A B S T R A C T

WILLIAMS, KELLY CALDWELL. Subcritical Water and Chemical Pretreatments of Cotton Stalk for the Production of Ethanol. (Under the direction of Ratna R. Sharma-Shivappa.)

The objective of this study was to explore the potential of sodium hydroxide, sodium percarbonate and subcritical water as pretreatments for cotton stalk to aid ethanol production. Sodium hydroxide was tested at concentrations of 0, 4 and 8 % (w/v) and sodium percarbonate was tested at concentrations of 0, 1, 2, 4 and 8 % (w/v). Cotton Stalks were pretreated with the chemicals by autoclaving 10% solids slurry at 121°C and 15psi for 30, 60 or 90 minutes. Both chemical pretreatments used a factorial experimental design where each time was run with each concentration. NaOH degraded more lignin with the maximum being 63.4% at 4% concentration and 30-minute treatment time. The maximum lignin degradation by Na-percarbonate was 42.0% at 8% concentration and 90-minute treatment time. Higher concentrations of both chemicals degraded more lignin. Based on the HPLC carbohydrate analysis, NaOH produced significantly higher xylan solubilization than Na-percarbonate. The maximum solubilizations for NaOH and Na-percarbonate, respectively, were 82.7% with 8% concentration and 90-minute treatment time and 59.0% with 2% concentration and 30-minute treatment time. Higher concentrations of NaOH produced higher xylan solubilizations but the solubilization values did not change significantly ($p>0.05$) for concentrations above 0% Na-percarbonate. Subcritical water was tested at temperatures of 230, 275 and 320°C, holding times of 2, 6 and 10 minutes and ground particles sizes of 1/8, 3/16 and 1/4 inches using a response surface model experimental design. Whole and smashed cotton stalks were also pretreated with subcritical water for each time-temperature

combination. Enzymatic hydrolysis was performed on the three ground sample combinations showing the highest lignin degradation and the three with the highest percent total sugars. Lignin analysis was done on all pretreated samples and sugars were analyzed using DNS assay for the subcritical water pretreatments and HPLC for the chemical pretreatments. The highest percent total sugar of 46.3% was found for 230°C, 10 minutes and 3/16 inches and the highest percent lignin reduction of 36.7% was found for 320°C, 2 minutes and 3/16 inch particle size. This suggests that lower temperatures produce more total sugars and higher temperatures produce higher lignin degradation. Response surface models were developed for percent total sugars and percent lignin reduction as a function of time, temperature and ground particle size with R-squared values of .6048 and .5112, respectively. The maximum percent total sugar for the whole cotton stalks was 44.8% for the 230°C, 10 minutes combination and for the smashed stalks it was 22.6% for the 320°C, 2 minutes combination. Except in one case, smashing the stalks did not increase the percent total sugars and did not increase the lignin reduction in any cases when compared to the whole stalks. Also, the ground stalks did not show any significant increase in lignin degradation when compared to whole and smashed but there was an increase in the percent total sugars for two of the whole sample sets and four of the smashed sample set. Pure lignin and cellulose samples were pretreated with subcritical water revealing that interaction between the components of lignocellulosic biomass affects the effectiveness of subcritical water pretreatment.

SUBCRITICAL WATER AND CHEMICAL PRETREATMENTS OF COTTON STALK FOR THE PRODUCTION OF ETHANOL

by
KELLY CALDWELL WILLIAMS

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Chair of Advisory Committee

B I O G R A P H Y

Kelly was born in Wilmington, NC as the first child of Hugh and Linda Caldwell. She lived in Wilmington until she moved to Raleigh, NC to attend North Carolina State University in 1999. She graduated in December 2003 with a BS in Mechanical Engineering and married the love of her life in January 2004. She spent the spring looking for a mechanical engineering job that wasn't completely boring but found nothing. It was at this time she decided that it was time for a change. She applied to the Biological and Agricultural Engineering department at the end of the spring and by some miracle they had funding for her to start in August. Kelly had so much fun getting her Masters that she decided to stay in school and get her PhD. Although NC State was wonderful she decided that it was time to do something really crazy, so she and her supportive husband will be moving to California in the fall of 2006 so she can attend UC Davis and earn her PhD.

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Chapter 1: Introduction

1.1 Background

A rise in the world's demand for energy has increased the need for alternate fuel sources. Biofuels are being explored as a solution to this problem; two of the most predominant being biodiesel and bioethanol. Biodiesel is most commonly made through transesterification of vegetable oils and animal fats and has the potential to be economically competitive with conventional diesel if made from waste vegetable oil (Zhang et al., 2003). The technology for bioethanol production from corn is well defined, however, production from other feedstocks such as lignocellulosic biomass still requires extensive research to develop a feasible production method. For this reason, this study is focused on technology to convert lignocellulosic biomass to ethanol.

Ethanol is made from the fermentation of sugars. Glucose, one of the most simple sugars, can be obtained from the cellulose found in plant matter, particularly lignocellulosic biomass. This biomass, as evident from its name, contains cellulose, lignin and hemicellulose. The challenge in using lignocellulosic biomass as an ethanol source is removing the lignin and hemi cellulose from the cellulose. The basic steps for ethanol production from lignocellulosic feedstocks are pretreatment, hydrolysis and fermentation. Pretreatment reduces cellulose fiber crystallinity and increases porosity and enzymatic accessibility. It can be achieved through physical, physio-chemical, chemical and biological pretreatments (McMillan, 1994).

Physical pretreatment methods include mechanical comminution and pyrolysis. Mechanical comminution, which includes grinding, milling and chipping, requires a high energy input. Pyrolysis involves heating the substrate at a high temperature causing decomposition of the substrate and works best when used in conjunction with other methods such as acid hydrolysis. Physio-chemical methods, such as steam explosion, require treatment of the biomass with high-pressure steam and then a quick reduction in the pressure. This rapid pressure drop causes the biomass to "explode". This method requires a low energy input but does not always completely separate the cellulose. Chemical pretreatments among others include ozonolysis, which is effective but expensive, and acid, alkali and oxidative delignification, which are effective, but require non-corrosive reactors in addition to problems with disposal of waste chemicals. Biological pretreatments involve microorganisms to break down the undesirable lignin and hemicellulose. This is an environment friendly and inexpensive method that involves lignin-degrading enzymes. However, it requires prolonged pretreatment intervals (Sun & Cheng, 2002).

After pretreatment, the next step in the production of ethanol from lignocellulosic biomass is hydrolysis or the break down of the sugar polymers into monomers. This degradation is usually done by enzymes or acid. After hydrolysis, the glucose is fermented into ethanol. Simultaneous saccharification and fermentation (SSF) may be employed to combine the two processes of hydrolysis and fermentation into one by using enzymes to convert cellulose and hemicellulose into reducing sugars parallel to a fermentation process occurring in the same reactor. The optimal temperatures for hydrolysis and fermentation are different, but this process has potential if all the incompatibilities can be worked out (Sun & Cheng, 2002). An efficient pretreatment method can enhance hydrolysis and therefore provide more sugars to be fermented into ethanol. Thus, an effective pretreatment method is thus critical for successful biomass to ethanol conversion.

In this study two chemical pretreatment methods involving sodium hydroxide (NaOH) and sodium percarbonate ($2Na_2CO_3 \cdot 3H_2O_2$) were explored. Sodium hydroxide has been shown to reduce lignin in cotton stalks by up to 65 percent at 2% w/v concentration for 90 minutes at 120 C (Silverstein, 2004). Sodium hydroxide has also been shown to increase the amount of glucose released by enzymatic hydrolysis from around 1 g/mL of glucose for untreated corn stover to 6 g/mL of glucose for 1.0N sodium hydroxide treated corn stover (Li et al., 2004). Burdette (2004) investigated the use of sodium percarbonate during pretreatment and found that the amount of available sugars increases in corn stover when treated with 2.5% sodium percarbonate for 24 hrs at 80 C (Burdette et al., 2004).

This study also investigated a less explored subcritical water pretreatment method. This process involves treating the biomass with water that is below the critical point. A substance is said to be supercritical when its temperature and pressure are above its critical point. For water this point is at 374C and 218 atm. Subcritical water would be any temperature pressure-combination that is below the critical point but still above the boiling point. In its supercritical state, water has the properties of both a liquid and a gas giving it powerful dissolving power (Aurand, 2001). Water has be shown to display qualities characteristic of supercritical water down to temperatures of 300°C (Sealock et al., 1993).

The lignocellulosic biomass for both of these pretreatments was cotton stalk. Cotton stalk has not been experimented with as an ethanol source and is readily available in North Carolina as a farming byproduct. Furthermore, farmers need a way to dispose of the cotton stalk that is left in the field following the harvest of the cotton crop to prevent pest infestation.

1.2 Objectives

Dilute acid is currently accepted as a viable method of pretreatment (Lee et al., 1999) and corn stover is the feedstock being extensively researched by the National Renewable Energy Lab (NREL) (Aden et al., 2002). Dilute acid pretreatment of corn stover using 1 weight % sulfuric acid at a temperature of 190°C has been found to solubilize over 90% xylose and provide 90% cellulose digestibility (Tucker et al., 2003). Schell (2003) reported that using 1.16 (w/w) % sulfuric acid on corn stover for 6 minutes at 179°C yielded 68% xylose solubilization and 87% cellulose conversion in SSF. However, the efficacy of acid pretreatment varies with factors such as feedstock composition, location, harvest time and germplasm. Besides, corn is predominantly grown in the midwestern part of the country while other regions have a wide variety of feedstocks at their disposal. Therefore, this project was undertaken to study the efficacy of various pretreatment techniques on cotton stalks, which are predominant in the southeastern United States, bearing in mind other implications such as overall cost of the process, environmental concerns and feedstock availability.

