years. According to the 2005 Annual Energy Outlook, US annual ethanol production from corn reached 2.8 billion gallons in 2003, 3.4 billion gallons in 2004, and is projected to increase to 4.5 billion gallons by 2025.

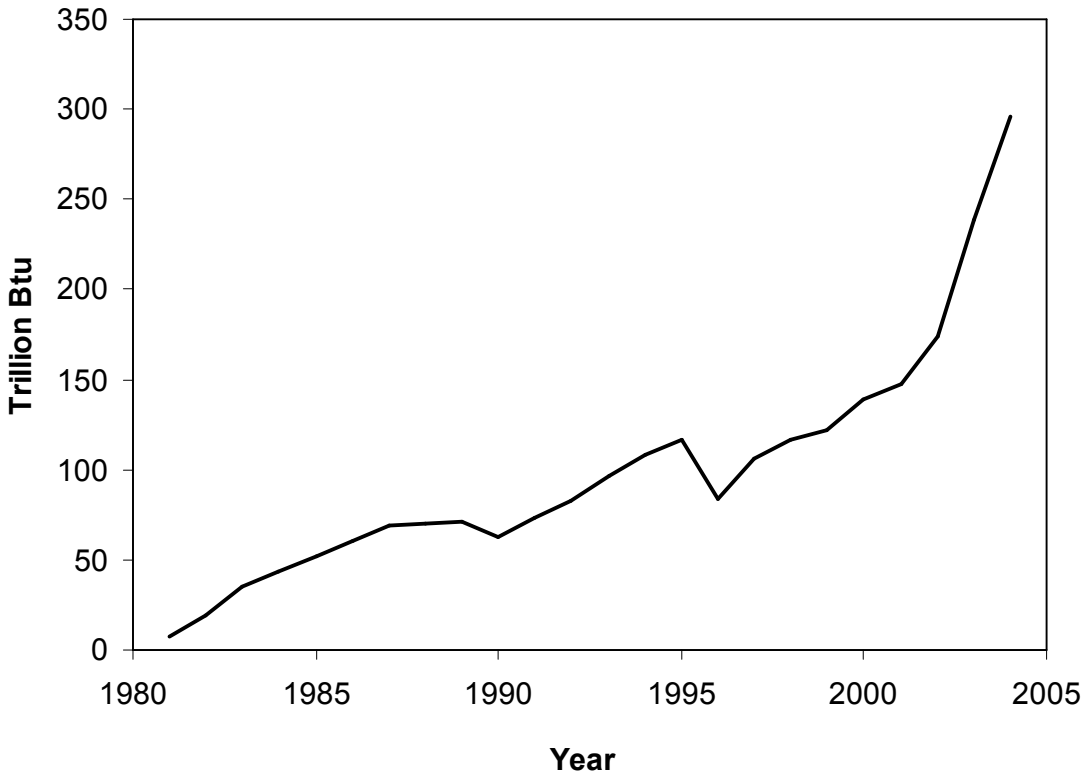


Figure II-I: Transportation sector ethanol consumption, 1981-2004

Note: Data from Energy Information Administration Annual Energy Review 2004.

Corn stover as a feedstock for fuel ethanol production

There are many possible biomass sources which can be utilized to produce renewable fuels and chemicals including dedicated energy crops as well as waste biomass such as agricultural residues. Kim and Dale (2003) estimated that there is enough biomass from wasted crops and crop residues to potentially replace 32% of the global gasoline consumption with bioethanol.

Corn stover and wheat straw, together comprising 61% of the total US crop residue energy potential, are excellent candidates for biological conversion to ethanol because of their great abundance and their high energy potentials as estimated by Klass in 1998. The residue-to-grain ratio for corn as estimated by Heid (1984) is between 1.0 and 1.5 lbs/lbs, which means that about 1.0 to 1.5 pounds of corn stover are produced for every pound of grain harvested. Kadam and McMillan estimated in 2002 that about 80 million dry t/yr of corn stover, which represents 80% of the total US agricultural residue, should be available for ethanol production with a theoretical yield of 19 billion L of ethanol annually.

Composition of corn stover

Corn stover is the agricultural waste that remains after corn harvest and consists of the stalk (internodes and nodes), leaf, sheath, and some tassel. Around 70% of the corn stem by mass is associated with vascular bundles which are thick-walled cells that are rich in cellulose, while the remainder of the stem is made of thin-walled parenchyma cells (Himmel et al., 2005). Corn stover typically contains about 35% to 50% cellulose,

20% to 35% hemicellulose, and 30% lignin, which are insoluble structural polymers (Wyman et al., 1996).

Cellulose

Cellulose is a linear homopolymer of D-glucose units linked by β–(1,4)-glycosidic bonds and predominantly exists in the crystalline form in which every other glucose molecule is rotated 180º. Cellulose is extremely resistive to both acid and enzymatic hydrolysis due to the structure of microfibrils surrounded by protective layers of lignin and hemicellulose. Recent evidence shows that cellulose crystallinity may have a lesser effect on enzymatic hydrolysis (Wyman, 1996).

Plant cell walls are predominantly made up of crystalline cellulose microfibrils that are embedded in a matrix of hemicellulose, pectin and a variety of proteins, and the cell walls collectively constitute an extra-cellular matrix (Mansfield and Saddler, 2003). All of the cellulose in plants is contained in the microfibrils (Himmel et al., 2005)

Commercial Avicel microcrystalline cellulose is used as a reference in the experiments in this paper because it is readily available in a standardized form and makes comparison of results from each experiment easier. Avicel is produced by limited acid hydrolysis of native cellulose to yield highly-ordered forms of cellulose by removing most of the amorphous regions. However, microcrystalline substrates such as Avicel have complex ultrastructures that interfere with enzymatic attack on the microcrystals (Saddler and Penner, 1995).

Hemicellulose

Hemicellulose is a highly complex, branched polymer made up of different monomeric sugars attached through different linkages. The main component of hemicellulose is a homopolymeric xylan backbone which is β-(1,4) linked β-D-xylopyranose that has only the position 2- and 3-carbons available for *O*-linked substitution. Hemicellulose is effectively hydrolyzed into soluble, monomeric sugars by dilute-acid pretreatment (Wyman, 1996). In 1997, Hansen and Björkman examined the ultrastructure of the wood fiber wall and found that, while lignin and cellulose had no affinity, hemicelluloses had affinity for both and therefore acted as a type of surfactant binding cellulose and lignin together (Glasser, 2000). It has been reported that during alkaline extraction, hemicellulose is partially degraded to aliphatic acids (Glasser, 2000).

Lignin

Lignin, second only to cellulose in natural abundance, is a relatively inexpensive and structurally appealing aromatic biopolymer with a high molecular weight that is environmentally friendly (Hu, 2002). All bioethanol processes have lignin residue which is typically combusted for energy. However, as a fuel source lignin is very inefficient, producing less than ¼ as much energy as middle distillate fuels, according to Hu (2002). By creating a market for the lignin residue, the competitiveness of lignocellulosic ethanol process can be enhanced (Lignin-Derived Co-Products, 2005).

There are several commercial applications of lignin including use in dispersants, adhesives and binders, emulsifiers, and surfactants, according to the Lignin Institute (Lignins – Products With Many Uses, 2005). Lignin can act as a dispersant by attaching

to particle surfaces thus preventing clumping in cement mixes, dyes and pigments, and pesticides. As a binder, lignin can be used in soil stabilizers, animal feed pellets, plywood, ceramics, and on unpaved roads to reduce airborne dust. Lignins can stabilize emulsions of immiscible liquids such as asphalt or wax emulsions, pesticides, and in pigments and dyes. NREL has investigated potential converting lignin into a hydrocarbon that can be used as a high-octane automobile fuel additive (Montague, 2003).

Lignin can also be used as a carbon source for carbon fibers. Composite materials which are lightweight, fatigue resistant and possess high strength, modulus, and stiffness, use carbon fibers as a reinforcing material. Otani et al. produced the first lignin-based carbon fibers in 1969 using thermal spinning, and satisfactory lignin fibers have been formed from kraft lignin, alkali lignin and lignosulfonate (Hu, 2002). More recently, carbon fibers have been manufactured from a commercial kraft lignin using thermal spinning followed with carbonization by J.F. Kadla et al. in 2002 for possible use in general purpose composite fiber applications.

Commercial lignins, such as those isolated from alkaline, acidic, or organic solvent-based processes which have undergone extensive fragmentation and degradation, have limited utility in applications that demand a constant well-defined feedstock due to their inherent chemical and molecular weight inhomogeneity and would therefore be used only for general purpose applications (Hu, 2002). These types of carbon fibers may be well suited for use in the automotive industry which requires a low-cost, high-volume, mid-range-performance material (Compere et al., 2002). Use of lignin-based carbon fiber composite materials could dramatically reduce fuel consumption by lowering the overall weight of the vehicle, and a study in 2002 showed that using 10% of US lignin can

produce enough carbon fiber to replace half of the steel used in domestic passenger vehicles (Griffith et al, 2003).

Since the discovery of lignin by Schulze around 1840, there have been an estimated 10,000 to 12,000 scientific papers published relating solely to lignins (Glasser, 2000). Lignin is probably the most influential factor causing native cellulose recalcitrance to enzyme attack, and a positive correlation between digestibility / fermentability and delignification extent has been identified (Wyman, 1996). The correlation levels off at 40%-50% delignification, according to Gharpuray et al. and Gould, but not according to Shimizu, who showed digestibility increasing up to 90% delignification (Wyman, 1996). However, because most delignification techniques result in significant solubilization of hemicellulose, studies that attribute digestibility increase to delignification may not be showing the effect of delignification alone (Wyman, 1996). Because the NREL PCS starting material used in this present work already has most of the hemicellulose removed, more insight can be gained relating the effect of lignin content to enzymatic hydrolysis and SSF fermentation.

Current biomass pretreatment methods

Native biomass is resistant to the enzymatic attack due to its crystallinity, presence of lignin and hemicellulose, acetylation of hemicellulose, and inaccessible surface area (Wyman, 1996). Biomass pretreatment methods are generally aimed at improving the enzymatic hydrolysis primarily by increasing pore volume of the substrate, which increases accessibility of enzyme to the inner microfibrils. Chemical pretreatments such as acid, alkali, and oxidation are effective and economical (Wyman, 1996).

Laureano-Parez et al. determined in 2005 that the initial hydrolysis rate is most influenced by cellulose crystallinity and lignin content is the main influence on the extent of hydrolysis.

Recently, Wyman et al. (2005) reviewed leading biomass pretreatment technologies and compared the sugar recovery data, while Eggeman and Elander (2005) analyzed the economics of these technologies using a full bioethanol facility model.

Cellulase enzymes

Cellulase enzymes are biological catalysts that selectively convert cellulose into glucose monomers, and include three distinct classes of enzymes: 1) the endo-1,4-β-D-glucanases, which act randomly on soluble and insoluble 1,4-β-glucan; 2) the exo-1,4-β-D-glucanases, which liberate D-glucose from 1,4-β-D-glucans and hydrolyze D-cellobiose slowly; and 3) the β-D-glucosidases, which hydrolyze cellobiose into D-glucose and soluble cellodextrins, as well as an assortment of glycosides (Wyman, 1996). Recently, evidence has shown that most cellulases bind only to the 1,0,0 face of the cellulose inside the microfibril (Himmel et al., 2005).

Cellulases are environmentally benign and biodegradable and do not require heavy metals or other toxic inorganic cofactors for its activity (Leatham and Himmel, 1991). Cellulase systems are naturally produced by a variety of aerobic and anaerobic bacteria, white rot fungi, soft rot fungi, and anaerobic fungi, and current commercial production is dominated by the *Trichoderma reesei* species of soft-rot fungi (Wyman, 1996).

Cellulose conversion and the amount of enzyme used are related but not directly proportional because the cellulose surface starts to become saturated with enzyme rendering additional enzyme loading ineffective (Elshafei et al., 1991). Himmel et al. reported in 2005 that the rate of cellulose conversion by cellulase slows at the latter part of the reaction due to the recalcitrant nature of biomass.

Fermentation organism

The *Saccharomyces cerevisiae* strains are the most commonly used ethanologens for starch and cellulosic biomass sugars and have several advantages for bioethanol production including the efficient fermentation of glucose to ethanol with essentially no side products, superior ethanol tolerance, and rapid fermentation rates under acidic conditions (Wyman, 1996). In an aerobic setting, glucose is fermented to produce yeast cell mass, while anaerobic conditions are used to ferment glucose to ethanol.

III. CELLULOSE DIGESTIBILITY OF PCS, HWI-PCS, AND VARIOUS NaOH-EXTRACTED PCS AND HWI-PCS SAMPLES (1% w/v GLUCAN LOADING)

ABSTRACT

The NREL PCS and HWI-PCS have been extracted with dilute sodium hydroxide in batch using a hot caustic rinse and hot water wash after the extraction. The results from enzymatic hydrolysis (Fig. III-1) show that the treated samples have good cellulose digestibility with glucose yields of 86% to 96% after 24 hours with 7 FPU/g glucan Spezyme CP loading (Lot no. 301-003480257), as compared with the Avicel microcrystalline cellulose reference which has 53% digestibility at 24 hours.