The goal of this study was to explore two pretreatment methods for cotton stalks: chemical and subcritical water pretreatments that would produce results comparable to those reported from acid pretreatment of corn stover. The objective of the chemical pretreatments was to find the combination of treatment time and chemical concentration that would yield the most lignin degradation and sugar availability for two chemicals: sodium hydroxide and sodium percarbonate. The objective of the subcritical water pretreatments was to find the combination of temperature, holding time and particle size that would yield the maximum lignin degradation and sugar availability using response surface methodology. The objectives were based on the hypothesis that the chemical and subcritical water pretreatments can breakdown lignin-carbohydrate interactions thereby reducing cellulose crystallinity.

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Chapter 2: Literature Review

2.1. Need for Biofuels

Energy is one of the most crucial resources in the world. It is an integral part of both industry and domestic life and it is hard to imagine life without it. The demand for energy has steadily increased over the past ten years while the amount of fossil fuel available to the world is predicted to begin decreasing before 2010 (Sun & Cheng, 2002). These predictions imply that fossil fuels will eventually run out and therefore it is imperative that alternate sources be made feasible.

Many alternative fuel sources have been explored. Two of the biofuels that have received most attention are biodiesel and bioethanol. Both of these fuel sources are made from renewable feedstocks. Biodiesel is made from vegetable oils or animal fats (Ma & Hanna, 1999) and ethanol is made from plant sugars (Wheals et al., 1999). Corn, sugar cane and lignocellulosic biomass have been explored as the major substrates for ethanol production. In this study ethanol from lignocellulosic sources will be investigated because an economically feasible production method has not been developed, while processes for conversion of corn and sugar cane to ethanol are well established (Wheals et al., 1999).

Apart from the issue of sustainability of fuel sources, the environment is gradually deteriorating due to the increased energy usage. Ethanol has potential to lower carbon dioxide emissions, air pollutants, and the United States' dependence on foreign petroleum sources (Dien et al., 1999). The carbon dioxide that is released in the combustion of ethanol can be equated to the amount that is consumed by plant sources, thus making the net carbon dioxide release zero (Wheals et al., 1999). The recycling of carbon dioxide can reduce smog. When compared to gasoline, ethanol gives better performance due to its higher octane content. It also has a higher heat of vaporization that gives higher power output, although, ethanol contains 33% less energy per gallon than gasoline (Zaldivar et al., 2001).

2.2. Biomass Sources

Ethanol can be produced from a variety of sources such as corn, sugar cane and lignocellulosic biomass (Wheals et al., 1999). Lignocellulosic biomass is a plentiful and economical resource that can serve as a source for ethanol production on a large scale (Dien et al., 1999), (Wheals et al., 1999). Lignocellulosic biomass sources include: “agriculture waste (wheat straw, corn stalks, soy bean residues, sugar cane bagasse), industrial waste (pulp and paper industry), forestry residues and municipal solid waste” (Zaldivar et al., 2001). Cotton stalk is an example of a lignocellulosic agricultural waste. In North Carolina there were 770 thousand harvested acres of cotton crop in 2003 (North Carolina Agricultural Statistics (NCAS)). Since “cotton stalk is a byproduct of cotton crop” (Guhagarkar, 1997) North Carolina has an abundance of this lignocellulosic biomass source. Cotton stalk contains 45.5% cellulose (Guhagarkar, 1997), which can be broken down into glucose for use in ethanol production. The cotton stalk that is left behind after the cotton harvest often becomes infested with pests such as boll weevils (OBWEO, 2006; TBWEF, 2006). Therefore farmers are in need of a way to get rid of the cotton stalk. Using this byproduct for ethanol production would help address the issue of disposing waste residue and turning it into something high value.

2.3. Lignocellulosic Biomass

Lignocellulosic biomass makes up about 50% of all biomass. It is made up of cellulose, hemicellulose and lignin. Cellulose is composed of monomers of glucose, a hexose sugar, linked by β(1,4)-glycosidic bonds. The structure of cellulose is shown in Figure 2.2.1.

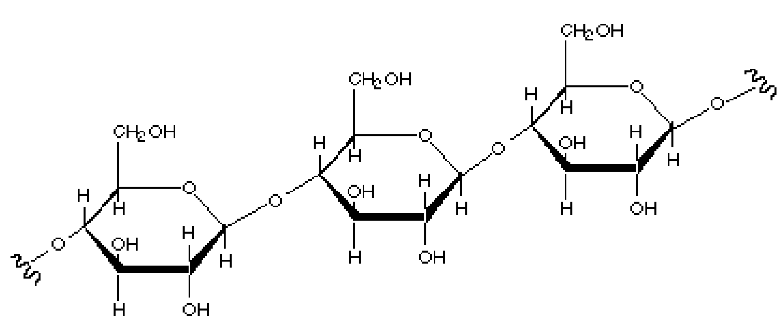

Figure 2.1. Cellulose structure

Hemicellulose is a highly branched carbohydrate and composed of both hexose and pentose sugars. An example of hemicellulose structure is shown in Figure 2.2.2 (Pérez et al., 2002).

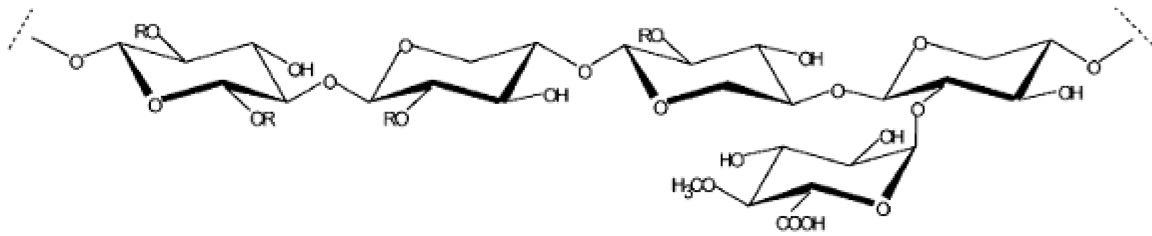

Figure 2.2. Hemicellulose Structure of Angiosperm

Lignin is a macromolecule with phenolic character (Zaldivar et al., 2001). It is helical and contains ether and carbon-carbon linkages (Siasu et al., 2003). A typical lignin structure is shown in Figure 2.2.3(J. Lee, 1997).

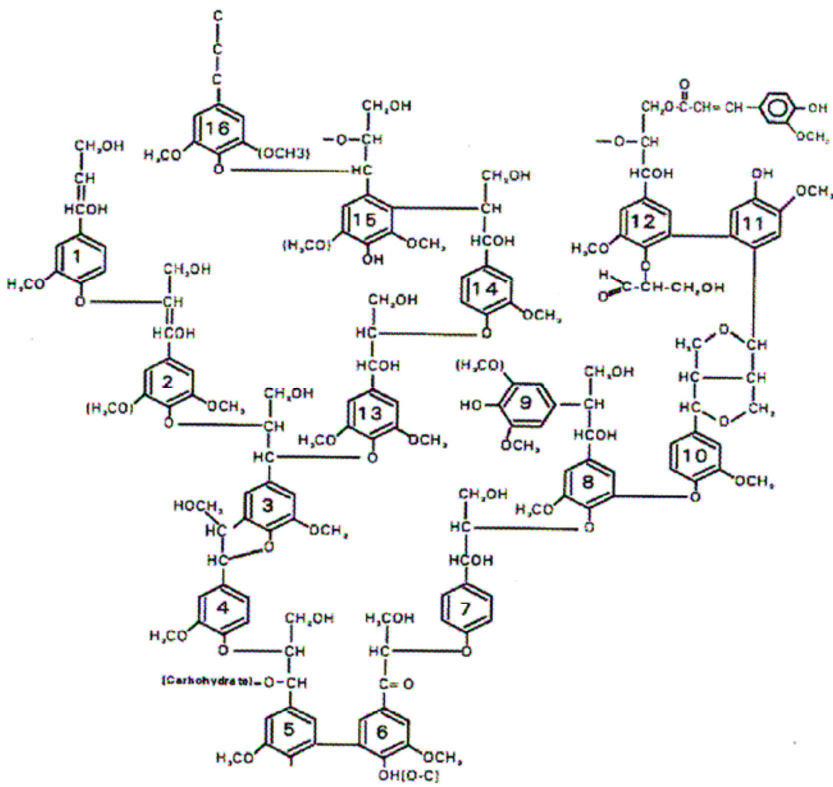

Figure 2.3. Lignin Structure

As shown in Figure 2.4 the cellulose and hemicellulose are imbedded in the lignin (Bidlack et al., 1992)

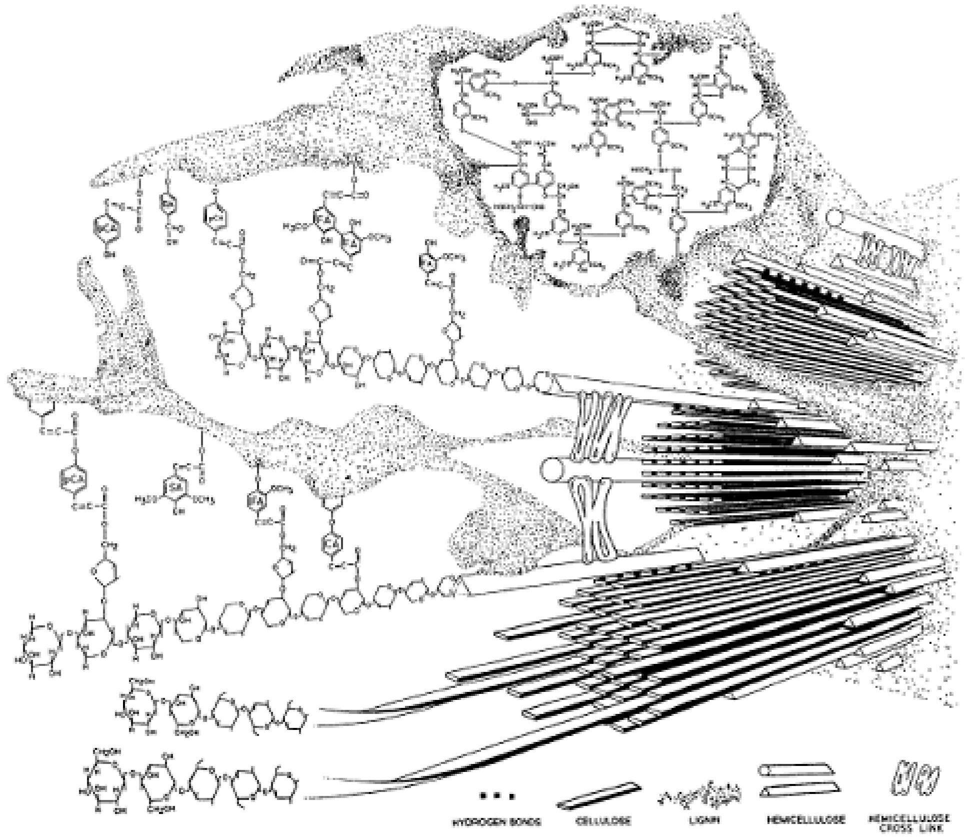

Figure 2.4. Secondary Cell Wall Structure of Plants

2.4. Ethanol Processing Methods

There are three main steps in the production of ethanol from lignocellulosic substrates. First, there is a pretreatment step to increase porosity and reduce cellulose fiber crystallinity (McMillan, 1994). Several methods may be employed for pretreatment as discussed in the subsequent sections. After pretreatment the polysaccharides are broken down into their monomers by a process called hydrolysis. Finally, the sugars are fermented to ethanol by yeast or bacteria.

2.5. Pretreatment

There are many ways of pretreating feedstocks for use in ethanol production. The methods fall into the general categories of biological, chemical, physical, physio-chemical and others. Since the current study focuses on lignocellulosic biomass, the pretreatment methods will be discussed in light of their applications for this type of biomass. The goal of any lignocellulosic biomass pretreatment is to degrade the lignin, increase porosity and decrease fiber crystallinity (McMillan, 1994).

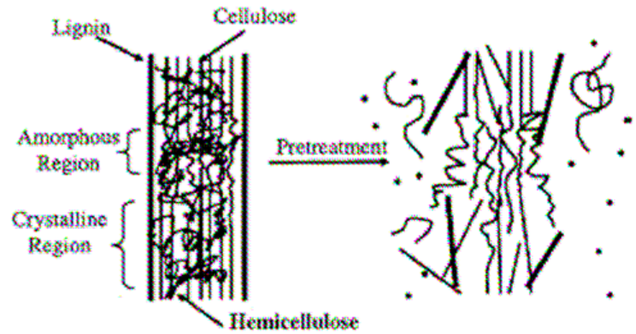

Figure 2.5. Effect of Pretreatment

A schematic representation of the effect of pretreatment is shown in Figure 2.5 (Mosier et al., 2005). Pretreatment breaks down the lignin allowing access to the cellulose and hemicellulose. The hemicellulose and cellulose are also detangled from each other allowing for more efficient access by hydrolysis enzymes.

2.5.1. Biological Pretreatment

The main biological pretreatments include fungi and enzymes. Certain kinds of fungi produce enzymes that can break down lignin and/or hemicellulose and leave the cellulose. The advantages of these biological pretreatments are that they require little energy input and are environmentally friendly. However, the rate of lignin and hemicellulose breakdown is very slow in most processes and still needs to be optimized to make it an effective pretreatment method (Sun & Cheng, 2002). White rot fungi, *Phanerochaete chrysosporium*, is the most commonly used microbe for delignification and was found to degrade 48.58% of lignin, 5.3% of cellulose and 19.72% of hemicellulose in grape cluster stems over the course of 10-12 days (Couto et al., 2003). Keller et al. (2003) found that *Phanerochaete* did not have any effect on the enzyme digestibility of raw corn stover. However, another fungi, *Cyathus sp.,* was found to increase digestibility by 3 to 6.9 times the control values over 29 days (Keller et al., 2003). These are reasonable amounts but over a long period of time.

2.5.2. Chemical Pretreatment

Some of the many chemical pretreatment methods are acid and alkaline delignification, ammonia recycled percolation (ARP) and ozonolysis. Ozonolysis involves using ozone gas to break down the lignin and hemicellulose. This method is effective under mild conditions and environmentally friendly since ozone does not leave residues because of its short half-life. However, the large amount of ozone required and the need for its onsite production makes it expensive (Sun & Cheng, 2002). Ozonolysis has been shown to break down 49% of lignin in corn stalks and 55-59% of lignin in autohydrolyzed (hemicellulose free) corn stalks (Queseda et al., 1999).

2.5.2.1. Acid Pretreatment

Acid delignification is the addition of an acid, such as sulfuric acid, to break down the undesirable materials. This method has proven to be effective, but also has some significant drawbacks. Firstly, acids are very corrosive and the reaction requires a non-corrosive treatment container. Also, the acid must be removed from the process and recycled to be economically feasible (Sun & Cheng, 2002). In addition, some acids, such as H_2SO_4, produce fermentation inhibitors. This means that a microbe that can tolerate these inhibitory products must be developed for subsequent hydrolysis and fermentation (Mohagheghi et al., 2004). Microbes, such as *Coniochaeta ligniaria* C8, have been found to consume the inhibitory compounds over a period of about 40 hours (Lopez et al., 2004). This method has potential for success but adds a time consuming step to the ethanol production process. Acid pretreated corn stover solids washed to remove the inhibitory compounds before fermentation produced approximately 85% ethanol in simultaneous saccharification and fermentation (Tucker et al., 2003), (Schell et al., 2003).

2.5.2.2 Alkaline Pretreatment

Alkaline delignification involves the addition of a base, such as sodium hydroxide, to a biomass to break down the lignin and hemicellulose. The success of this method depends on the amount of lignin in the biomass. This method has had considerable success with corncobs and switch grass (Sun & Cheng, 2002). Pretreatment of rye straw with 2% H_2O_2 at a pH of 11.5 for 20-60 hours produced 52-88% delignification respectively (Sun et al., 2000). Sodium Hydroxide has also been tested as a chemical pretreatment. It has been shown to increase the sugar yield of enzymatic hydrolysis. The amount of sugars increases as the concentration increases from 0 to 1.0 N NaOH (Li et al., 2004) and from 0 to 2% w/v (Silverstein, 2004). Sodium Percarbonate has also been shown to increase sugar yields in biomass. Samples of corn fiber, distiller's dried grains, sugarcane bagasse and spent barley malt had 38.3, 25.6, 60.5 and 40.8% sugar release respectively upon treatment with 1.0% sodium percarbonate (Burdette et al., 2004).

2.5.3. Physical Pretreatment

Physical pretreatment processes include mechanical comminution and pyrolysis. Mechanical comminution is simply breaking the biomass into smaller pieces mechanically. This can include grinding, chipping and milling. These processes reduce the particle size as well as cellulose crystallinity. The reduction in crystallinity is what makes the method partially successful. The power input required for mechanical processing depends on the method and final particle size (Sun & Cheng, 2002). The smaller the particle needs to be, the more energy input required. In some cases, this energy can be up to one-third the energy input for the whole production process (Van Walsum et al., 1996). However, some amount of mechanical comminution is required before most pretreatments to achieve success.

Pyrolysis is another method of physical pretreatment and involves heating the biomass. At high temperatures the cellulose decomposes and produces a gas and an unstable product. When the process is run at lower temperatures the reaction goes much slower and does not produce any harmful substances. This method is usually only successful in combination with others, such as acid pretreatment (Sun & Cheng, 2002). The amount of phenolic substances produced by pyrolysis can be minimized with small particles, short reaction time and lower pressure and temperature (Amen-Chen et al., 2001).

2.5.4. Physio-chemical Pretreatment

One of the most common physio-chemical pretreatment methods is steam explosion. This process involves treatment with high pressure and temperature steam and then quick release of the pressure causing the biomass to undergo an explosion. The advantages of this method are the low energy input and negligible environmental impact. However, steam explosion does not always break down all the lignin, requires small particle size and can produce compounds that may inhibit subsequent fermentation. Despite these drawbacks, steam explosion is currently the most popular method for separating lignin and hemicellulose from cellulose (Sun & Cheng, 2002). Steam explosion pretreatment of red oak wood chips removed 10-20% of the lignin and 40-60% of other carbohydrates besides cellulose (Ibrahim & Glasser, 1999). Steam explosion of soft wood chips achieved a maximum theoretical sugar yield of 50% at 210C and 4 minutes (Ballesteros et al., 2000).