The samples exhibit similar hydrolysis trends, and the majority of the hydrolysis is complete after 24 hours for each of the samples tested while the digestibility profile of the Avicel reference is still increasing at 96 hours. The HWI-PCS has the highest overall cellulose digestibility and performs better than the caustic-extracted samples tested after 24 hours. For the delignified samples, both the overall cellulose digestibility and initial hydrolysis rates increase with increasing delignification. The PCS extracted directly by 0.8% NaOH at 70°C has similar digestibility as HWI-PCS extracted with 0.4% NaOH at 80°C since these two samples have similar compositions. Because the PCS is acidic with a pH of approximately 1.5, a portion of the NaOH is neutralized during the caustic extraction for the PCS sample that has not been first washed with water.

INTRODUCTION

The cellulose digestibility of delignified NREL PCS was studied for several caustic-extracted PCS and HWI-PCS samples. This study is important because it is possible to recover a high-purity lignin from the biomass prior to enzymatic hydrolysis and fermentation. This process therefore offers a clean downstream since the lignin is removed first.

MATERIALS AND METHODS

Materials

The NREL standard pretreated corn stover (PCS) was provided by Abengoa Bioenergy R&D and was received in April 2004. The composition of the NREL PCS is shown in Table III-1 as determined by the NREL standard laboratory analytical procedure (LAP). Avicel PH-101 (Fluka; Cat. No.11365; Lot No. 1094627) was used as a microcrystalline cellulose reference. Cellulase enzyme, Spezyme CP (Genencor, Lot No. 301-00348-257), was obtained from NREL and has an average activity of 31.2 FPU/mL as determined by NREL. Novozyme 188 β-glucosidase (Novo Inc., Lot no. 11K1088) has an activity of 750 CBU/mL. A New Brunswick Scientific (Edison, NJ) Series 25 incubator shaker was used for agitation and temperature control.

Experimental procedures

HWI-PCS preparation

To prepare the hot-water-insoluble PCS (HWI-PCS), 15 g PCS dry basis (29.9% TS) were loaded into a 1" by 10" tubular reactor and preheated to 70 ºC for twenty

minutes in a forced-air convection oven. Hot DI water at 70 ºC and 3 mL/min was pumped through the biomass for 25 min with a liquid to solid ratio of 5 mL water / g dry PCS in order to remove more than 90% of the total soluble material as previously determined. An 80 mesh stainless steel filter was used at the reactor exit to prevent any insoluble solids from passing through, and the reactor was operated under atmospheric pressure. The solids were collected using a Buchner funnel and vacuum filtration. The procedure was repeated about twelve times, and the resulting HWI-PCS was combined before compositional analysis and subsequent NaOH extractions.

NaOH extraction

The NREL PCS and HWI-PCS have been extracted using caustic solution in batch mode using 1 L Erlenmeyer flasks in an incubated shaker at 150 rpm. The liquid to solid ratio used was 15 mL NaOH / g biomass (dry basis). The HWI-PCS was extracted at 80°C using 0.4%, 0.8%, and 1.5% NaOH concentrations. Additionally, PCS was directly extracted with 0.8% NaOH at 70°C.

After the 30 minute extraction, the biomass cake was vacuum-filtered using a Buchner funnel, and the cake was rinsed with caustic at the same concentration and temperature as the extraction, followed by water washing at the same temperature. The liquid to solid ratios used for the hot NaOH rinse and hot water wash were 20 and 50 mL liquid / g biomass, respectively. The NaOH rinse was added in approximately three stages to remove excess soluble lignin. An additional 3 L of DI water at room temperature were needed to completely neutralize the biomass cake.

The biomass was stored in wet state (approximately 25% TS) under refrigeration to await the digestibility test. Small portions of the samples were freeze-dried overnight, crushed to smaller particle size, and analyzed for composition in duplicate using NREL standard methods. Prior to compositional analysis, the samples were freeze-dried since it was previously determined that oven-drying the NaOH-extracted samples at 45°C causes inaccuracy due to incomplete glucan hydrolysis because the biomass forms a very rigid structure upon heating which could not be completely hydrolyzed even when grinded to a fine particle size.

Cellulose digestibility

The cellulose digestibility experiment was conducted in duplicate in 250 mL shake flasks with a 100 mL working volume. All components added to the flask were assumed to have a density of 1 g/mL. The basis for each flask was 1 g glucan (1% w/v). To prevent any organism growth, 0.4 mL tetracycline (10 mg/mL in 70% ethanol) and 0.3 mL cyclohexamide (10 mg/mL in distilled water) were added. Sodium citrate buffer (0.05 M) was used to bring the final working volume to 100 mL. Substrate blanks were run with 2 mL buffer solution instead of enzyme to account for any glucose contribution from the samples.

The flasks were allowed to preheat for 1 hour before the addition of 1 mL Spezyme CP cellulase (Genencor, Lot No. 301-00348-257) and 1 mL Novozyme 188 β-glucosidase at 7 FPU/g and 2 CBU/FPU, respectively. The enzyme solutions were prediluted to 7 FPU/mL and 14 CBU/mL just prior to their addition into the flasks. The flasks were maintained in an incubated shaker at 50°C and 150 rpm throughout the

course of the experiment. Samples were taken at times 0, 6, 12, 24, 36, 48, 72, and 96 hours, boiled for 5 min to denature the enzyme, centrifuged, and analyzed for glucose and cellobiose using HPLC. The cellulose digestibility was calculated by the following equation,

$$\text{Cellulose Digestibility} = \frac{\text{Cellulose digested (g)}}{\text{Initial cellulose (g)}} \times 100\%$$

where the cellulose digested was calculated by the sum of glucose and equivalent glucose from cellobiose measured by HPLC times the correction factor 0.9 to convert glucose to cellulose.

Analytical methods

NREL standard laboratory analytical procedures (LAP) were used as the basis for all analyses including the biomass compositional analysis and digestibility test. Carbohydrates were analyzed by HPLC P-column at 85ºC column temperature by refractometric detection using DI water at 0.55 mL/min as the mobile phase.

RESULTS AND DISCUSSION

The cellulose remains intact during the sodium hydroxide treatment and is not solubilized. This could be due to the fact that the PCS starting material has already been pretreated with dilute-acid which would release any easily-soluble amorphous cellulose leaving behind only crystalline cellulose which is resistant to the caustic treatment. The 0.4%, 0.8%, and 1.5% NaOH extractions result in 85%, 90%, and 93% delignification respectively. The 0.8% NaOH treatment is considered an optimal concentration.

It is apparent from the data (Fig. III-1) that all of the PCS treatment conditions tested have good cellulose digestibilities in the range of 91% to 96% at 36 hours as compared with the microcrystalline Avicell reference which has 59% digestibility at 36 hours. Also, each condition tested exhibits quick hydrolysis within the first 24 hours.

Interestingly, the delignified samples as a group have lower digestibility than the HWI-PCS. This possibly happens because extracted lignin is precipitated onto the surface of the biomass after the extraction and could adsorb enzyme. This possibility was minimized by rinsing the extracted solids with hot caustic solution to remove excess soluble lignin that is trapped in the biomass. A large amount of caustic rinse was not used to keep the experiment practical, though completely removing the soluble lignin from the biomass by using more caustic wash might positively affect the cellulose digestibility.

Generally, the digestibility increased with increasing NaOH concentration. This is likely due to increased accessibility of cellulose to the enzyme with the removal of lignin which increases the pore size.

It should also be noted that the HWI-PCS data show complete hydrolysis after 48 hours, whereas the delignified samples do not achieve total theoretical hydrolysis but rather level off at around 93-95% of the theoretical value. This shows that some of the cellulose cannot be hydrolyzed which might be due to collapsed pores upon delignification rendering the cellulose inaccessible to the enzyme. Another possible explanation is that a portion of the extracted lignin reattaches to the cellulose surface which would also prevent the enzyme from accessing the cellulose.

CONCLUSIONS

The HWI-PCS exhibits good digestibility after delignification by dilute sodium hydroxide, and the process of first fractionating the lignin from NREL PCS prior to enzymatic digestibility shows promising results. Initial hydrolysis rates and overall conversion correlate positively with the amount of lignin removal. As a group, the delignified samples have about 5% unhydrolyzed glucan while the HWI-PCS hydrolyzes completely.

Table III-1: Compositional analysis of 2004 NREL PCS insoluble solids.

Component	NREL PCS [%]
Glucan	57.0 ± 0.6
Xylan	1.8 ± 0.0
Galactan	0.0 ± 0.0
Arabinan	0.9 ± 0.1
Mannan	0.5 ± 0.1
Acid-Insoluble Lignin	26.0 ± 0.1
Acid-Soluble Lignin	2.1 ± 0.0
Ash	9.2
Acetyl	0.3
Total	97.8 ± 0.9

Note: 1. Values are expressed as mean and standard deviation (n=3).
2. The NREL PCS was washed with room-temperature water to remove the soluble portion.

Table III-2: Compositional analysis of PCS, HWI-PCS, and various NaOH-insoluble HWI-PCS operated in batch with a liquid to solid ratio of 15 mL NaOH / g dry HWI-PCS.

Component	PCS 0.8%NaOH 70°C [%]	HWI-PCS [%]	HWI-PCS 0.4%NaOH 80°C [%]	HWI-PCS 0.8%NaOH 80°C [%]	HWI-PCS 1.5%NaOH 80°C [%]
Glucan	91.1 ± 0.9	60.2 ± 0.5	90.4 ± 1.7	94.7 ± 0.3	96.1 ± 0.3
Xylan	2.0 ± 0.0	2.0 ± 0.1	2.0 ± 0.0	2.0 ± 0.1	1.9 ± 0.0
Galactan	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Arabinan	0.5 ± 0.0	0.9 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.6 ± 0.1
Mannan	0.5 ± 0.1	0.3 ± 0.4	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.1
Acid-Insoluble Lignin	6.0 ± 2.0	28.2 ± 0.5	6.4 ± 0.0	4.3 ± 0.4	3.1 ± 0.3
Acid-Soluble Lignin	0.5 ± 0.0	1.5 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
Ash	1.2 ± 0.0	8.5 ± 0.3	1.3 ± 0.0	0.9 ± 0.0	0.7 ± 0.0
Acetyl	0.8 ± 0.1	0.6 ± 0.2	0.4 ± 0.1	0.2 ± 0.1	0.2 ± 0.0
Total	102.6 ± 3.1	102.2 ± 2.1	101.8 ± 1.9	103.7 ± 1.0	103.8 ± 0.8
Solid Remaining [2]	44.9	--	60.8	57.9	55.8
Delignification [3]	73.5 ± 8.0	--	85.1 ± 1.0	90.2 ± 0.8	93.0 ± 0.6

Note: 1. Values are expressed as mean and standard deviation (n=2).
2. Solid remaining is the amount of insoluble material remaining after the NaOH extraction.
3. Delignification values are calculated on corresponding PCS or HWI-PCS dry basis.

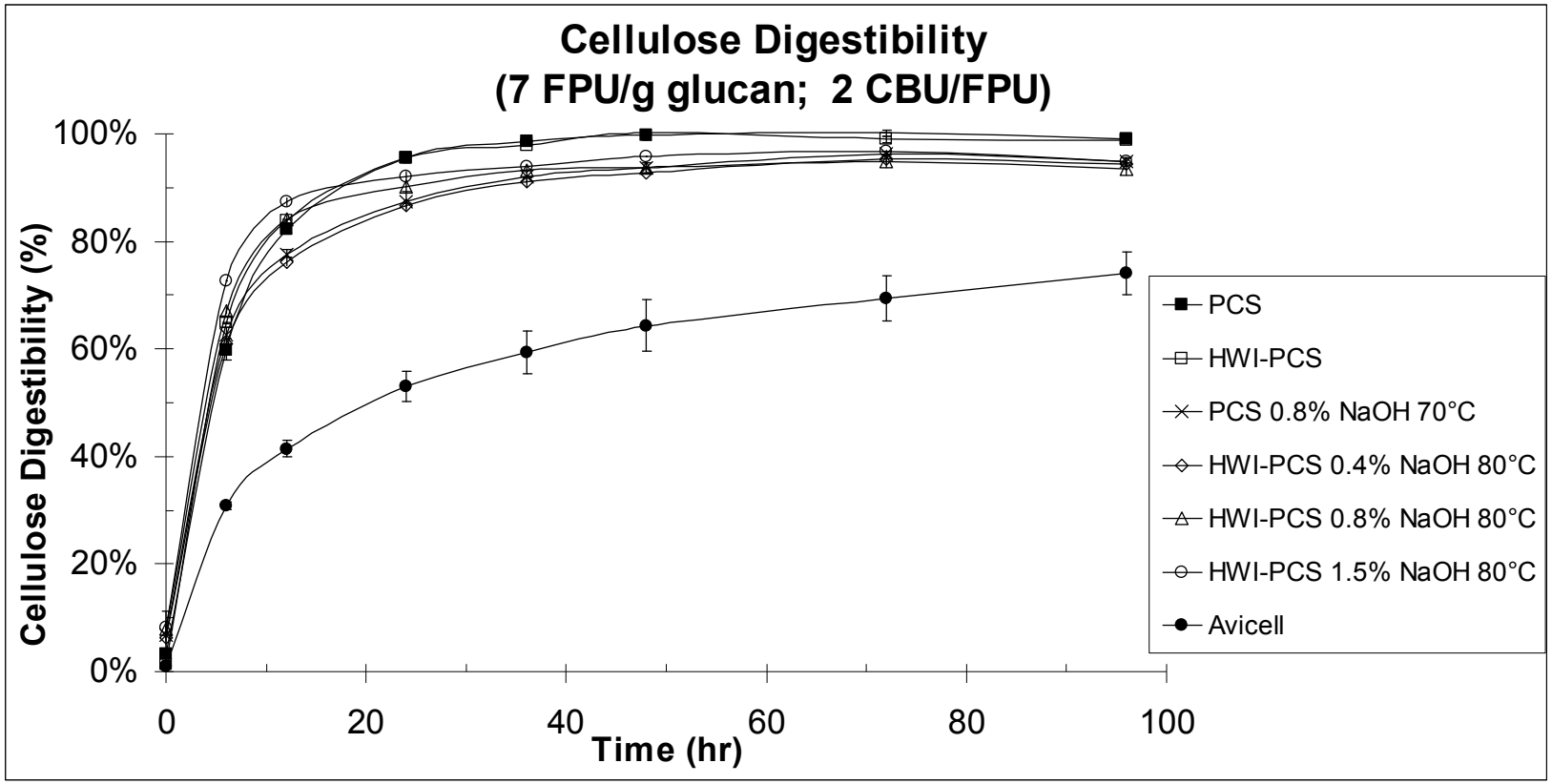


Figure III-I: Cellulose digestibility of various treated PCS samples based on 1% glucan loading.