Lignocellulosic materials can also be exploded using ammonia and carbon dioxide. The process is the same as steam explosion but involves a different chemical. Ammonia explosion does not produce products that may inhibit fermentation but it requires that the ammonia be recycled for economic and environmental reasons. Carbon dioxide explosion also does not produce fermentation inhibiting products, but it is much less effective than both the steam and ammonia explosion (Sun & Cheng, 2002).

2.5.5. Other Pretreatments

Other pretreatment methods include radiation and supercritical carbon dioxide. 240W microwave irradiation pretreatment of ground rice straw was found to release 2-4% of reducing sugars (Kitchaiya et al., 2003). 100MR irradiation pretreatment increased the effectiveness of enzymatic and acid hydrolysis two and four times, respectively, in sugar cane bagasse (Kumakura et al., 1982). Supercritical carbon dioxide is carbon dioxide above its critical point of 31C and 73 atm (Aurand, 2001). Supercritical carbon dioxide had no significant effect on the yield of reducing sugars or enzymatic digestibility of aspen lignocellulosic biomass (Kim & Hong, 2001).

2.6. Hydrolysis

Hydrolysis, the next step in the ethanol production process following pretreatment is the breakdown of polysaccharides into monosaccharides. Cellulose is broken down to glucose and hemicellulose is broken into pentose and hexose sugars, mainly xylose. The two main hydrolysis methods are acid and enzymatic. Acid hydrolysis involves using acids to break down the cellulose into glucose (Sun & Cheng, 2002). It is a relatively fast method that produces high concentrations of glucose (Kumakura et al., 1982) but it also has drawbacks similar to those of acid delignification. Byproducts are formed and the acid must be recovered after the hydrolysis reaction occurs (Kumakura et al., 1982).

Enzymatic hydrolysis uses cellulase enzymes produced by bacteria and fungi to break down cellulose. Bacteria and fungi produce these cellulase enzymes. The yield of hydrolysis by cellulase can be increased by increasing the substrate concentration up to a certain extent. After this point the high substrate concentration could act as an inhibitor to the reaction. The reaction yield can also be increased to a point by increasing the amount of cellulase (Sun & Cheng, 2002). Enzymes are expensive and difficult to recover for reuse, but considerable research is being conducted by Novozymes and Genencor to overcome this hurdle. Collaboration between Novozymes and the Department of Energy resulted in a 30-fold reduction in the enzyme cost in a corn stover to ethanol conversion process (Barba, 2005).

Certain substances may slow down the hydrolysis reaction using cellulase. The inhibitory characteristic of lignin on hydrolysis therefore makes pretreatment an invaluable step in the production process. Glucose can also act as an inhibitor. As the cellulose is broken down into glucose, the hydrolysis reaction slows down because the high concentration of glucose inhibits the cellulase.

2.7. Fermentation

Fermentation is the final step in the ethanol production process. It is a reaction that produces ethanol from sugars in an anaerobic environment. Carbon dioxide is also released as a byproduct. Fermentation may be performed by yeast (fungi) or bacteria (NWICC, 2001). The three main bacterial microorganisms developed to ferment sugars into ethanol are *Escherichia coli*, *Klebsiella oxytoca*, and *Zymomonas mobilis*. The former two are able to ferment a variety of sugars to ethanol while the latter gives high yields of ethanol but is specific to glucose and fructose sugars. Research has been done to try and expand the capabilities of *Z. mobilis* to include xylose and other sugars (Dien et al. 2003). *E. coli*, *K. Oxytoca* and *Z. mobilis* have all been genetically engineered to produce ethanol from all pentose and hexose sugars (Becker & Boles, 2003).

Yeast can also be used to ferment sugars to ethanol. *Saccharomyces Cervisiae* provides high yields of ethanol at a fast rate. It also has a relatively high ethanol tolerance. It naturally only utilizes hexose sugars, but has been genetically engineered to use D-Xylose and L-arabinose, both pentose sugars, to produce ethanol (Becker & Boles, 2003), (Ho et al., 1998).

An alternative to the separate hydrolysis and fermentation steps is simultaneous saccharification and fermentation (SSF) which attempts to solve the problem associated with inhibition of enzymatic hydrolysis. During SSF the glucose is fermented before it is able to inhibit the hydrolysis reaction. SSF has many advantages including increased hydrolysis yield with less enzyme, shorter reaction time and less reactor volume since the two reactions are combined (Sun & Cheng, 2002). However, there are also significant problems with this method. The temperature requirements for hydrolysis and fermentation reactions are different causing a conflict of interest. The optimal temperature for hydrolysis is 50C while the optimum temperature for fermentation is 35C (Krishna & Chowdary, 2003). Also, the ethanol produced in the fermentation reaction can be intolerable to the hydrolysis microbes and act as an inhibitor of the enzymes (Sun & Cheng, 2002).

2.8. Challenges in Ethanol Production

The lignin present in lignocellulosic biomass lowers the hydrolysis rate (Miyakawa et al., 1986) and makes the conversion process inefficient and costly (Sun & Cheng, 2002). Thus, finding a microorganism that can utilize both pentose and hexose sugars (Becker & Boles, 2003) and overcoming the presence of lignin are the main problems facing lignocellulosic biomass as a substrate for ethanol production (Zaldivar et al., 2001). Perhaps the biggest challenge in ethanol production is making it economically viable; businesses will not be interested in investing in something that is not.

The aforementioned pretreatment and hydrolysis methods have had significant success with some low-lignin biomass but there is still a lot of research needed to find an economically feasible method of ethanol production from lignocellulosic biomass. The properties of supercritical fluids give them great potential as a solution.

2.9. Supercritical Fluid Applications

Supercritical fluids have many applications that can be divided into five general categories: extraction, conversion, particle production, material processing and analytical applications. These applications span over a variety of industries from food processing to dry cleaning. The five applications are discussed in detail below.

2.9.1. Extraction

Supercritical extraction is the use of a supercritical fluid to extract or remove a specific component from a mixture. Carbon dioxide is the primary solvent used in these applications. It can be applied to separate oils for use in industries such as food, cosmetics and pharmaceuticals. Also, supercritical carbon dioxide can be used to separate caffeine from coffee. Fat and cholesterol can be reduced in dairy products and eggs, and cocoa butter can be extracted from cocoa beans using supercritical carbon dioxide (Mohamed & Mansoori, 2002). Tocoferols, free fatty acids and squalene can be extracted from soybeans using supercritical carbon dioxide, leaving behind a high concentration of stereols. (Chang et al., 2000). Supercritical carbon dioxide has also been used to extract natural vitamin E from wheat germ (Ge et al., 2002).

Supercritical ethane and methanol have also been used as extraction fluids. Supercritical ethane has been used to extract cholesterol from foods (Mohamed & Mansoori, 2002) Supercritical methanol has been used to extract bio-oil from *Prangmites australis* (Kucuk & Agirtas, 1999). Antioxidants from Oak bark have also been extracted using 80% (v/v) supercritical methanol mixed with water (Andrensek et al., 2004). Overall, supercritical fluids, especially carbon dioxide can be used to extract high end products from various materials.

2.9.2. Oxidation

Materials can also be converted or broken down into other products using supercritical fluids. This process is also called oxidation or gasification. Supercritical water can be used to break down toxic organic waste into carbon dioxide, water and gaseous nitrogen (Mohamed & Mansoori, 2002). Supercritical water oxidation has also been shown to break down plastics such as polyethylene tetrephthalate (PET), polyurethane and nylon, cellulose, lignin and hydrogenate hydrocarbons (Yesodharan, 2002). Less char is produced in supercritical oxidative reactions run with air or oxygen as the oxidant (Hong & Spritzer, 2002). (Lin & Wang, 2000) reported that toxic byproducts were reduced in supercritical water oxidation of 2-Chlorophenol. The dissolving power of supercritical fluids has been shown to break down both organic and non-organic substances.

2.9.3. Particle Production

Supercritical water is highly useful in particle production. The process employed to produce the particles used in drugs, flavors and fragrance agents can be improved by supercritical water. Supercritical water expands rapidly through small holes and nozzles allowing for fine particle size in powders for use in drugs (Mohamed & Mansoori, 2002). Small particle production also has applications in protein purification, coatings and semi-conductors (Mohamed & Mansoori, 2002). Other applications of supercritical water particle production are being explored in the synthesis of inorganic nanoparticles (Teja & Eckert, 2000).

2.9.4. Material Processing

The area of material processing has many applications for supercritical water. Supercritical water can be used as either a solvent or a reactant in polymerization reactions and allows the microstructure and morphology to be controlled as the reaction is occurring. The ability to control the reaction in this way allows many new materials to be created in the laboratory. Supercritical water lowers the viscosity of molten polymers or coatings allowing for new methods in applying and producing films, coatings, membranes and composites, just to name a few (Teja & Eckert, 2000).

2.9.5. Analytical Applications

Supercritical fluids have been found to have analytical applications. They have been used in chromatographic techniques for almost forty years. Supercritical extraction techniques are also used in sample preparation. The analytical applications of supercritical water have been explored extensively and still hold a lot of potential as technology continues to develop (Teja & Eckert, 2000).