Table III-3: Average cellulose digestibility with cellulase loading of 7 FPU/g glucan and 2 CBU/FPU (1% glucan basis).

	Sampling time (hr) / Cellulose digestibility (%)							
PCS	0	6	12	24	36	48	72	96
Average	3.2%	59.9%	82.2%	95.5%	98.5%	99.7%	100.2%	99.2%
Standard deviation	1.1%	2.0%	0.7%	0.3%	0.0%	0.9%	0.6%	0.5%
HWI-PCS	0	6	12	24	36	48	72	96
Average	1.4%	64.9%	83.8%	95.5%	97.9%	100.2%	99.0%	98.9%
Standard deviation	0.5%	0.1%	0.4%	0.8%	0.1%	0.1%	0.6%	0.2%
PCS 0.8% NaOH 70°C	0	6	12	24	36	48	72	96
Average	6.8%	62.6%	77.7%	87.5%	92.1%	93.8%	96.3%	94.8%
Standard deviation	4.4%	1.4%	0.9%	1.8%	1.2%	0.8%	0.4%	0.9%
HWI-PCS 0.4% NaOH 80°C	0	6	12	24	36	48	72	96
Average	6.2%	61.1%	76.2%	86.6%	91.0%	92.7%	95.2%	94.3%
Standard deviation	0.7%	N/A	0.8%	0.4%	0.8%	N/A	0.7%	0.3%
HWI-PCS 0.8% NaOH 80°C	0	6	12	24	36	48	72	96
Average	7.9%	67.2%	84.1%	90.2%	93.3%	93.8%	94.8%	93.4%
Standard deviation	1.4%	0.6%	0.7%	0.8%	0.6%	0.9%	1.1%	1.0%
HWI-PCS 1.5% NaOH 80°C	0	6	12	24	36	48	72	96
Average	8.1%	72.7%	87.4%	92.0%	91.7%	95.9%	96.7%	94.8%
Standard deviation	1.1%	0.1%	0.6%	1.1%	3.3%	N/A	0.4%	0.5%
Avicel	0	6	12	24	36	48	72	96
Average	1.0%	30.9%	41.4%	53.1%	59.3%	64.3%	69.4%	74.0%
Standard deviation	0.1%	0.7%	1.5%	2.9%	4.0%	4.7%	4.3%	4.0%

Note: Values are expressed as mean and standard deviation (n=2).

Table III-4: Average glucose released (g/L) with cellulase loading of 7 FPU/g glucan and 2 CBU/FPU (1% glucan basis).

	Sampling time (hr) / Glucose concentration (g/L)							
PCS	0	6	12	24	36	48	72	96
AVERAGE	0.1	5.0	7.9	10.4	10.8	11.0	11.0	11.0
STDEV	0.0	0.2	0.1	0.0	0.0	0.1	0.1	0.1
HWI-PCS	0	6	12	24	36	48	72	96
AVERAGE	0.1	5.8	8.4	10.3	10.8	11.0	11.0	11.0
STDEV	0.0	0.1	0.1	0.1	0.0	0.0	0.1	0.0
PCS 0.8% NaOH 70°C	0	6	12	24	36	48	72	96
AVERAGE	0.2	5.9	8.0	9.5	10.1	10.3	10.6	10.5
STDEV	0.1	0.1	0.1	0.2	0.1	0.1	0.0	0.1
HWI-PCS 0.4% NaOH 80°C	0	6	12	24	36	48	72	96
AVERAGE	0.2	5.8	7.9	9.4	10.0	10.2	10.5	10.4
STDEV	0.0	--	0.1	0.1	0.1	--	0.1	0.0
HWI-PCS 0.8% NaOH 80°C	0	6	12	24	36	48	72	96
AVERAGE	0.2	6.1	8.6	9.9	10.3	10.4	10.5	10.3
STDEV	0.0	0.0	0.0	0.1	0.1	0.1	0.1	0.1
HWI-PCS 1.5% NaOH 80°C	0	6	12	24	36	48	72	96
AVERAGE	0.2	6.4	9.0	10.0	10.1	10.6	10.7	10.5
STDEV	0.0	0.0	0.1	0.1	0.4	--	0.0	0.1
Avicel	0	6	12	24	36	48	72	96
AVERAGE	0.1	3.2	4.4	5.8	6.5	7.1	7.7	8.2
STDEV	0.0	0.1	0.1	0.3	0.4	0.5	0.5	0.4

Note: Values are expressed as mean and standard deviation (n=2).

IV. CELLULOSE DIGESTIBILITY OF PCS, HWI-PCS, AND VARIOUS NaOH-EXTRACTED PCS AND HWI-PCS SAMPLES (6% TOTAL SOLIDS LOADING)

ABSTRACT

The cellulose digestibility for various delignified PCS and HWI-PCS samples was investigated at 6% total solids and cellulase loading of 5 FPU/g glucan with 2 CBU/FPU β-glucosidase supplementation. The high total solids loading caused high glucose concentrations upon enzymatic digestion of glucan, and therefore resulted in decreased hydrolysis rates due to product inhibition. It appears that there is a tradeoff between achieving fast hydrolysis rates and high glucose concentrations. Glucose concentrations ranged between about 20-30 g/L at 24 hours and reached about 37-48 g/L by 96 hours.

The trend of the enzymatic digestibility follows the same general order as in the 1% glucan loading case from section III of this paper, however the hydrolysis rate is considerably slower and the differentiation between the samples is greater. The hydrolysis is still slowly proceeding at 96 hours with 6% TS loading, while hydrolysis was nearly complete by 24 hours for the previous 1% glucan loading case.

INTRODUCTION

There is a need to achieve high glucose concentrations in order to produce high ethanol concentrations, therefore the enzymatic hydrolysis was tested using 6% total solids basis.

MATERIALS AND METHODS

Materials

The NREL standard pretreated corn stover (PCS) was provided by Abengoa Bioenergy R&D and was received in April 2004. The composition of the as-received PCS is shown in Table IV-1 as determined by NREL standard procedures. Avicel PH-101 (Fluka; Cat. No.11365; Lot No. 1094627) was used as a microcrystalline cellulose reference. Cellulase enzyme, Spezyme CP (Genencor, Lot No. 301-00348-257), was obtained from NREL and has an average activity of 31.2 FPU/mL as determined by NREL. Novozme 188 β-glucosidase (Novo Inc., Lot no. 11K1088) has an activity of 750 CBU/mL. A New Brunswick Scientific (Edison, NJ) Series 25 incubator shaker was used for agitation and temperature control.

Experimental procedures

HWI-PCS preparation

To prepare the hot-water-insoluble PCS (HWI-PCS), 20 g PCS dry basis (30.5% TS) were loaded into a 1" by 10" tubular reactor and preheated to 70ºC for twenty minutes in a forced-air convection oven. Hot DI water at 70ºC and 3 mL/min was pumped through the biomass for 33.3 min having a liquid to solid ratio of 5 mL water / g

dry PCS as previously determined to remove greater than 90% of the hot-water extractable portion. An 80 mesh stainless steel filter was used at the reactor exit to prevent any insoluble solids from passing through, and the reactor was operated under atmospheric pressure. The procedure was repeated about twelve times, and the resulting HWI-PCS was combined before compositional analysis and subsequent NaOH extractions.

NaOH extraction

The NREL PCS and HWI-PCS have been extracted using caustic solution in batch mode using 1 L Erlenmeyer flasks in an incubated shaker at 150 rpm following the same procedure as in section III of this thesis. The liquid to solid ratio used was 15 mL NaOH / g biomass (dry basis) which was previously determined to remove greater than 90% of the total NaOH extractable solids. The HWI-PCS was extracted at 80°C using 0.4%, 0.8%, and 1.5% NaOH concentrations. PCS was also directly extracted at 70°C with 0.8% NaOH.

After the 30 minute extraction, the biomass cake was vacuum-filtered using a Buchner funnel, and the cake was rinsed with caustic at the same concentration and temperature as the extraction, followed by water washing at the same temperature. The liquid to solid ratios used for the hot NaOH rinse and hot water wash were 20 and 50 mL liquid / g biomass, respectively. An additional 3 L of DI water at 22°C were needed to completely neutralize the biomass cake. The samples were freeze-dried overnight since it was previously determined that oven-drying the NaOH-extracted samples will cause

inaccurate compositional analysis. The samples were crushed to smaller particle size and analyzed for composition using NREL standard methods.

Cellulose digestibility

The cellulose digestibility experiment was conducted in duplicate in 250 mL shake flasks with a 100 mL working volume. All components added to the flask were assumed to have a density of 1 g/mL. The basis for each sample was 6% w/v total solids. Tetracycline and cyclohexamide were added to prevent any organism growth in the standard amounts of 0.4 mL and 0.3 mL, respectively. Sodium citrate buffer (0.05 M) was used to bring the volume to 100 mL. Substrate blanks were run with 2 mL buffer solution instead of enzyme to account for any glucose contribution from the samples.

The flasks were allowed to preheat for 1 hour before the addition of 1 mL Spezyme CP cellulase (Lot No. 301-00348-257) and 1 mL β-glucosidase at 5 FPU/g glucan and 2 CBU/FPU, respectively. The flasks were maintained in an incubated shaker at 50°C and 150 rpm throughout the course of the experiment. Samples were taken at times 0, 6, 12, 24, 48, 72, and 96 hours and analyzed using HPLC P-column and RI detector.

It must be noted that the digestibility data for HWI-PCS is based on a cellulase loading of 4.4 FPU/g glucan. This is because of a correction that had to be made due to an incomplete hydrolysis of the glucan during the compositional analysis due to 45°C oven drying and grinding. It was previously believed that only the delignified samples required freeze-drying to obtain accurate compositional analysis, but it seems that oven drying can cause error in glucan/lignin content even for the HWI-PCS sample. It seems

that after oven-drying, even at moderate temperatures, the biomass can become rigid and is not easily hydrolyzed completely by the 72% sulfuric acid. This is possibly due to collapsed pores and strong hydrogen bonding between the tightly-packed cellulose fibers. After freeze-drying the sample and grinding with a coffee grinder, the sample completely hydrolyzed, thus accurate glucan content was obtained and the cellulase loading value adjusted. The cellulose digestibility was calculated by the following equation,

$$\text{Cellulose Digestibility} = \frac{\text{Cellulose digested (g)}}{\text{In itial cellulose (g)}} \times 100\%$$

where the cellulose digested was calculated by the sum of glucose and equivalent glucose from cellobiose measured by HPLC times the correction factor 0.9 to convert glucose to cellulose.

RESULTS AND DISCUSSION

The HWI-PCS shows the best cellulose conversion which agrees with the result from section III. All of the samples perform noticeably better than the Avicel microcrystalline reference. The difference between the HWI-PCS and delignified samples is greater with 6% total solids loading than with 1% glucan loading. For the delignified HWI-PCS samples, the digestibility increases with increasing delignification. This confirms the same general trend that was found in section III of this thesis.

There is a tradeoff between achieving high glucose concentrations and achieving high levels and rates of cellulose conversion. This is likely due to product inhibition, though non-productive enzyme binding could also play a slight role. It is believed that substrate inhibition is not present, and Elshafei et al. (1991) mention that substrate

loading between 0.5-5% total solids has only minimal effect on cellulose conversion though there is a substantial effect on hemicellulose conversion for alkaline treated corn stover samples; however in our case, there is very little hemicellulose to begin with. Varga et al. (2002) have reported that product inhibition starts to occur around 15 g/L glucose.

In our case, the glucose concentration reached 48 g/L for the 1.5% NaOH treated HWI-PCS sample, and was 37 g/L for the HWI-PCS after 96 hours. It is believed that there is an extreme effect of product inhibition here as a result. Because the experiment was based on total solids loading, the glucose concentrations increase according to increasing cellulose content of biomass samples.

CONCLUSION

Increasing the total solids content could prove beneficial for achieving higher ethanol concentrations. As a result of glucose inhibition, the hydrolysis rates were slower than the previous case with 1% glucan loading. If the total solids content is low, the glucose concentration will also be low and conversion rates high; however, if the total solids content is high, the glucose concentration can be increased though conversion will be slower due to product inhibition. This inhibitory effect should not be present if the fermentation is operated in simultaneous saccharification and fermentation (SSF) mode because any available glucose will be rapidly consumed by the yeast.