2.9.6. Conversion of biomass

Conversion of biomass is a specific application of supercritical fluids that falls into the oxidation category. It is discussed separately because it is a novel application. Supercritical fluids can be used to break down lignin (Ehara & Saka, 2002), (Drews et al., 2000), (Kim & Hong, 2001) or to break down cellulose into glucose in very short time (Sasaki et al., 1998). Supercritical carbon dioxide has been explored as a pretreatment method for lignocellulosic biomass. (Kim & Hong, 2001) showed that the percent of theoretical sugar yield from aspen and southern yellow pine was 75% at 73% moisture content when treated with supercritical carbon dioxide at 3100psi and 165C for 60 minutes. The percent went up as the moisture content increased. (Aurand, 2001) showed that biomass can be completely gasified in supercritical water at around 600°C and 350 atm.

Supercritical water has been explored as a method to break down cellulose. When looking at cellulose conversion over a range of temperatures, a sharp increase in the rate of cellulose conversion into glucose occurs around the critical temperature of water making it much faster than the decomposition of glucose into other monomers. This means that glucose is being produced faster than it is degraded. Some glucose was converted to other sugars such as erythrose, fructose, glyceraldehydes and others at temperatures of 320C – 400C and 25MPa but a significant amount of glucose remained after the reaction (Sasaki et al., 1998). However the cellulose decomposition occurred within ten seconds at the lowest temperature and even faster at higher temperatures. This is very efficient conversion, but could make the process hard to control with such small treatment times.

Supercritical water is also used to produce gaseous and liquid fuels from biomass (Sealock et al., 1993). Some supercritical water gasification studies are listed in Table 2.1 (Hong & Spritzer, 2002).

Table 2.1. Prior Lab-Scale Supercritical Water Gassification Results

Reference	Feedstock	T (C)	P (psi)	Catalyst	Rxn Time (min)	% C Gassified	g H gas/100 g feed
Modell, et al., 1978	Glucose	374	3200	Mixed Metallic	30	23	1.4
	Cellulose					18	0.3
Woermer, 1976	Maple Sawdust	374	3200	None	30	88	2.3
Sealock and Elliot, 1991	Cellulose	400	4000	Ni/Cs_2CO_3	15	76	6.5
	Holocellulose					70	5
	Lignin					37	4.1
	Wood					74	7.6
Yu et al., 1993	Glucose	600	5140	None	0.5	86	11.3
Xu et al., 1996	Glucose	600	5140	Activated C	0.3	99	8.1
	Bagasse			Activated C	1.4	100	3.3
	Glycerol			None	0.75	100	11.8
Antal, 1996	Cellobiose	600	5140	Activated C	0.3	100	5.2
	Water Hyacinth					100	9.4
Antal and Xu, 1998	Corn Starch (CS)	650	4170	Activated C	0.25	100	9.1
	Sewage Sludge + CS					94	8.8
	Sawdust + CS					100	9.6

2.10. Supercritical Fluid Catalysts

In addition to the various applications of super- and subcritical fluids, the effects of various catalysts on super- and subcritical fluid reactions have also been explored. Catalysts in general have been found to increase the efficiency of these reactions by increasing conversion and yield and allowing for lower reaction temperatures without sacrificing conversion. Sodium carbonate has been shown to be an effective catalyst in supercritical water oxidation of chlorinated wastes. Alkali materials and nickel are effective for oil production reactions with supercritical water. Organic materials can be completely broken down with supercritical water at 380-450C with the addition of MnO2/CeO2 catalyst (Yesodharan, 2002). Copper (II) cations have been shown to increase the oxidation of 2-Cholrophenol in supercritical water (Lin & Wang, 2000). Ethanol has been used as a catalyst in supercritical carbon dioxide extraction of useful substances from ginkgo biloba and beta carotene and lycopene from tomato paste waste (Baysal et al., 2000).

2.11. Supercritical Carbon Dioxide vs. Water

Carbon dioxide and water both have advantages and disadvantages as players in lignocellulosic biomass conversion. “Water is non-toxic, inexpensive and easy to recycle” (Song et al., 2004). When using supercritical carbon dioxide, products do not have to be separated from the solvent since the carbon dioxide goes back to its gaseous state after returning to its normal state (Yang et al., 2002). Another advantage of carbon dioxide is its low critical point. Carbon dioxide is supercritical above 72.8 atm and a temperature of 31.1°C compare to water at 218 atm and a temperature of 374°C (Aurand, 2001). One of the main advantages of water is that samples do not have to be dried. Unlike carbon dioxide, wet samples can be treated with supercritical water. (Song et al., 2004), (Yoshida & Oshima, 2004), (Yoshida & Matsumura, 2001). This is important since up to 95% of biomass has a high moisture content (Kruse et al., 2003) and drying adds another step to the process and increases energy input requirements.

2.12. Water as a Solution

The properties of water at standard temperatures and pressure including hydrogen bonding found in water the make it a good polar (non-organic) solvent. Hydrogen bonds form between the hydrogen and oxygen atoms of different water molecules. If a molecule has the ability to make hydrogen bonds then it will easily dissolve into the water and create hydrogen bonds with the water. Most organic molecules do not form hydrogen bonds easily and therefore do not readily dissolve in water (Horton et al., 2002).

Water is a polar molecule implying that there is a charge differential within the molecule. One end has a relatively positive charge and the other has a negative charge. Polar molecules are attracted to water and therefore dissolve easily. Conversely, molecules that are not polar, most organic molecules, are unable to dissolve into the water (Horton et al., 2002).

2.13. Properties of Supercritical Water

A substance is said to be supercritical when it is above its critical point. For water, this point is at a temperature of 374C and a pressure of 218 atm (3204 psi). (Aurand, 2001). As water nears its critical point, its properties become very different from those of water at standard temperature and pressure. At or near this state water has high density, high diffusivity, low viscosity, high compressibility (Aurand, 2001) and a break down of hydrogen bonding (NIAIST, 2004).

Density is a measure of the amount of mass per volume. High density gives the substance a better ability to dissolve (Aurand, 2001). Diffusivity is a substance’s ability to diffuse or equally distribute properties such as heat or concentration. This especially important when the reaction is diffusion rate limited (University of Leeds, 2004). Viscosity is a liquid’s ability to resist movement or sheer forces. High diffusivity and viscosity promote quick chemical reactions (Aurand, 2001). Compressibility is a substance’s ability to be compressed or made to occupy a smaller volume. The high compressibility of supercritical water means that small changes in temperature and/or pressures can produce dramatic changes in fluid density and therefore dramatic changes in dissolving ability. This property is important because the reaction rate can be changed and optimized over a small range of temperatures and pressures (University of Leeds, 2004).

The hydrogen bonding is also disrupted at high temperature and pressures (NIAIST, 2004). This disruption causes water to behave in a less polar manner (Marrone, n/d) thus increasing its ability to dissolve organic materials. The dissolving power found in these properties of supercritical and near supercritical water give it great potential for use in ethanol production from lignocellulosic biomass.

2.13.1. Supercritical Water Conversion Mechanism

The high complexity of cellulose and its linkages with hemicellulose and lignin make it resistant to hydrolysis by traditional methods (Zaldivar et al., 2001). The reaction of biomass in supercritical water comprises pyrolysis and hydrolysis subreactions (Song et al., 2004). The major reaction pathways for cellulose in both super- and subcritical water are hydrolysis, retro-aldol condensation, keto-enol tautomerism and dehydration (Sasaki et al., 2004). Sasaki et al. (2004) also proposed that supercritical water causes the cellulose to swell and dissolve explaining the quick increase in cellulose liquefication around the critical point. The mechanism of lignin degradation in supercritical water is hydrolysis and dealkylation. The yield of tetrahydrofuran insoluble compounds decreased and the molecular weight of tetrahydrofuran soluble compounds increased as water density increased during lignin degradation (Siasu et al., 2003)

2.14. Subcritical vs. Supercritical Water

Subcritical water has similar potential as supercritical but with less expense. High temperature liquid water has ‘potential to be more manageable, less corrosive and have wider application to industry than supercritical water systems’ (Sealock et al., 1993). Subcritical water requires less energy input since the temperatures and pressures are lower that those of supercritical water. Water has been shown to display unique qualities characteristic of supercritical water down to temperatures of 300°C (Sealock et al., 1993). Overall, subcritical water has potential as an effective alternative to supercritical water.

2.15. Applications of Subcritical Water

The applications of subcritical water can be divided into two main categories: extraction and conversion of biomass.

2.15.1. Extraction

Subcritical water extraction is similar to that of supercritical water except that the state of the water is different. Subcritical water can be used to extract antioxidant compounds from rosemary plants (Ibanez et al., 2003) as well as to extract whitening agents and azo dyes in paper samples (Santos et al., 2005). Anthocyanins can also be extracted from red grape skin using subcritical water (Yu & Howard, 2005). Additionally, soil can be remediated through subcritical water extraction of dioxins (Hashimoto et al., 2004).

2.15.2. Conversion of Biomass

Subcritical water can be used to break down organic materials at temperatures ranging from 250-374 with pressurization. It has been found that cellulose reacts 2-3 times faster than lignin in liquid hot water (Sealock et al., 1993). Subcritical water can be used to release glucose from cellulose. The maximum glucose released from pure cellulose was 20% at 25Mpa and 320°C for 7 seconds, 25% at 25 Mpa and 375°C for 3.5 seconds and 25% at 25Mpa and 400°C for .05 seconds (Sasaki et al., 2004). It appears that longer reaction times allow for similar yields at reduced temperatures.