Table IV-1: Compositional analysis of 2004 NREL PCS insoluble solids.

Component	NREL PCS [%]
Glucan	57.0 ± 0.6
Xylan	1.8 ± 0.0
Galactan	0.0 ± 0.0
Arabinan	0.9 ± 0.1
Mannan	0.5 ± 0.1
Acid-Insoluble Lignin	26.0 ± 0.1
Acid-Soluble Lignin	2.1 ± 0.0
Ash	9.2
Acetyl	0.3
Total	97.8 ± 0.9

Note: 1. Values are expressed as mean and standard deviation (n=3).
2. The NREL PCS was washed with room-temperature water to remove the soluble portion.

Table IV-2: Compositional analysis of PCS, HWI-PCS, and various NaOH-insoluble HWI-PCS operated in batch with a liquid to solid ratio of 15 mL NaOH / g dry HWI-PCS.

Component	PCS 0.8%NaOH 70°C [%]	Hot-water insoluble PCS [%]	HWI-PCS 0.4%NaOH 80°C [%]	HWI-PCS 0.8%NaOH 80°C [%]	HWI-PCS 1.5%NaOH 80°C [%]
Glucan	85.1 ± 0.6	60.5 ± 0.6	84.4 ± 0.2	87.3 ± 0.4	87.2 ± 0.3
Xylan	1.9 ± 0.1	1.7 ± 0.1	1.9 ± 0.0	1.9 ±0.0	1.8 ± 0.0
Galactan	0.0 ± 0.0	0.3 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Arabinan	0.5 ± 0.0	0.8 ± 0.0	0.5 ± 0.0	0.6 ± 0.0	0.6 ± 0.1
Mannan	0.6 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	0.7 ± 0.0	0.7 ± 0.1
Acid-Insoluble Lignin	5.7 ± 0.9	25.6 ± 0.4	6.3 ± 0.5	3.8 ± 0.0	3.0 ± 0.5
Acid-Soluble Lignin	0.5 ± 0.0	1.4 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.4 ± 0.0
Ash	6.5	8.3 ± 0.5	6.5	5.3	4.9
Acetyl	1.0 ± 0.1	1.6 ± 0.0	1.1 ± 0.0	1.6 ± 0.0	2.1 ± 0.3
Total	101.8 ± 1.8	100.7 ± 1.9	101.7 ± 0.7	101.7 ± 0.4	100.7 ± 1.3
Solid Remaining [2]	43.0	--	69.0	66.5	65.8
Delignification [3]		--	84.5 ± 1.2	90.6 ± 0.0	92.6 ± 1.1

Note: 1. Values are expressed as mean and standard deviation (n=2).
2. Solid remaining is the amount of insoluble material remaining after the NaOH extraction.
3. Delignification values are based on dry HWI-PCS.

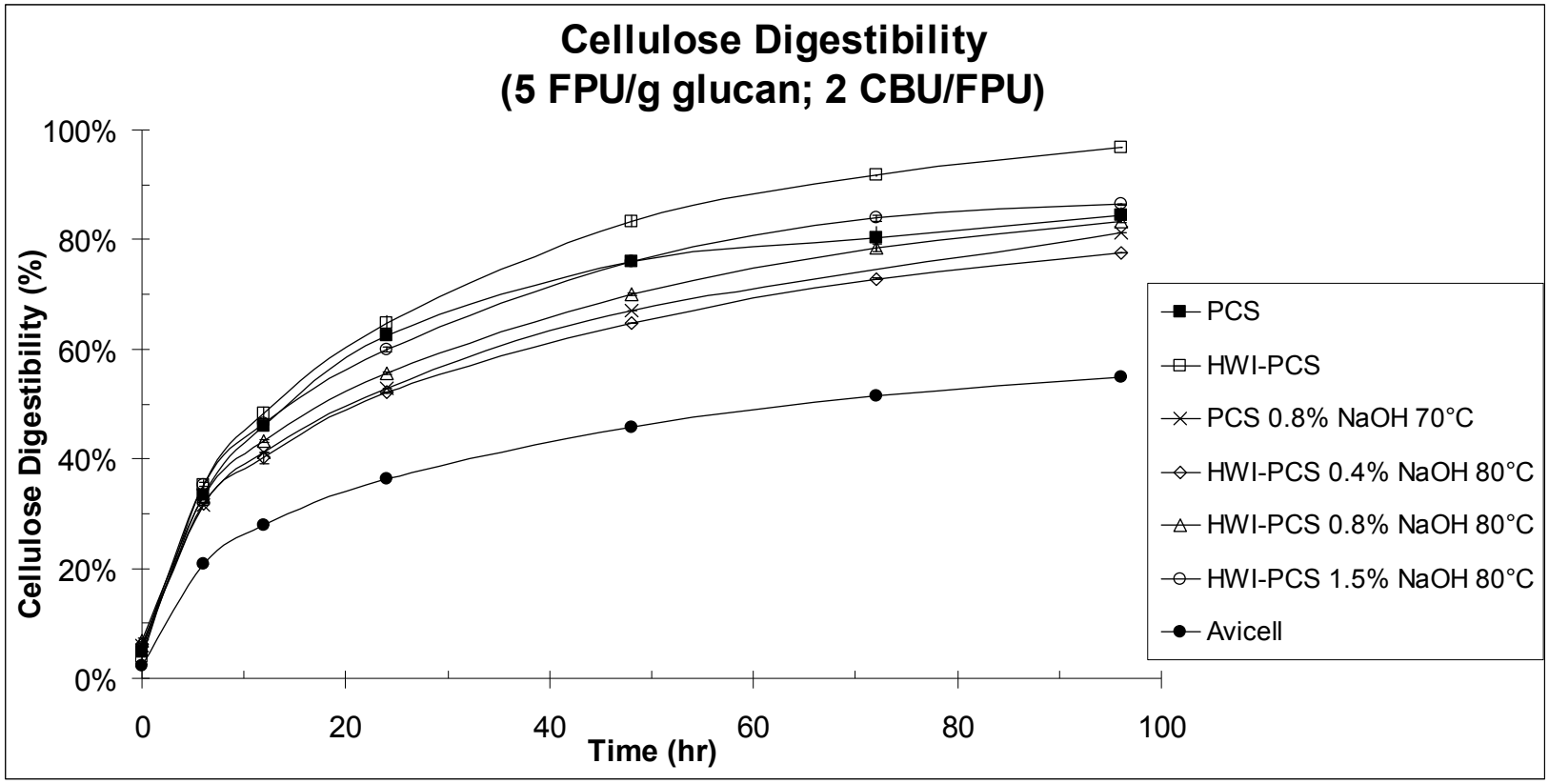


Figure IV-I: Cellulose digestibility for various treated PCS samples based on 6% TS loading.

Table IV-3: Average cellulose digestibility with cellulase loading of 5 FPU/g glucan and 2 CBU/FPU (6% TS basis).

	Sampling time (hr) / Cellulose digestibility (%)						
PCS	0	6	12	24	48	72	96
Average	4.9%	33.4%	45.9%	62.4%	76.0%	80.3%	84.5%
Standard deviation	0.1%	0.1%	0.0%	0.2%	0.8%	2.1%	1.3%
HWI-PCS	0	6	12	24	48	72	96
Average	3.9%	35.2%	48.2%	64.8%	83.4%	91.7%	96.7%
Standard deviation	0.6%	0.8%	1.2%	1.4%	1.0%	0.2%	0.0%
PCS 0.8% NaOH 70°C	0	6	12	24	48	72	96
Average	5.3%	35.3%	46.4%	59.9%	75.9%	83.9%	86.4%
Standard deviation	0.9%	0.3%	0.3%	0.5%	0.3%	0.6%	0.1%
HWI-PCS 0.4% NaOH 80°C	0	6	12	24	48	72	96
Average	5.9%	31.6%	41.2%	52.9%	67.0%	0.0%	81.3%
Standard deviation	--	--	--	--	--	--	--
HWI-PCS 0.8% NaOH 80°C	0	6	12	24	48	72	96
Average	6.1%	31.9%	40.2%	52.1%	64.8%	72.8%	77.6%
Standard deviation	0.2%	0.1%	1.1%	0.2%	0.0%	0.1%	0.1%
HWI-PCS 1.5% NaOH 80°C	0	6	12	24	48	72	96
Average	7.0%	33.3%	43.3%	55.7%	70.0%	78.4%	83.3%
Standard deviation	0.4%	0.3%	0.3%	0.2%	0.2%	0.7%	0.3%
Avicell	0	6	12	24	48	72	96
Average	2.3%	20.8%	27.8%	36.3%	45.7%	51.6%	54.9%
Standard deviation	0.2%	0.0%	0.0%	0.1%	0.1%	0.1%	0.2%

Note: 1. Values are expressed as mean and standard deviation (n=2).
2. Cellulase loading was 4.4 FPU/g glucan for the HWI-PCS.

Table IV-4: Average glucose released (g/L) with cellulase loading of 5 FPU/g glucan and 2 CBU/FPU (6% TS basis).

	Sampling time (hr) / Glucose concentration (g/L)						
PCS	0	6	12	24	48	72	96
AVERAGE	0.2	4.8	8.2	12.9	18.1	19.8	21.1
STDEV	0.0	0.1	0.1	0.1	0.1	0.6	0.3
HWI-PCS	0	6	12	24	48	72	96
AVERAGE	0.5	9.1	14.3	21.3	29.8	34.8	36.8
STDEV	0.0	0.2	0.4	0.5	0.5	0.3	0.1
PCS 0.8% NaOH 70°C	0	6	12	24	48	72	96
AVERAGE	0.9	12.6	18.6	26.0	34.7	0.0	43.8
STDEV	--	--	--	--	--	--	--
HWI-PCS 0.4% NaOH 80°C	0	6	12	24	48	72	96
AVERAGE	1.0	13.1	18.4	25.8	33.7	38.7	41.7
STDEV	0.0	0.1	0.6	0.1	0.0	0.0	0.1
HWI-PCS 0.8% NaOH 80°C	0	6	12	24	48	72	96
AVERAGE	1.1	13.7	20.1	28.0	37.3	43.0	46.3
STDEV	0.1	0.2	0.2	0.2	0.2	0.4	0.1
HWI-PCS 1.5% NaOH 80°C	0	6	12	24	48	72	96
AVERAGE	0.8	14.0	20.9	29.5	40.0	45.9	48.2
STDEV	0.1	0.2	0.2	0.3	0.3	0.4	0.0
Avicell	0	6	12	24	48	72	96
AVERAGE	0.6	10.6	15.4	21.3	27.9	32.0	34.4
STDEV	0.0	0.0	0.0	0.0	0.0	0.1	0.1

Note: Values are expressed as mean and standard deviation (n=2).

V. SIMULTANEOUS SACCHARAFICATION AND FERMENTATION OF PCS, HWI-PCS, AND VARIOUS NaOH-EXTRACTED PCS AND HWI-PCS SAMPLES (6% TS LOADING)

ABSTRACT

Surprisingly, the result from simultaneous saccharification and fermentation (SSF) does not follow the trend expected from enzymatic hydrolysis result. The best SSF ethanol yield is from the PCS directly extracted by 0.8% NaOH at 70ºC. The lowest SSF ethanol yield from the samples tested was the HWI-PCS, which had good enzymatic hydrolysis in sections III and IV of this paper. The ethanol yield of the 0.4%, 0.8%, and 1.5% NaOH extracted HWI-PCS samples did not correspond with the results from the enzymatic hydrolysis either.

The delignified samples have better SSF ethanol yield than the standard NREL PCS. The ethanol concentrations for the delignified samples reached between 16-23 g/L at 96 hours.

INTRODUCTION

The SSF test generally followed NREL standard analytical method, but was based on 6% total solids rather than 6% glucan.

MATERIALS AND METHODS

Materials

The NREL standard pretreated corn stover (PCS) was provided by Abengoa Bioenergy R&D and was received in April 2004. The composition of the as-received PCS is shown in Table V-2. Avicel PH-101 (Fluka; Cat. No.11365; Lot No. 1094627) was used as a microcrystalline cellulose reference. Cellulase enzyme, Spezyme CP (Genencor, Lot No. 301-04075-054), was obtained from NREL and has an average activity of 59 FPU/mL as determined by NREL. Novozyme 188 β-glucosidase (Novo Inc., Lot no. 11K1088) has an activity of 750 CBU/mL. *Saccharomyces cerevisiae* ATCC® 200062 (NREL-D_5A) was used as the fermentation organism for the SSF procedure, and the yeast extract (Cat. No. Y-0500) and peptone (Cat. No. P-6588) used for the growth medium were purchased from Sigma. A New Brunswick Scientific (Edison, NJ) Series 25 incubator shaker was used for agitation and temperature control.

Experimental procedures

Hot-water extraction

The HWI-PCS was prepared in batch using three 1 L Erlenmeyer flasks in an incubator (New Brunswick Scientific, Series 25) maintained at 70ºC and 150 rpm for 30 minutes. A predetermined liquid to solid ratio of 10 mL DI water per g dry PCS was used in order to remove more than 90% of the total soluble components for batch operation.

Predetermined amounts of water and NREL PCS were preheated separately. To start the extraction, the 70°C water was poured into the flask containing the biomass. After the hot-water extraction the samples were vacuumed-filtered, rinsed with room-

temperature DI water, and the samples were combined for compositional analysis and subsequent NaOH extraction. The solid remaining after the hot-water extraction was 65.4%.