Subcritical, or liquid hot, water can also be used for liquification of biomass into bio oil and other liquid fuels. Liquification of feedstocks has been found to occur at temperatures in the lower end of the 250-450°C range (Sealock et al., 1993). Subcritical water has also been used in liquification of corn stalks to bio-oil. The maximum cellulose weight loss from corn stalks occurred at 600K and 25MPa with 2.4% loss/K or 80% loss overall. The maximum hemicellulose weight loss occurred at 550K and 25Mpa with 2.4% loss/K or 50% loss overall. The maximum lignin weight loss occurred at 600K and 25Mpa with .3% loss/K or 25% loss overall (Song et al., 2004). Temperature has been found to play an important role in the liquification of various feedstocks (Sealock et al., 1993).

2.16. Subcritical Water Catalysts

Like other supercritical fluids, adding catalysts can also enhance the subcritical water reaction. For example, liquification of biomass can be improved by adding an alkali metal catalyst (Sealock et al., 1993). $NaCO_3$ will produce two peaks, instead of one, in the overall weight loss/K curve for the liquification of biomass (Song et al., 2004). Also, the addition of an alkali catalyst has also been shown to reduce the amount of furans produced in the biomass liquification reaction (Nelson et al., 1984).

2.17. Subcritical Water Reaction Mechanism

The major reaction pathways for cellulose in both super- and subcritical water are hydrolysis, retro-aldol condensation, keto-enol tautomerism and dehydration (Sasaki et al., 2004). The hydrolysis reaction is important in the production of monomeric sugars as the cellulose and hemicellulose are hydrolyzed into glucose and xylose monomers, respectively. The main products of the subcritical reaction are hydrolysis products, aqueous degradation products of glucose and organic acids (Sasaki et al., 2004). The subcritical liquification of pure cellulose produces phenols, cyclopentanones and hydroquinones (Nelson et al., 1984).

2.18. Economics

Lignocellulosic biomass is an attractive substrate for ethanol production because it is relatively cheap and readily available (von Sivers & Zacchi, 1996). The overall profitability of an ethanol production plant is increased if a coproduct, such as a chemical, is produced concurrently (Kaylen et al., 2000). As of the year 1995, the predicted cost of ethanol ranged from 18 to 151 cents/liter depending on what economic assumptions were put in place. The most important factor in determining economic success was the overall ethanol yield (von Sivers & Zacchi, 1996). Currently, dilute acid pretreatment is one of the most researched methods for pretreatment of lignocellulosic biomass. In 2002 the National Renewable Energy Lab (NREL) reported the results from an economic model on a dilute acid pretreatment process followed by enzymatic hydrolysis. This model also predicted the cost of ethanol per gallon to be most sensitive to the fermentation yield. It predicted that a 10%

decrease in glucose yield would increase the cost per gallon five cents and a 5% decrease would increase the cost by three cents. Also, if the reactor cost was decreased by 50% the cost per gallon of ethanol would decrease by four cents (Aden et al., 2002). This suggests that using subcritical water pretreatment may have the potential to parallel dilute acid pretreatment economically. The yields may be lower but the cost of the reactor may be less since it does not have to be resistant to acid corrosion. Also the NREL model is based on a reaction temperature of 190°C (Aden et al., 2002) which is only 30°C lower than the lowest running temperature of 230°C for the current experiments so the energy input may not be significantly higher. Other additional costs incurred in acid pretreatment that would be reduced or eliminated in subcritical water pretreatment are acid purchase and disposal and neutralization of the acid before fermentation. In order for ethanol to be economically feasible, the production price must be less than the targeted selling price, which the department of energy (DOE) has chosen to be $1.07(Aden et al., 2002). Overall, subcritical water has the potential to rival dilute acid pretreatment but further economic and experimental analysis is needed.

2.19. Conclusion

Biofuels such as ethanol have great potential to serve as an alternate fuel source. Ethanol can be produced from lignocellulosic biomass such as cotton stalk, which is plentiful in North Carolina. Subcritical water is an emerging pretreatment method for lignocellulosic biomass because of it’s potential to break down lignin and convert cellulose into glucose. Economically, subcritical water has potential to rival the commonly accepted dilute acid method because it is less corrosive and more environmentally friendly. This study will explore the potential of subcritical water, sodium hydroxide and sodium percarbonate as pretreatment methods for the production of ethanol from a cotton stalk substrate.

Chapter 3: Methods and Procedures

The focus of this project was to measure sugar and lignin quantities before and after the alkali and subcritical water pretreatments. The cotton stalks used in this project were obtained from Cunningham Research Station in Kinston, NC. The stalks, consisting of stalks, leaves and cotton residue, were harvested in October 2003. They were delivered to NC State University in Raleigh, NC after being shredded and baled in the field.

3.1. Initial Composition

Initial measurements of moisture and DNS reducing sugar content of the cotton stalks were made in triplicate. This data and other existing composition data served as a starting point with which to compare the pretreated samples.

3.1.1. Moisture Content

Moisture content was determined using Lab Analytical Procedure number 001 (LAP-001) from National Renewable Energy Lab (NREL) (Ehrman, 1994). It was measured in triplicate for both shredded and 40 mesh cotton stalk samples. Samples weighing three grams were placed in the convection oven overnight (or until constant weight was achieved) at 105°C and reweighed. The percent moisture was calculated with the following formula:

$$\%\ \text{Moisture} = 100\% - \left(\frac{W_2 - W}{W_1 - W}\right) \times 100 \qquad (3.1)$$

Where: W = weight of dish
W_1= initial sample weight + dish weight (g)
W_2 = dried sample weight + dish weight (g)

3.2. Sample Analysis

Each pretreated sample was analyzed for insoluble lignin and sugars using either high performance liquid chromatography or DNS assay.

3.2.1. Acid Insoluble Lignin Analysis

The insoluble lignin content was analyzed using LAP-003 from NREL (Templeton & Ehrman, 1995). Crucibles were preignited at 500°C in the furnace and weighed. Samples of oven dried pretreated cotton stalks weighing 0.3 grams were placed in small beakers. Three milliliters of 72% H_2SO_4 was added to each beaker. The samples were mixed until thoroughly wet and then set at room temperature for two hours to hydrolyze. After two hours, the samples were transferred to serum bottles and diluted to 4% acid by adding 84 mL deionized water. Bottles were sealed, crimped and placed in the autoclave for 1 hour at 121°C. The seals were then removed after cooling for 20 minutes and the samples vacuum filtered. The filtrate was saved for carbohydrate analysis. The liquid samples being saved for carbohydrate analysis by HPLC were neutralized to pH 4-5 with barium hydroxide, filtered through a 0.2 μm filter and placed in the -80°C freezer. The remaining particles in the serum bottle were washed out with deionized water. The crucible and its contents were dried at 105°C for 2 hours and then cooled in the dessicator and weighed. This was the weight of the crucible, acid insoluble lignin and ash, W_2. The crucible and contents were then placed in the furnace at 500°C for three hours and then cooled and weighed. This was the weight of the crucible and ash, W_3. The percent acid insoluble lignin was calculated with the following formula:

$$\%\ \text{Acid-insoluble lignin} = \frac{W_2 - W_3}{W_1} * 100 \qquad (3.2)$$

Where: W_1 = initial sample weight
W_2 = weight of crucible, acid-insoluble lignin, and acid-insoluble ash
W_3 = weight of crucible and acid-insoluble ash

3.2.2. Acid Soluble Lignin Analysis

The acid soluble lignin was measured for the subcritical water pretreatment liquid for the pure lignin samples. The acid soluble lignin procedure was taken from NREL LAP-004

(Ehrman, 1996). The absorbance of filtrate from the acid insoluble lignin analysis was measured at 205 nm using a quartz cuvette and a spectrophotometer (PharmaSpec UV-1700, Shimadzu). The spectrophotometer was blanked with 4% sulfuric acid and calibrated before use. Samples were dilute as needed to obtain an absorbance reading less than 1.0. The acid soluble lignin was calculated using the following formula:

$$\text{Acid Soluble Lignin} = \left(\frac{\frac{A}{b \times a} \times df \times V \times \frac{L}{1000mL}}{W} \right) \times 100$$

Where: A = absorbance at 205nm
b = cell path length (1cm)
a = absorptivity (110L/g-cm)
df = dilution factor
V = sample volume (mL)
W = dry weight of initial sample (g)

3.2.3. Carbohydrate Analysis

Two methods were utilized to quantify the amount of reducing sugars found in samples. High performance liquid chromotagraphy was used for the chemical pretreatment experiments and a DNS assay was used for the subcritical water experiments.

3.2.3.1. High Performance Liquid Chromatography (HPLC)

Carbohydrate analysis for the chemical pretreatments was done using high performance liquid chromatography (HPLC). The LAP-002 procedure from NREL (Ruiz & Ehrman, 1996) was modified for use with a Dionex DX-300 Chromatography System with a CarboPac™ PA10 anion exchange column, CarboPac™ PA10 guard column, gradient pump, automated sample and pulse amperometric detector with gold working electrode. The samples were first neutralized to pH between 4 and 5 with 2.3 grams of barium hydroxide per 16 mL and vortexed. They were then centrifuged at 7500 rpm for 10 minutes to pellet the solids and supernatant was then filtered through a 0.2 μm filter to remove any remaining unwanted solids. The samples were diluted 1:10 with deionized water and run through the HPLC system. The concentration of xylose and glucose were measured for each sample. The amount of each sugar in the sample was calculated with the following formula:

$$\% \text{ Sugar} = \frac{\frac{C}{1000mL/L} \times df \times \frac{1g}{1000mg} \times V}{W_1 \times \frac{T_{final}}{100\%}} \times 100 \tag{3.3}$$

Where: W_1 = initial weight of sample (g)
V = volume of filtrate (87.0 mL)
C = Concentration of sugar in hydrolyzed sample (mg/L)
df = dilution factor
T_{final} = % total solids content of shredded sample on a 105°C dry weight basis

3.2.3.2. DNS Assay

The DNS procedure used was a modified version of LAP-006 from NREL (Adney & Baker, 1996). LAP-006 is a procedure for cellulase enzyme activity so only the parts relevant to DNS assay itself were referenced. First, the DNS Reagent was prepared by mixing 400mL of distilled water with 10.0g of 3,5-Dinitrosalicylic acid and 20.75 mL of 19.3N (50% w/w) sodium hydroxide. Three hundred grams of Rochelle salts (sodium potassium tartrate tetrahydrate) were added and dissolved. Deionized water was added up to 1 L. The solution was stored in the dark by wrapping the bottle with aluminum foil.