NaOH Extractions

The caustic extractions were carried out following the same procedure as described in section III. The NREL PCS and HWI-PCS have been extracted using caustic solution in batch mode using 1 L Erlenmeyer flasks in an incubator shaker at 150 rpm. The liquid to solid ratio used was 15 mL NaOH / g biomass (dry basis). The HWI-PCS was extracted at 80°C using 0.4%, 0.8%, and 1.5% NaOH concentrations. PCS was also directly extracted at 70°C with 0.8% NaOH.

After the 30 minute extraction, the biomass cake was vacuum-filtered using a Buchner funnel, and the cake was rinsed with caustic at the same concentration and temperature as the extraction, followed by water washing at the same temperature. The solid to liquid ratios used for the hot NaOH rinse and hot water wash were 1:25 and 1:50 g biomass / mL liquid, respectively. An additional 3 L of DI water at 22°C were needed to completely neutralize the biomass cake. The samples were freeze-dried overnight since it was previously determined that oven-drying the NaOH-extracted samples will cause inaccurate compositional analysis. The samples were crushed to smaller particle size and analyzed for composition using NREL standard methods.

SSF Procedure

A slightly modified version of the Simultaneous Saccharification and Fermentation Procedure NREL LAP-008 was used with a total solids loading of 6% TS.

Inoculum preparation

The *Saccharamyces cerevisiae* D_5A inoculum was aerobically prepared in two growth stages using 50 mL YPD medium (10 g/L yeast extract; 20 g/L peptone; 20 g/L dextrose) in 250 mL shake flasks. The second stage growth was inoculated with 10% v/w from the first stage growth flask. At the end of the second growth stage a portion of the inoculum was analyzed for ethanol by HPLC using H-column, glucose using YSI analyer, pH, and dry cell mass as shown in Table 1.

Table V-1: Second stage inoculum of *S. cerevisiae* D_5A in YPD medium.

Ethanol (g/L)	10.8
Glucose (g/L)	4.1
pH	3.8
Dry Cell Mass (g/L)	9.4

SSF preparation

In each 250 mL SSF flask, 6 g biomass (dry basis) were added along with 10 mL of 10x YP medium (100 g/L yeast extract; 200 g/L peptone) and the appropriate amount of water to bring the final working volume to 100 mL. The flasks were weighed and sterilized at 121°C for 30 min, and any evaporated moisture was added back as sterile DI water upon cooling. Using a sterile pipette, 10 mL of the *S. cerevisiae* D_5A inoculum were added to the SSF flasks.

The cellulase was the last to be added to the flask. Spezyme CP (Genencor, Lot No. 301-04075-054) has an activity of 59 FPU/mL for the stock solution and was sterilized by filtration. This is a new batch of cellulase from that which was used in sections III and IV of this paper. The enzyme was diluted to 19 FPU/mL before adding 9.5 FPU/g glucan to the SSF flasks. The flasks were incubated at 38°C and 150 rpm for 120 hours using bubble traps to maintain anaerobic conditions. There was no pH control, and the pH gradually decreased from approximately 5.5 to 3.5 after 120 hours.

Samples were aseptically taken in a laminar hood at 12, 24, 48, 72, 96, and 120 hours. Microcentrifuge tubes with screw cap were used to prevent ethanol evaporation. The centrifuge tubes were heated at 85°C for 25 min to stop the SSF. Lactic acid, glycerol, acetic acid, and ethanol were measured using an HPLC H-column at 60ºC column temperature by refractometric detection using 0.1M H_2SO_4 at 0.55 mL/min as the mobile phase. Cellobiose and glucose were measured by HPLC with equipped with P-column at 85ºC column temperature by refractometric detection using DI water at 0.55 mL/min as the mobile phase. The ethanol yield is calculated as % theoretical maximum,

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

where the factor 0.511 is the conversion factor for glucose to ethanol based on stoichiometric biochemistry of yeast.

RESULTS AND DISCUSSION

The as-received PCS from NREL was recently reanalyzed, and it was found that there was no significant change in composition during 1 year in cold storage. The NREL PCS has a fraction insoluble solids (%FIS) of 64.0% ± 0.8% and has 19.4% ± 0.2% insoluble solids (%IS) as determined by NREL LAP-018 using the filtration method.

The ethanol yields were not as high as expected, but they are still better than the Avicel reference. The NREL PCS extracted with 0.8% NaOH at 70°C has the best ethanol yield (78% at 120 hr) of the samples tested, which is surprising because it had lower enzymatic hydrolysis of the samples tested in sections III and IV of this paper. Also unexpectedly, the HWI-PCS showed relatively poor ethanol yield, though it had one of the highest cellulose digestibilities out of the samples tested.

Glucose concentrations remained low between about 0.1 g/L and 0.2 g/L signifying that the yeast was rapidly consuming any available glucose. Also, high levels of lactic acid were detected ranging from about 7-13 g/L for the samples except for the PCS for which lactic acid stayed below 2.5 g/L. Glycerol concentrations were around 0.5 g/L, and acetic acid was between about 1 g/L and 2 g/L.

CONCLUSIONS

The SSF ethanol result was not as good as expected from the enzymatic hydrolysis data; however the samples still performed better than the Avicel reference. The best condition overall is probably the 0.4% NaOH extracted HWI-PCS sample since it has good ethanol yield (72% at 120 hrs) and requires lower NaOH concentration.

Table V-2: Compositional analysis of as-received and washed NREL PCS.

Component	As-received NREL-PCS [%]	Washed NREL-PCS [%]
Glucan and	36.5 ± 0.6	57.0 ± 0.6
Equivalent glucan[2]	5.2	--
Xylan	1.2	1.8 ± 0.0
Equivalent Xylan[2]	19.0 ± 0.3	--
Galactan	1.7 ± 0.1	0.0 ± 0.0
Arabinan	3.1 ± 0.0	0.9 ± 0.1
Mannan	0.7 ± 0.1	0.5 ± 0.1
Acid-Insoluble Lignin	17.0 ± 0.1	26.0 ± 0.1
Acid-Soluble Lignin[3]	NA	2.1 ± 0.0
Ash	6.1	9.2
Acetyl	5.9	0.3
Total	96.4 ± 1.2	97.8 ± 0.9

Note: 1. Values are expressed as mean and standard deviation (n=2).
2. Equivalent glucan and xylan is the theoretical amount obtained from monomers.

Table V-3: Compositional analysis of NaOH extracted PCS and HWI-PCS samples

Component	PCS 0.8%NaOH 70°C [%]	Hot-water insoluble PCS [%]	HWI-PCS 0.4%NaOH 80°C [%]	HWI-PCS 0.8%NaOH 80°C [%]	HWI-PCS 1.5%NaOH 80°C [%]
Glucan	82.1 ± 3.0	56.5 ± 0.4	82.5 ± 0.5	85.5 ± 0.6	87.9± 0.2
Xylan	2.0 ± 0.0	2.4 ± 0.0	2.0 ± 0.0	1.9 ± 0.0	1.0 ± 1.2
Galactan	0.0 ± 0.0	0.0 ±0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Arabinan	0.6 ± 0.0	1.0 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.1
Mannan	0.6 ± 0.1	0.6 ± 0.0	0.6 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
Acid-Insoluble Lignin	5.2 ± 1.0	25.1 ± 0.3	4.6 ± 0.1	4.0 ± 0.0	3.6 ± 0.9
Acid-Soluble Lignin	0.5 ± 0.0	1.7 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	0.5 ± 0.0
Ash	5.4 ± 0.0	8.2 ± 0.6	5.7 ± 0.1	4.9 ± 0.4	4.3 ± 0.1
Acetyl	0.0 ± 0.0	0.9 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Total	96.4 ± 4.1	96.4 ± 1.6	96.4 ± 0.7	97.7 ± 1.0	98.3 ± 2.5
Solid Remaining [2]	43.4	65.4	69.4	68.7	67.6
Delignification [3]	85.7 ± 2.6	--	86.1 ± 0.3	87.8 ± 0.1	89.0 ± 2.5

Note: 1. Values are expressed as mean and standard deviation (n=2).
2. Solid remaining is the amount of insoluble material remaining after the NaOH extraction.
3. Delignification values are based on dry PCS and HWI-PCS.

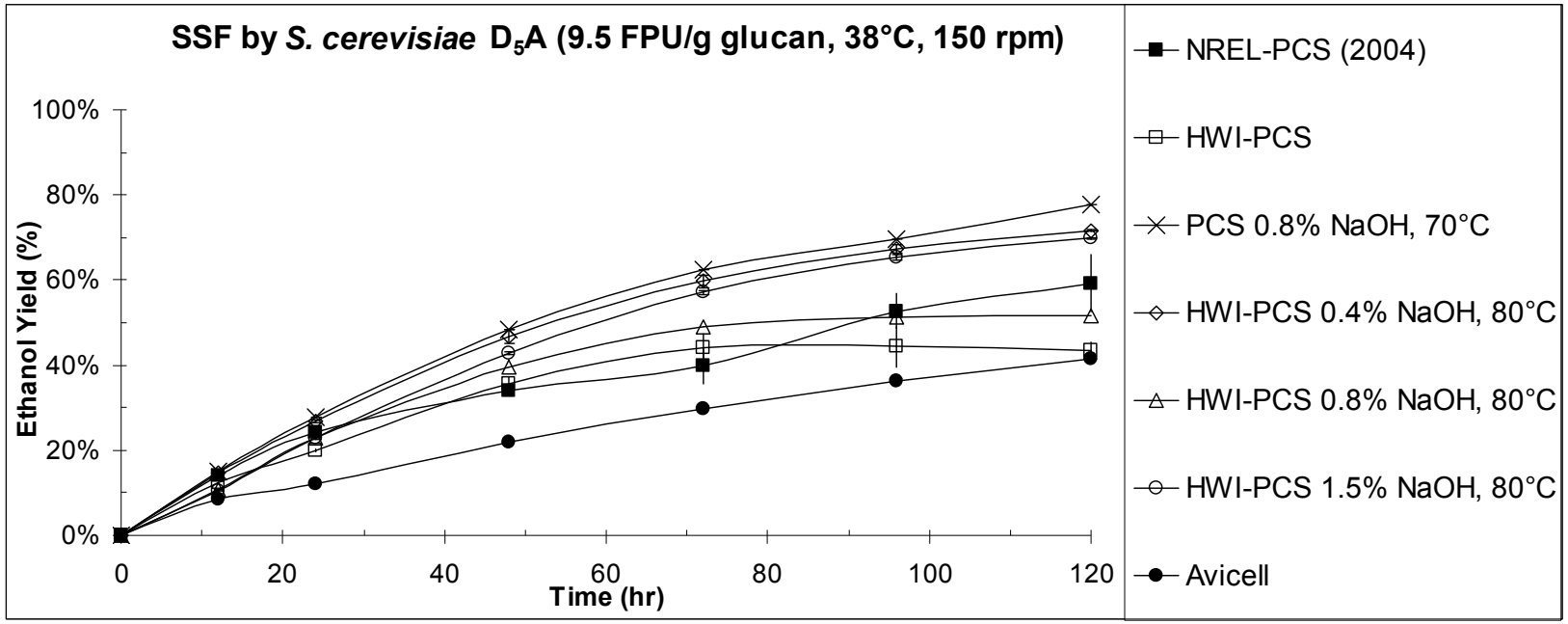


Figure V-I: SSF ethanol yield for various treated NREL PCS samples at 6% TS by *S. cerevisiae* D_5A.

Table V-4: SSF ethanol yield for various treated NREL PCS samples at 6% TS by *S. cerevisiae* D_5A (9.5 FPU/g glucan).

	Sampling time (hr) / Ethanol Yield (%)						
NREL-PCS	0	12	24	48	72	96	120
AVERAGE	0.0%	14.1%	24.0%	34.0%	39.8%	52.8%	59.3%
STDEV	0.0%	1.1%	--	0.9%	4.2%	4.0%	6.7%
HWI-PCS	0	12	24	48	72	96	120
AVERAGE	0.0%	12.4%	20.0%	35.5%	44.1%	44.3%	43.6%
STDEV	0.0%	0.1%	0.2%	1.8%	3.4%	4.6%	1.9%
NREL PCS 0.8% NaOH, 70°C	0	12	24	48	72	96	120
AVERAGE	0.0%	14.9%	27.7%	48.4%	62.3%	69.7%	77.8%
STDEV	--	--	--	--	--	--	--
HWI-PCS 0.4% NaOH, 80°C	0	12	24	48	72	96	120
AVERAGE	0.0%	14.7%	26.9%	46.8%	59.7%	67.3%	71.6%
STDEV	0.0%	--	0.4%	1.7%	1.5%	1.0%	0.1%
HWI-PCS 0.8% NaOH, 80°C	0	12	24	48	72	96	120
AVERAGE	0.0%	10.9%	23.0%	39.6%	48.9%	51.4%	51.8%
STDEV	0.0%	0.3%	0.9%	3.2%	4.3%	4.8%	7.1%
HWI-PCS 1.5% NaOH, 80°C	0	12	24	48	72	96	120
AVERAGE	0.0%	10.5%	23.0%	42.7%	57.1%	65.3%	69.8%
STDEV	0.0%	0.1%	0.3%	0.3%	0.5%	0.7%	0.3%
Avicell	0	12	24	48	72	96	120
AVERAGE	0.0%	8.3%	12.0%	21.8%	29.8%	36.2%	41.4%
STDEV	0.0%	0.1%	0.2%	0.4%	0.7%	0.6%	--

Note: 1. Values are expressed as mean and standard deviation (n=2).