The Citrate Buffer was prepared by mixing 210 grams of citric monohydrate with 750 mL of Deionized water. The solution was diluted to 1 L and the pH was checked. The pH was adjusted to 4.5 by adding sodium hydroxide solution. The buffer was then diluted to 0.05M by adding deionized water at 1:19 buffer to water ratio.

Glucose standards were prepared for each analysis. A stock solution was prepared by dissolving 0.4 grams of glucose to 200mL of citrate buffer and the glucose standards were prepared as follows: 0.5 mL + 0mL buffer = 1.0mg/0.5mL, 400uL + 100 uL buffer = 0.8mg/0.5mL, 300uL + 200 uL buffer = 0.6mg/0.5mL, 200uL +300uL buffer =

0.4mg/0.5mL, 100 uL + 400 uL buffer = 0.2 mg/0.5mL. Each standard was then diluted 1:2 by adding 0.5 mL of the standard to 1.0mL buffer.

Samples were prepared by adding 0.5 mL of sample to 1 mL of buffer. Three mL of DNS reagent was added to each sample and standard tube. All tubes were boiled for 5.0 minutes in vigorously boiling water. After 5.0 minutes, the tubes were transferred to an ice-cold water bath. After cooling, 1.5 mL from each tube was placed in a cuvette and the absorbance was read at 540 nm. The sugar concentrations of the samples were found by comparing to the standard curve values such as that shown in Figure 3.2.

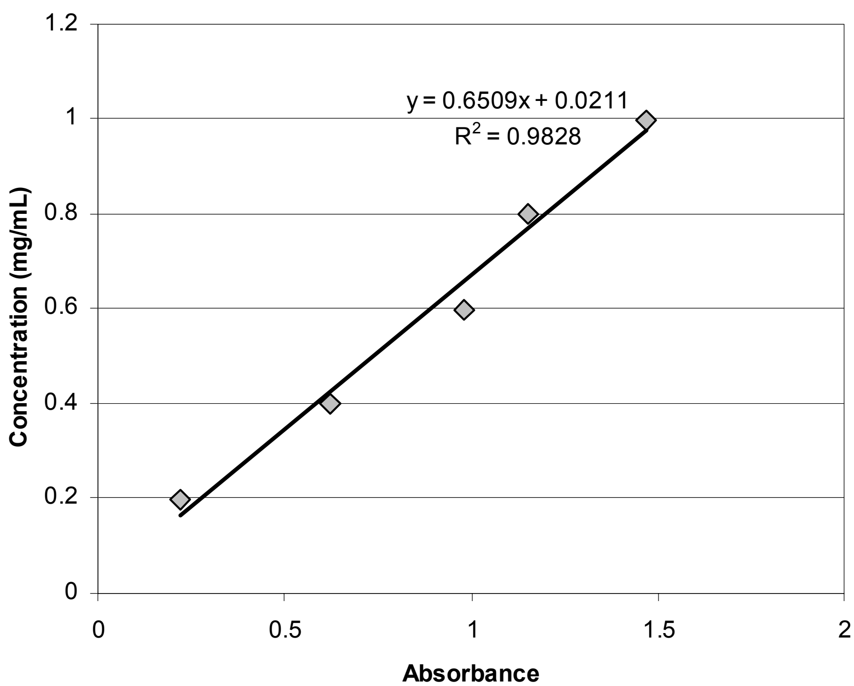

Figure 3.1. DNS Standard Curve

3.3. Chemical Pretreatment Experiments

A flow chart of the chemical pretreatment experiments is shown in Figure 3.2.

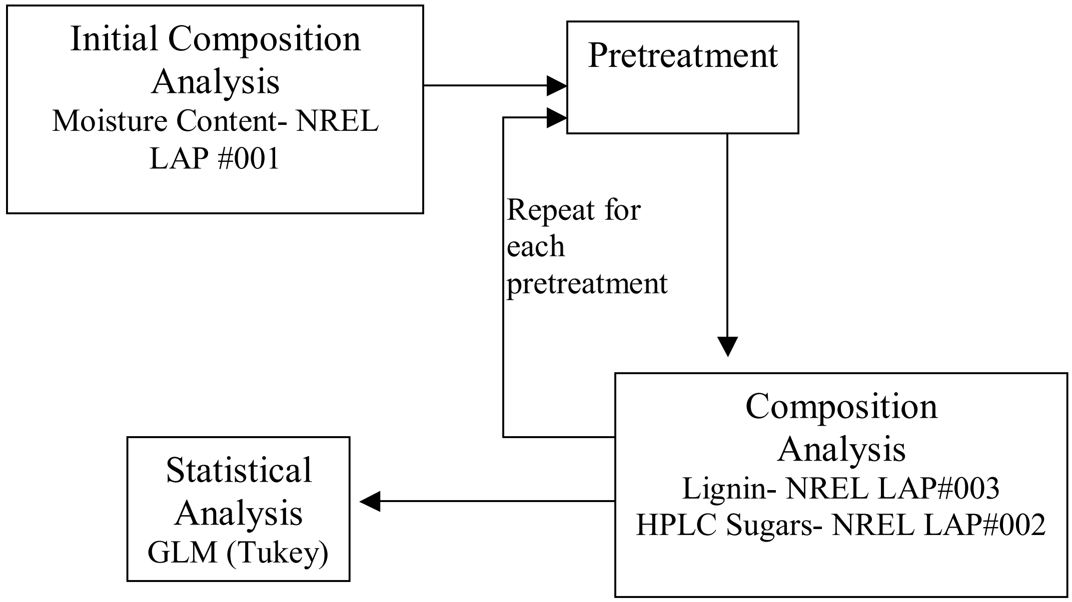

Figure 3.2. Chemical Pretreatment Flow Chart

Cotton stalks ground to 40 mesh were pretreated with 0, 0.5, 1, 2, 4 and 8% w/v Sodium percarbonate and 0, 4 and 8% w/v Sodium Hydroxide. Each pretreatment was done in triplicate at 10% solids loading. Samples with moisture content of approximately 6% and weighing 10 g each were put into a serum bottle. One hundred milliliters of chemical solution was added to the serum bottle and the bottles were autoclaved at 121°C and 15psi for 30, 60 or 90 minutes. A total of 54 pretreatments (3 times x 6 concentrations x 3 replicates) were conducted for sodium percarbonate and 18 pretreatments (3 times x 2 concentrations x 3 replicates) were conducted for NaOH. The bottles were allowed to cool and the samples were removed from the bottle, rinsed and placed in the oven at 105°C. Lignin analysis and carbohydrate analysis by HPLC was completed as explained above after the samples were dry.

3.3.1. Analysis Calculations

After all the lignin and carbohydrate data was collected, the lignin reduction and hemicellulose solubilization were calculated. The lignin reduction is a measure of the difference in the amount of lignin between the untreated and treated samples and was calculated using the following formula:

$$\% \text{ Lignin Reduction} = \left(\frac{W_1 - W_2}{W_2}\right) \times 100 \qquad (3.4)$$

Where: W_1 = weight of lignin in untreated sample
W_2 = weight of lignin in treated sample

The hemicellulose solubilization is an indication of the amount of hemicellulose that was converted into xylan and other sugar chains and was calculated as follows:

$$\% \text{ Hemicellulose Solubilization} = \left(\frac{W_1 - W_2}{W_2}\right) \times 100 \qquad (3.5)$$

Where: W_1 = weight of hemicellulose sugars in untreated sample
W_2 = weight of hemicellulose sugars in treated sample

3.3.2. Statistical Analysis

The concentration, time, lignin reduction, glucose and hemicellulose solubilization values for each sample were entered into SAS (version 9.1.3, Cary, NC) and a GLM procedure was run. The effects of concentration, time and the concentration-time interaction were explored at 95% confidence interval. A Tukey means comparison was also run within the GLM procedure. Each chemical was run as a separate SAS program and then together for chemical comparisons.

3.4. Subcritical Water Pretreatment Experiments

Cotton stalk samples were also pretreated with subcritical water. The parameters tested were temperature, holding time and stalk particle size. A flow chart for the experiments is shown in Figure 3.3.