Table V-5: SSF ethanol concentration (g/L) various treated NREL PCS samples at 6% TS by *S. cerevisiae* D_5A (9.5 FPU/g glucan).

	Sampling time (hr) / Ethanol concentration (g/L)						
NREL-PCS	0	12	24	48	72	96	120
AVERAGE	1.08	2.73	3.90	5.07	5.74	7.27	8.03
STDEV	0.00	0.13	--	0.10	0.50	0.47	0.79
HWI-PCS	0	12	24	48	72	96	120
AVERAGE	1.08	3.46	4.93	7.91	9.57	9.61	9.47
STDEV	0.00	0.01	0.04	0.36	0.65	0.89	0.37
PCS 0.8% NaOH, 70°C	0	12	24	48	72	96	120
AVERAGE	1.08	5.25	8.84	14.62	18.52	20.59	22.85
STDEV	--	--	--	--	--	--	--
HWI-PCS 0.4% NaOH, 80°C	0	12	24	48	72	96	120
AVERAGE	1.08	5.21	8.63	14.25	17.86	20.01	21.22
STDEV	0.00	N/A	0.12	0.47	0.42	0.28	0.04
HWI-PCS 0.8% NaOH, 80°C	0	12	24	48	72	96	120
AVERAGE	1.08	4.26	7.79	12.62	15.32	16.05	16.16
STDEV	0.00	0.08	0.25	0.93	1.26	1.40	2.06
HWI-PCS 1.5% NaOH, 80°C	0	12	24	48	72	96	120
AVERAGE	1.08	4.23	7.96	13.88	18.19	20.65	21.98
STDEV	0.00	0.04	0.09	0.10	0.16	0.20	0.08
Avicell	0	12	24	48	72	96	120
AVERAGE	1.08	3.82	5.01	8.23	10.85	12.97	14.65
STDEV	0.00	0.03	0.07	0.14	0.25	0.19	--

Note: 1. Values are expressed as mean and standard deviation (n=2).

VI. SIMULTANEOUS SACCHARAFICATION AND FERMENTATION OF PCS, HWI-PCS, AND VARIOUS NaOH-EXTRACTED PCS AND HWI-PCS SAMPLES (6% TS LOADING)

ABSTRACT

In this section, the SSF ethanol yield was investigated for HWI-PCS that has been extracted by 0.4% at 50ºC. The SSF biomass loading was 6% TS, and Avicel and HWI-PCS were also tested at 3% TS loading. The effect of increasing the amount of total solids is more noticeable for the HWI-PCS than for the Avicel.

INTRODUCTION

SSF of 2004 NREL PCS (received July 2005), HWI-PCS, and 0.4% NaOH-extracted HWI-PCS at 6% TS loading. Avicel and HWI-PCS were also tested at 3% total solids to see if there is any effect of solids concentration on the ethanol yield.

MATERIALS AND METHODS

Materials

The 2004 NREL standard pretreated corn stover (PCS) was provided by Abengoa Bioenergy R&D and was received in July 2005. The composition of the as-received PCS solids is shown in Table VI-2 and that of the liquor is shown in Tables VI-3 and VI-4 as

determined by the NREL standard analytical procedures. Avicel PH-101 (Fluka; Cat. No.11365; Lot No. 1094627) was used as a microcrystalline cellulose reference. Cellulase enzyme, Spezyme CP (Genencor, Lot No. 301-00348-257), was obtained from NREL and has an activity of 59 FPU/mL. Novozyme 188 β-glucosidase (Novo Inc., Lot no. 11K1088) has an activity of 750 CBU/mL. *Saccharomyces cerevisiae* ATCC® 200062 (NREL-D_5A) was used as the fermentation organism for the SSF procedure, and the yeast extract (Cat. No. Y-0500) and peptone (Cat. No. P-6588) used for the growth medium were purchased from Sigma. A New Brunswick Scientific (Edison, NJ) Series 25 incubator shaker was used for agitation and temperature control.

Experimental procedures

Hot-Water Extraction

The HWI-PCS was prepared in batch using six 1 L Erlenmeyer flasks in an incubator (New Brunswick Scientific, Series 25) maintained at 70ºC and 150 rpm for 30 minutes. In order to remove more than 90% of the total soluble components for batch operation, 10 mL DI water was added per gram PCS (dry basis).

Predetermined amounts of water and NREL PCS (received July 2005 from Nature Works) were preheated separately. To start the extraction, the 70°C water was poured into the flask containing the biomass. After the hot-water extraction the samples were vacuumed-filtered, rinsed with room-temperature DI water, and the samples were combined for compositional analysis and subsequent NaOH extraction. The solid remaining after the hot-water extraction was 65.0%.

NaOH Extractions

The caustic extractions were carried out following a similar procedure as described in section III of this paper but with a temperature of 50°C. The HWI-PCS has been extracted using 0.4% NaOH solution in batch mode using 1 L shake flasks in an incubated shaker at 50°C and 150 rpm. The liquid to solid ratio used was 15 mL NaOH / g biomass (dry basis).

After the 30 minute extraction, the biomass cake was rinsed with caustic at the same concentration and temperature as the extraction in a vacuum-filtering flask, followed by water washing also at 50ºC. The solid to liquid ratios used for the hot NaOH rinse and hot water wash were 1:25 and 1:50 g biomass / mL liquid, respectively. An additional 3 L of DI water at room temperature were needed to completely neutralize the biomass cake. A portion of the samples were freeze-dried overnight, and analyzed for composition using standard NREL methods. The rest of the biomass was stored under refridgeration in wet condition (approximately 20% TS) for the SSF test.

SSF Procedure

A slightly modified version of the Simultaneous Saccharification and Fermentation Procedure NREL LAP-008 was used with a total solids loading of 6% TS. Avicel and HWI-PCS were also tested at 3%TS loading.

Inoculum preparation

The Saccharamyces cerevisiae D_5A inoculum was aerobically prepared in two growth stages using 50 mL YPD medium (10 g/L yeast extract; 20 g/L peptone; 20 g/L

dextrose) in a 250 mL shake flask for the first stage. The second stage growth was inoculated with 10% v/w from the first stage growth flask. At the end of the second growth stage a portion of the inoculum was analyzed for ethanol by HPLC using H-column, glucose using YSI analyzer, pH, and dry cell mass as shown in Table 1.

SSF preparation

In each 250 mL SSF flask, 6 g biomass (dry basis) were added along with 10 mL of 10x YP medium (100 g/L yeast extract; 200 g/L peptone) and the appropriate amount of water to bring the final working volume to 100 mL. The flasks were weighed and sterilized at 121°C for 30 min, and any evaporated moisture was added back as sterile DI water upon cooling. Using a sterile pipette, 10 mL of the S. cerevisiae D_5A inoculum were added to the SSF flasks.

The cellulase was the last to be added to the flask. New Spezyme CP (Genencor 301-04075-054) has an activity of 59 FPU/mL for the stock solution (as-received) and was sterilized by filtration. The enzyme was diluted to 19 FPU/mL before adding 9.5 FPU/g glucan to the SSF flasks. The flasks were incubated at 38°C and 150 rpm for 120 hours using bubble traps to maintain anaerobic conditions. There was no pH control, and the pH gradually decreased from approximately 5.5 to 3.5 after 120 hours.

Samples were aseptically taken in a laminar hood at 24, 48, 72, 96, and 120 hours. Microcentrifuge tubes with screw cap were used to prevent ethanol evaporation. The centrifuge tubes were heated at 85°C for 25 min to stop the SSF. Lactic acid, glycerol, acetic acid, and ethanol were measured using an HPLC H-column. Cellobiose and glucose were measured by HPLC with a P-column. After the 120 hour sampling, 1 mL of

70% sulfuric acid were added to each flask to decrease the pH to approximately 1.5, and the SSF slurries were frozen in plastic containers for future compositional analysis. The ethanol yield is calculated as % theoretical maximum,

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

where the factor 0.511 is the conversion factor for glucose to ethanol based on stoichiometric biochemistry of yeast.

RESULTS AND DISCUSSION

The 0.4% NaOH extracted HWI-PCS sample has a good ethanol yield of about 77% at 120 hrs. The 2004 NREL PCS (received July 2005) has very poor SSF yield possibly due to the high acetic acid content. Strangely, the HWI-PCS performed better in this experiment as compared to Section V of this paper.

The ethanol concentration and yield for the HWI-PCS with 6% TS loading are 15 g/L and 69%, respectively, and are 6.5 g/L and 52% for the 3% TS case. Based on this result, it would be desirable to keep the total solids higher.

The as-received NREL PCS (received July 2005) from has a fraction insoluble solids (%FIS) of 62.8% ± 0.6% and has 18.6% ± 0.2% insoluble solids (%IS) as determined by NREL LAP-018 using the filtration method. The NREL PCS liquor was obtained by pressing the as-received PCS using cheesecloth and then centrifuged. The density of the pressate is 1.068 g/mL.

CONCLUSIONS

The dilute-caustic treatment HWI-PCS using 0.4% NaOH at 50°C appears a feasible treatment to fractionate the lignin prior to use in the process of biomass conversion for bioethanol production. It is believed that increasing the amount cellulase would have a positive effect on the ethanol yield. By increasing the total solids content from 3% to 6%, the ethanol yield also increased.

Table VI-1: Second stage inoculum of *S. cerevisiae* D_5A in YPD medium.

Ethanol (g/L)	12.3
Glucose (mg/L)	11.2
pH	4.9
Dry Cell Mass (g/L)	7.3

Table VI-2: Compositional analysis of as-received and washed NREL PCS (received July 2005).

Component	As-received NREL PCS [%]	Washed NREL PCS [%]
Glucan	37.4 ± 0.1	59.5 ± 0.3
Equivalent glucan	4.6	--
Xylan	0.8	2.4 ± 0.2
Equivalent Xylan	18.3 ± 0.0	--
Galactan	1.3 ± 0.4	1.4 ± 0.0
Arabinan	3.1 ± 0.3	1.2 ± 0.1
Mannan	0.6 ± 0.1	0.8 ± 0.0
Acid-Insoluble Lignin	17.7 ± 0.1	26.7 ± 0.1
Acid-Soluble Lignin	2.7 ± 0.0	1.6 ± 0.1
Ash	5.2 ± 0.5	7.7 ± 0.2
Acetyl	1.3 ± 0.7	1.0 ± 0.4
Total	93.0 ± 2.2	102.3 ± 1.4

Note: Values are expressed as mean and standard deviation (n=2).
Equivalent glucan and equivalent xylan is the theoretical amount obtained from their monomers.

Table VI-3: Compositional analysis of as-received NREL PCS (received July 2005) liquor.

Component	Monomeric Sugars [g/L]	Total Sugars [g/L]
Cellobiose	1.2 ± 0.0	--
Glucose	16.6 ± 0.5	18.4 ± 0.0
Xylose	72.5 ± 0.0	64.3 ± 0.0
Galactose	5.2 ± 0.2	6.1 ± 0.0
Arabinose	8.9 ± 0.2	10.3 ± 0.0
Mannose	0.0 ± 0.0	0.0 ± 0.0

Table VI-4: Compositional analysis of as-received NREL PCS (received July 2005) liquor.

Component	Concentration [g/L]
Xylitol	0.0 ± 0.0
Succinic Acid	1.2 ± 0.0
Lactic Acid	0.6 ± 0.0
Glycerol	0.5 ± 0.0
Acetic Acid	14.8 ± 0.0
Ethanol	0.1 ± 0.0
HMF	0.5 ± 0.0
Furfural	4.5 ± 0.0

Table VI-5: Compositional analysis of as-received NREL PCS, HWI-PCS, and NaOH-extracted HWI-PCS samples.

Component	As-received NREL-PCS [%]	HWI-PCS [%]	HWI-PCS 0.4% NaOH 50°C [%]
Glucan	37.4 ± 0.1	58.8 ± 0.0	81.3 ± 2.1
Equivalent glucan[2]	4.6	--	--
Xylan	0.8	0.0 ± 0.0	0.0 ± 0.0
Equivalent Xylan[2]	18.3 ± 0.0	--	--
Galactan	1.3 ± 0.4	0.0 ± 0.0	0.0 ± 0.0
Arabinan	3.1 ± 0.3	0.7 ± 0.0	0.5 ± 0.0
Mannan	0.6 ± 0.1	0.5 ± 0.0	0.6 ± 0.1
Acid-Insoluble Lignin	17.7 ± 0.1	25.4 ± 0.5	8.1 ± 3.0
Acid-Soluble Lignin	2.7 ± 0.0	1.3 ± 0.0	0.5 ± 0.0
Ash	5.2 ± 0.5	8.7 ± 0.7	7.4 ± 0.9
Acetyl	1.3 ± 0.7	1.1 ± 0.4	0.1 ± 0.1
Total	93.0 ± 2.2	96.5 ± 1.6	98.5 ± 6.2
Solid Remaining [3]	--	65.0	71.1
Delignification [3]	--	--	75.9 ± 8.4

Note: 1. Values are expressed as mean and standard deviation (n=2).
2. Equivalent glucan and equivalent xylan is the theoretical amount obtained from their monomers.
3. Solid Remaining and Delignification values are based on dry PCS and HWI-PCS.

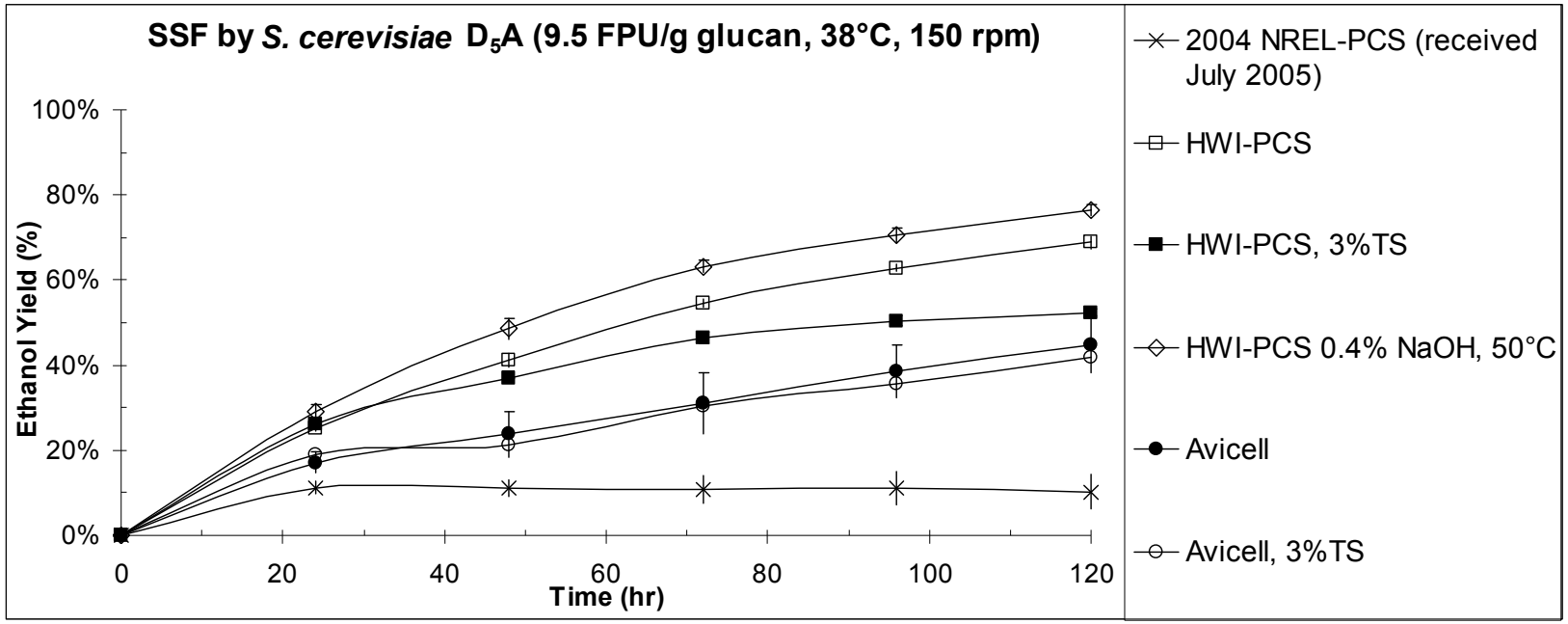


Figure VI-I: SSF ethanol yield for various treated NREL PCS samples by *S. cerevisiae* D_5A.

Table VI-6: SSF ethanol yield for various treated NREL PCS samples by *S. cerevisiae* D_5A with 6% TS biomass loading except as noted (9.5 FPU/g glucan).

	Sampling time (hr) / Ethanol Yield (%)					
2004 NREL-PCS (received July 2005)	0	24	48	72	96	120
AVERAGE	0.0%	11.3%	11.1%	10.8%	11.1%	10.2%
STDEV	0.0%	1.6%	1.9%	3.3%	3.9%	4.1%
HWI-PCS 0.4% NaOH, 50°C	0	24	48	72	96	120
AVERAGE	0.0%	28.9%	48.5%	63.2%	70.6%	76.5%
STDEV	0.0%	1.8%	2.4%	1.6%	1.6%	1.3%
HWI-PCS 6%TS	0	24	48	72	96	120
AVERAGE	0.0%	25.3%	41.1%	54.7%	62.9%	68.8%
STDEV	0.0%	0.9%	1.6%	0.9%	0.9%	1.4%
HWI-PCS 3%TS	0	24	48	72	96	120
AVERAGE	0.0%	26.1%	37.1%	46.4%	50.5%	52.4%
STDEV	0.0%	0.8%	0.3%	1.2%	1.2%	0.9%
Avicell 6% TS	0	24	48	72	96	120
AVERAGE	0.0%	17.1%	23.8%	31.2%	38.4%	44.9%
STDEV	0.0%	2.5%	5.4%	7.2%	6.2%	6.5%
Avicell, 3%TS	0	24	48	72	96	120
AVERAGE	0.0%	19.1%	21.4%	30.3%	35.6%	41.9%
STDEV	0.0%	1.8%	2.3%	2.1%	2.6%	2.6%

Table VI-7: SSF ethanol concentrations (g/L) for various treated NREL PCS samples by *S. cerevisiae* D_5A with 6% TS biomass loading except as noted (9.5 FPU/g glucan).

	Sampling time (hr) / Ethanol concentration (g/L)					
2004 NREL-PCS (received July 2005)	0	24	48	72	96	120
AVERAGE	1.23	2.67	2.65	2.60	2.65	2.53
STDEV	0.00	0.20	0.25	0.42	0.49	0.53
HWI-PCS 0.4% NaOH, 50°C	0	24	48	72	96	120
AVERAGE	1.23	9.25	14.68	18.73	20.80	22.43
STDEV	0.00	0.50	0.67	0.43	0.45	0.35
HWI-PCS 6% TS	0	24	48	72	96	120
AVERAGE	1.23	6.30	9.48	12.19	13.83	15.02
STDEV	0.00	0.17	0.33	0.19	0.18	0.28
HWI-PCS 3%TS	0	24	48	72	96	120
AVERAGE	1.23	3.84	4.94	5.88	6.29	6.48
STDEV	0.00	0.08	0.03	0.12	0.12	0.09
Avicell 6% TS	0	24	48	72	96	120
AVERAGE	1.23	6.86	9.03	11.46	13.84	15.96
STDEV	0.00	0.83	1.78	2.35	2.04	2.14
Avicell, 3%TS	0	24	48	72	96	120
AVERAGE	1.23	4.37	4.73	6.21	7.08	8.11
STDEV	0.00	0.29	0.37	0.35	0.43	0.43

VII. LIGNIN EXTRACTION AND RECOVERY

ABSTRACT

An experiment was conducted to determine the optimum condition to precipitate lignin from lignin-rich filtrate from the caustic extraction considering pH, temperature, and time of acid addition (before or after heating).

Lignin concentrations of 40 g/L were obtained by maintaining the pH of the solution around 12.5. Approximately 74% of the total lignin available in the HWI-PCS was extracted and precipitated.

INTRODUCTION

There is a desire to extract and recover lignin in relatively pure form as a coproduct of the bioethanol process. Typically lignin is combusted to produce steam and therefore power, but there is a growing market for lignin fibers in possible areas such as composite materials which use carbon-fibers that can be prepared from lignin.

By first removing the hemicellulose from corn stover by a dilute-acid process, lignin can be extracted in relatively pure form by a subsequent alkaline extraction. The lignin can then be precipitated out of the lignin-rich liquor by lowering the pH to around 1.5 to 2.0.

MATERIALS AND METHODS

Hot-water extraction

The HWI-PCS was prepared in batch using a 4 L Erlenmeyer flask in an incubated shaker (New Brunswick Scientific, Series 25) maintained at 70ºC and 150 rpm for 30 minutes. In order to remove more than 90% of the total soluble components for batch operation, 10 mL DI water was added per gram PCS (dry basis).

Predetermined amounts of water and NREL PCS (received July 2005) were preheated separately. To start the extraction, the 70°C water was poured into the flask containing the biomass. After the hot-water extraction the HWI-PCS was vacuumed-filtered, rinsed with room-temperature DI water, and freeze dried for lignin analysis and subsequent NaOH extraction. The solid remaining after the hot-water extraction was 65.1%.

NaOH extractions

Lignin was extracted from the HWI-PCS using 0.8% NaOH in a series of five consecutive batch extractions at 50ºC and 150 rpm. Exactly 60 g HWI-PCS dry basis (253.5 g wet) were loaded in each of five 2 L Erlenmeyer flasks. A liquid to solid ratio of 15 g 0.8% NaOH / g dry PCS was used; therefore, 900 g caustic were preheated to 50ºC and added to the first flask. After the 30 min extraction, the slurry was vacuum filtered using a Buchner funnel to recover the lignin-rich filtrate.

The biomass cake was pressed by using plastic wrap over the funnel to create a seal and allowing the vacuum to stretch the plastic wrap and press the cake to remove any free moisture. The pH of the liquor was adjusted to approximately 12.5 using 40% NaOH

and, after heating back to 50ºC, the filtrate was added to the second flask. This procedure was continued for the remaining flasks. The insoluble biomass cakes were washed thoroughly with water and analyzed for lignin content. The lignin analysis is shown in Table VII-1.

To prepare more of the filtrate in order to have enough for the following tests, the entire experiment was repeated on half-scale and the resulting filtrate was combined with the first set of extractions. The total volume of combined filtrate is 2.3928 L. The final pH of the combined filtrates is about 11.1 and the density is 1.0221 g/mL.

Titration test

The liquor was titrated using 10 N H_2SO_4 and 1 N H_2SO_4 to find the required amounts of acid to lower the pH to 1.5, 1.8, and 2.0 for the lignin precipitation tests. It was determined that in order to lower the pH of the filtrate to 1.5, 1.8, and 2.0 that 2 mL of 10 N H_2SO_4 must be added in addition to 14.25, 9.5, and 7.62 mL of 1 N H_2SO_4, respectively. The titration results are shown in Table VII-3.

Test A – Time of acid addition

To determine the best time for when to lower the pH for lignin precipitation, an experiment was conducted in which two beakers containing 100 mL of the dark-black filtrate by mass were heated to 85ºC and the pH lowered to 1.8. The pH was adjusted before heating for the first beaker and adjusted after heating for the second case.

#1 Acid addition before heating

A predetermined amount of sulfuric acid (2 mL 10 N, and 9.5 mL 1 N) was added to 100 mL filtrate at room temperature. A magnetic stirrer and digital thermocouple were used. After the pH was reduced to 1.8 lignin immediately precipitated, and the stir rate was increased to maximum due to the increased viscosity. The temperature was raised to 85ºC over 10 min using a hot-plate and kept between 84 - 87ºC for five minutes before filtering while still hot. The lignin precipitated uniformly fine particles. A Buchner funnel (Coors USA 60244) was used with Fisher P8 fast, coarse filter paper. The liquid filtered immediately as it was poured onto the filter. The lignin cake was rinsed with a small amount of DI water to remove the Na_2SO_4 salt. After drying at 45ºC, 3.913 g of lignin was recovered from the 100 mL filtrate.

#2 Acid addition after heating

The 100 mL filtrate was first heated to 80ºC, and then the required acid was added to lower the pH to 1.8. The filtrate was dark-black until the addition of acid when it quickly turned brown. Precipitation was allowed for five minutes at 85ºC before filtering. The lignin appeared to precipitate with a range of particle sizes between fines to around 1 mm. As with case #1, the liquid filtered immediately as it was poured onto the filter and had negligible filtering time. After drying at 45ºC, 4.004 g of lignin was recovered from the 100 mL filtrate.

Test B - pH

The lignin precipitation was tested at pH values of 1.5, 1.8, and 2.0. Sulfuric acid was added in amounts previously determined in the titration test to 100 mL of the lignin-rich filtrate, and the liquid was heated to 85°C using a hotplate with magnetic stirrer and digital thermocouple. The preheating time was approximately 10 minutes and the liquid was kept at 85°C for 5 minutes before filtering while hot. The filtration time was negligible, and the liquid was able to be filtered immediately upon pouring.

The amount of lignin precipitated from the 100 mL filtrate is 4.0259, 3.9220, and 3.8455 g, respectively. This result shows that as the pH is reduced further, more lignin is able to be precipitated. The pH of 1.8 was chosen for the following temperature test.

Test C – Temperature

Temperatures of 40, 60, and 80ºC were explored to see the effect of temperature on lignin precipitation. After adding the predetermined amount of sulfuric acid to 100 mL filtrate in order to lower the pH to 1.8, the liquid was heated to the temperature set point. The preheating time was kept to 10 minutes, and the mixture was kept at the set temperature for 5 minutes. For the 40, 60, and 80ºC cases, filtration time was 90, 60, and 5 seconds, respectively, while the amount of lignin precipitated was 3.9982, 4.1481, and 3.834 g, respectively. The lignin color after 45ºC drying was dark black for temperatures up to around 60ºC and is colored brown for temperatures around 80ºC and higher.

RESULTS AND DISCUSSION

Almost 75% of the original lignin in the HWI-PCS was extracted and precipitated. The NaOH is neutralized during the caustic extraction; therefore additional NaOH must be added to maintain the pH. Also, more silica is solubilized with increased pH especially around pH of 10 and higher. This result shows up as lower ash content for the samples with higher NaOH concentration in the solid analyses from the previous sections.

The amount of lignin extracted is related to the pH of the caustic solution; with higher pH levels, there is more capacity to solubilize lignin. Similarly, more lignin can be precipitated out of solution by further lowering the pH of the lignin-rich filtrate. It was found that there was not much difference in the amount of lignin precipitated by lowering the pH beyond 1.8.

CONCLUSIONS

Lignin can be effectively extracted and recovered using dilute-caustic treatment of the PCS to solubilize the lignin followed by acid-precipitation of the resulting slurry. Precipitation conditions including pH, time of acid addition, and temperature were investigated, and it was determined that quantitatively there is little difference between the conditions tested. However, there is a qualitative difference in the type of lignin precipitated over the temperature range of 25-85°C. Lignin color is dark black at temperatures of about 25°C, and the color gradually changes to brown around 45°C and progressively lighter shades of brown up to 85°C. Temperatures of around 80-90°C are optimum for fast filtration times.

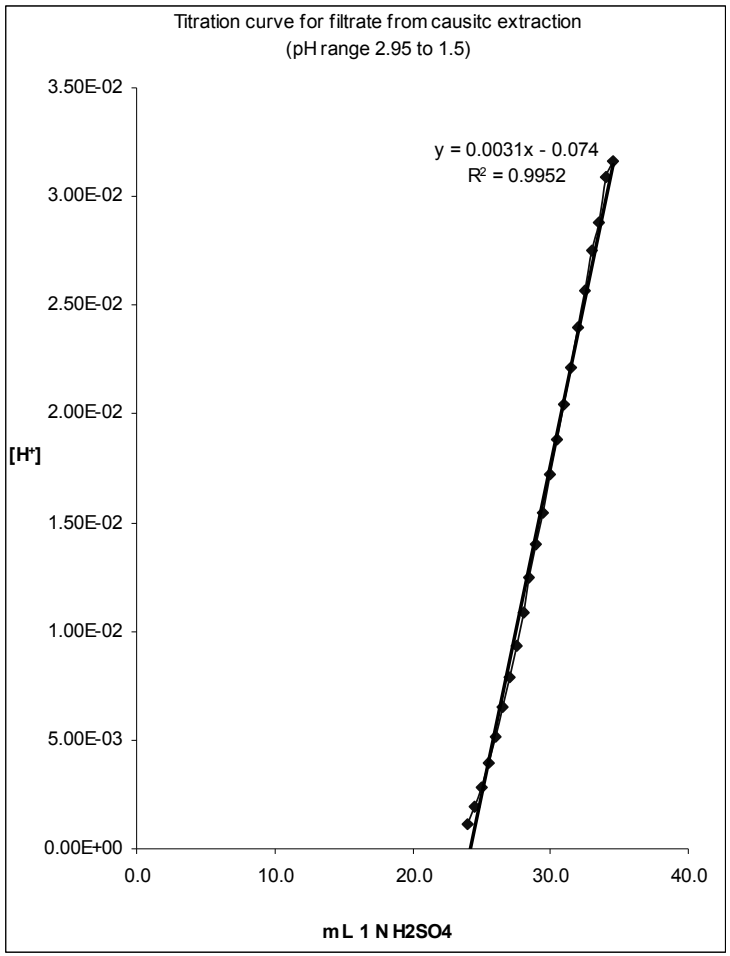
Table VII-1: Lignin analysis of the insoluble solids after caustic extraction.

	HWI-PCS	0.66 % NaOH, 50°C HWI-PCS 1st Flask	0.71 % NaOH, 50°C HWI-PCS 2nd Flask	0.81 % NaOH, 50°C HWI-PCS 3rd Flask	0.95 % NaOH, 50°C HWI-PCS 4th Flask	1.02 % NaOH, 50°C HWI-PCS 5th Flask
Acid-insoluble lignin	28.8 ± 0.1	5.3 ± 1.4	5.7 ± 0.3	7.1 ± 0.0	8.0 ± 0.5	9.5 ± 0.0
Solid-remaining	65.1	65.3	68.1	69.1	69.4	70.3
Delignification	--	87.9 ± 3.3	86.6 ± 0.8	83.0 ± 0.1	80.8 ± 1.3	76.9 ± 0.1

Note: Solid-remaining and delignification values are calculated on dry basis.
NaOH concentration is calculated considering water present in HWI-PCS.

Table VII-2: Filtrate pH for each of the consecutive NaOH extractions.

	0.8% NaOH	1st Flask	2nd Flask	3rd Flask	4th Flask	5th Flask
pH before extraction	13.27	--	12.54	12.51	12.47	12.49
pH after extraction	--	12.15	11.50	11.15	11.35	11.29
40% NaOH added (mL)	--	5.00	7.50	10	8	--
Adjusted pH	--	12.54	12.51	12.47	12.49	--



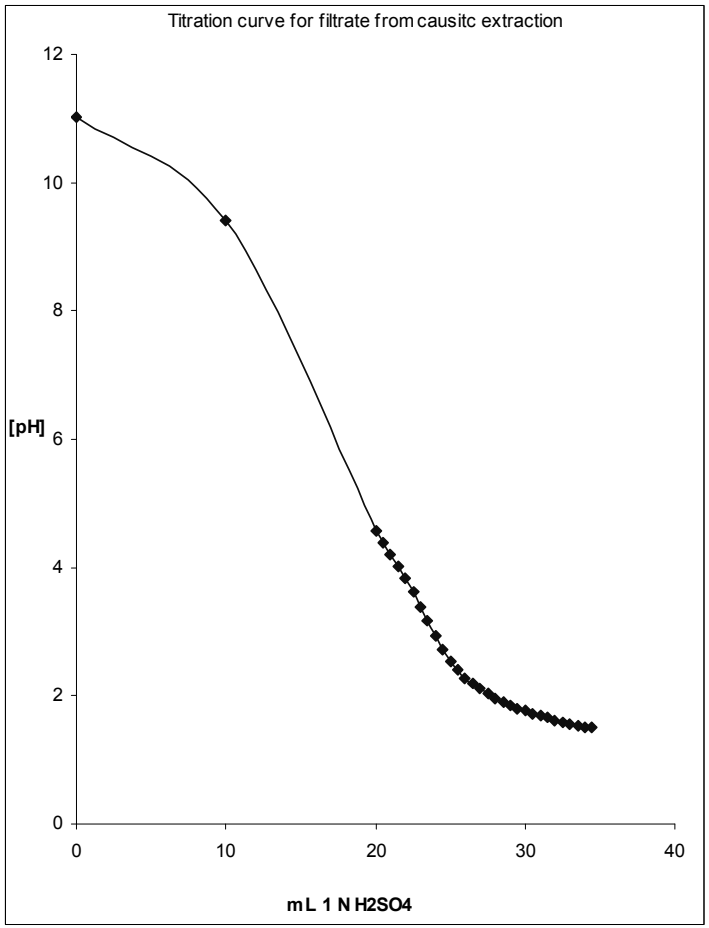


Table VII-3: Titration curves for 100 mL filtrate using sulfuring acid (average of duplicates)

BIBLIOGRAPHY

Bernton, H., Kovarik, W., Sklar, S., 1982. The Forbidden Fuel: Power Alcohol in the Twentieth Century. Boyd Griffen, New York.

Bordetsky, A., Hwang, R., Korin, A., Lovaas, D., Tonachel, L., 2005. Securing America: Solving Our Oil Dependence Through Innovation. National Resources Defense Council, New York. 5, 40-41.

Compere, A.L., Griffith, W.L., Leitten Jr., C.F., and Shaffer, J.T., 2002. Low Cost Carbon Fiber From Renewable Resources. Oak Ridge National Laboratory. Retrieved on August 20, 2005, from http://www.ornl.gov/~webworks/cppr/y2001/pres/111380.pdf

Eggeman, T., and Elander, R.T., 2005. Process and economic analysis of pretreatment technologies. Bioresource Technology, 96, 2019-2025.

Elshafei, A.M., Vega, L.L., Kalsson, K.T., Clausen, E.C., and Gaddy, J.L., 1991. The saccharification of corn stover by cellulase from *Penicillium funiculosum*. Bioresource Technology. 35, 73-80.

Ethanol Timeline. Retrieved August 20, 2005, from http://www.eia.doe.gov/kids/history/timelines/ethanol.html

Garrett and Grisham, 1999. Biochemistry. 2nd ed. Brooks/Cole.

Glasser, W.G., Northey, R.A., Schultz, T.P., 2000. Lignin: Historical, Biological, and Materials Perspectives. American Chemical Society, Washington, D.C.

Griffith, W.L., Compere, A.L., Leitten Jr., C.F., and Shaffer, J.T., 2003. Low-Cost, Lignin-based Carbon Fiber for Transportation Applications. ORNL

Hu Chemical Modification, Properties, and Usage of Lignin. Kluwer Academic / Plenum Publishers, New York, 121-137.

Heid, W.G., 1984. Turning Great Plains Crop Residues and Other Products into Energy. USDA Agricultural Economics Report No. 523.

Himmel, M., Vinzant, T., Bower, S., Jechura, J., 2005. BSCL Use Plan: Solving Biomass Recalcitrance. NREL/TP-510-37902.

Kadam, K.L., McMillan, J.D., 2002. Availability of corn stover as a sustainable feedstock for bioethanol production. Bioresource Technology. 88, 17-25.

Kadla, J.F., Kubo, S., Venditti, R.A., Gilbert, R.D., Compere, A.L., Griffith, W., 2002. Lignin-based carbon fibers for composite fiber applications. Carbon. 40, 2913-2920.

Kim, T.H., 2004. Bioconversion of Lignocellulosic Material Into Ethanol: Pretreatment, Enzymatic Hydrolysis, and Ethanol Fermentation. Thesis (Ph. D.)–Auburn University, 2004.

Kim, S. and Dale, B.E., 2004. Global potential bioethanol production from wasted crops and crop residues. Biomass and Bioenergy. 26, 361-375.

Klass, D.L., 1998. Biomass for Renewable Energy, Fuels, and Chemicals. Academic Press, San Diego.

Leatham, G.F., and Himmel, M.E., 1991. Enzymes in Biomass Conversion. ACS Symposium Series, Washington DC, 460.

Laureano-Perez, L., Teymouri, F., Alizadeh, H., Dale, B., 2005. Understanding Factors that Limit Enzymatic Hydrolysis of Biomass. Applied Biochem. Biotech., 121-124, 1081-1100.

Lignins – Products With Many Uses. Retrieved on October 1, 2005 from http://www.lignin.org/whatis.html

Lignin-Derived Co-Products. Retrieved on August 20, 2005, from http://www.eere.energy.gov/biomass/lignin_derived.html

Mansfield, S.D. and Saddler, J.N., 2003 Applications of Enzymes to Lignocellulosics. ACS Symposium Series, Washington, DC, 855.

McDonald, D.G., Bakhshi, N.N., Mathews, J.F. Roycowdhury, A., Bajpai, P., and Moo-Young, M., 1983. Alkali treatment of corn stover to improve sugar production by enzymatic hydrolysis. Biotech. Bioeng. 25, 2067-2076.

Montague, L., 2003. Lignin Process Design Confirmation and Capital Cost Evaluation. NREL/SR-510-31579.

NREL, 2004. Chemical Analysis and Testing Laboratory Analytical Procedures (CAT). National Reneewable Energy Laboratory, Golden, CO.

Otani, S., Fukuoka, Y., Igarashi, B., Sasaki, K., 1969. Method for producing carbonized lignin fiber. US Patent 3,461,082.

Renewable Fuels Association. Homegrown for the Homeland Ethanol Industry Outlook 2005.

Saddler, J.N., and Penner, M.H., 1995. Enzymatic Degradation of Insoluble Carbohydrates. ACS Symposium Series, Washington DC, 618.

Schell, D.J., Farmer, J., Newman, M., McMillan, J.D., 2003. Dilute-sulfuric acid pretreatment of corn stover in pilot-scale reactor. Applied Biochem. Biotechnol., 105-108, 69-85.

Schell, D.J., Riley, C.J., Dowe, N., Farmer, J., Ibsen, K.N., Ruth., M.F., Toon, S.T., Lumpkin, R.E., 2004. A bioethanol process development unit: initial operating experiences and results with a corn fiber feedstock. Biores. Technol., 91, 179-188.

Sun, Y., and Cheng, J., 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. Biores. Technol., 83, 1-11.

U.S. Department of Energy, 2004. Annual Energy Review, Energy Information Administration. Washington, DC, DOE/EIA-0384(2004).

U.S. Department of Energy, 2005. Annual Energy Outlook with Projections to 2025, Energy Information Administration. Washington, DC, DOE/EIA-0383(2005).

Varga, E., Szengyel, Z., Réczey, K., 2002. Chemical Pretreatments of Corn Stover for Enhancing Enzymatic Digestibility. Appl. Biochem. Biotechnol., 98-100, 73-87.

Varga, E., Réczey, K, Zacchi, G., 2004. Optimization of Steam Pretreatment of Corn Stover to Enhance Enzymatic Digestibility. Appl. Biochem. Biotechnol., 113-116, 509-523.

Wyman, C.E., 1996. Handbook on Bioethanol: Production and Utilization. Taylor & Francis, Washington D.C.

Wyman, C.E., Dale, B.E., Elander, R.T., Holtzapple, M., Ladisch, M.R., Lee, Y.Y., 2005. Coordinated development of leading biomass pretreatment technologies. Bioresource Technology. 96, 1959-1966.

Wyman, C.E., Dale, B.E., Elander, R.T., Holtzapple, M., Ladisch, M.R., Lee, Y.Y., 2005. Comparative sugar recovery data from laboratory scale application of leading pretreatment technologies to corn stover. Bioresource Technology. 96, 2026-2032.