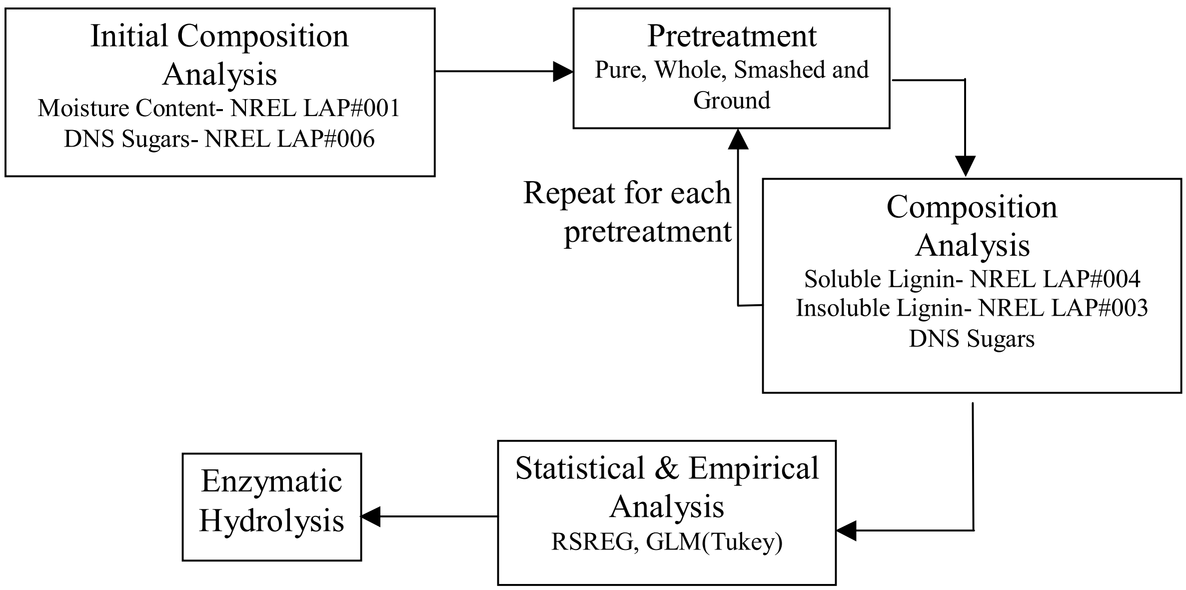

Figure 3.3. Subcritical Water Pretreatments

3.4.1. Subcritical Water System

The subcritical water system is depicted in Figure 3.4. Figure 3.4A. shows a schematic of the system and Figure 3.4B is an actual photograph of a part of the system. The system is similar to that used by Stikeleather and Singleton (2001).

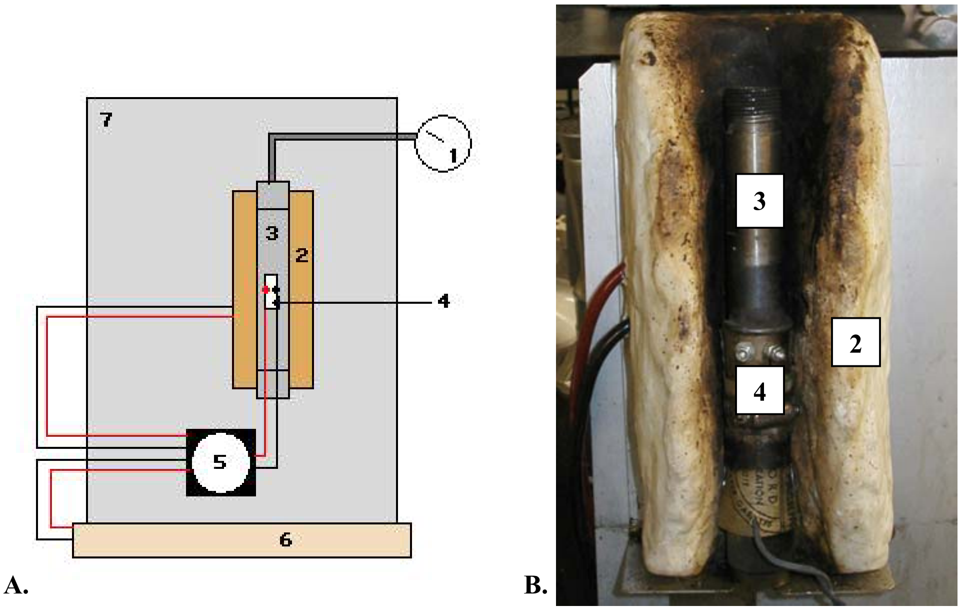

Figure 3.4. : Subcritical Water System

1- Pressure Gauge	2- Induction Coil in Ceramic
3- Reaction Vessel	4- Resistance Temperature Sensor (RTD)
5- Temperature Controller	6- Induction Heater Power Supply
7- Metal Support	

As listed in the caption of Figure 3.4., item one is the pressure gauge. The pressure was not measured for every run, but pressure readings were taken at every temperature and are listed in Table 3.1.

Table 3.1. Pressure Measurements

Temperature (°C)	Pressure (psi)
230	450
275	1100-2000
320	1750-4250

The pressure measurements varied for each temperature due to the variation in temperature over each run during the holding time as the controller was regulating the temperature.

Item two in Figure 3.4 is the induction coil. Current from the high frequency induction heater power supply (#6) was run through the coil creating a magnetic field in the 403 stainless steel reaction vessel (#3) causing it to heat up. The temperature was controlled with a RTD (#4) and controller (#5). The vessel, induction coil and controller were supported by a folded sheet of metal (#7).

3.4.2 Response Surface Model Experimental Design

Each parameter was tested at three levels using a Box Behnken experimental design using StatEASE software (version 7.0, New York, NY) and the optimal combination of parameters resulting in higher reducing sugars and lignin reduction was found using response surface optimization. Water with cotton stalk sample was heated to temperatures of 230, 275 and 320°C using an induction heating coil. Holding times of 2, 6 and 10 minutes were investigated. Preliminary experiments were run to confirm that the most successful holding time value would most likely fall within this range.

The particle sizes tested were .125 (1/8), .1875 (3/16) and .25 (1/4) inches. The levels that were used to design the experiment are shown in Table 3.2 and the combinations obtained through RSM experimental design that were tested are shown in Table 3.3.

Table 3.2: Subcritical Water Experimental Variables and Levels

	Temperature (°C)	time (min)	size (in)
-1	320	2	1/4
0	275	6	3/16
1	230	10	1/8

Table 3.3: Subcritical Water Experimental Design

Temperature (°C)	Time (min)	size (in)
230	2	3/16
320	2	3/16
230	10	3/16
320	10	3/16
230	6	1/8
320	6	1/8
230	6	1/4
320	6	1/4
275	2	1/8
275	10	1/8
275	2	1/4
275	10	1/4
275	6	3/16
275	6	3/16
275	6	3/16
275	6	3/16
275	6	3/16

Table 3.4: Experimental Design for Subcritical Water Pretreatment of Pure Lignin and Cellulose

Temperature (°C)	Time (min)
320	2
275	6
230	10

Smashed and Whole stalks were tested using the variables and levels shown in Table 3.1 but a factorial design was used for a total of 54 pretreatment experiments (3 temperatures x 3 times x 3 replicates x 2 sizes). Pure cellulose (cotton balls) and lignin were tested using the same variables and levels but only at the combinations shown in Table 3.4 were used.

3.4.3. Pure Components

Organosolv lignin (Sigma) and cotton balls were pretreated to represent pure lignin and cellulose, respectively. The structure of the organosolv lignin is shown in Figure 3.5.

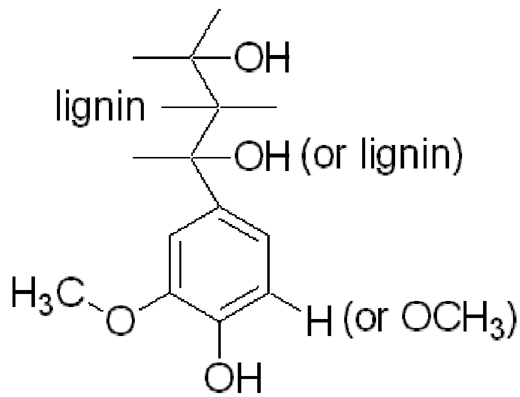

Figure 3.5. Structure of Organosolv Lignin

One gram of lignin was suspended in 25 mL of water and the solution poured into the treatment vessel. The samples were analyzed for solids recovery and soluble and insoluble lignin. One gram of cotton balls was stuffed into a treatment bag and placed with 25 mL of water in the treatment vessel. The solid samples were analyzed for DNS reducing sugars and solids recovery and liquid samples were analyzed for DNS reducing sugars.

3.4.4. Whole and Smashed Cotton Stalks

Whole and smashed cotton stalks were pretreated to evaluate the importance of mechanical comminution in relation to the subcritical water pretreatment. The smashed cotton stalks were smashed using a sheet metal roller and the whole stalks were not altered before pretreatment with subcritical water. For both smashed and whole stalks, one-gram samples were held in a silicon mesh bag and pretreated with 25 mL of subcritical water.

3.4.5. Pretreatment Protocol

For each ground sample pretreatment 1-gram sample was first loaded into the reaction cell. Twenty-five milliliters of water was then added to cover the sample. The reaction cell was then placed in the induction heater and the heater was turned on. It took between 3.5 and 6 minutes to heat up and the sample was then held for the specified holding time. After the holding time, the heater was turned off and the reaction cell was quenched in water. The sample was removed when the vessel was cool to touch.

3.4.6. Sample Analysis

Each one-gram sample was held in a preweighed silicon mesh bag as shown in Figure 3.6 except for the pure lignin samples which were suspended in the water inside the vessel.

Fig. 3.6. Silicon Mesh Bag w/ Sample

After treatment the sample and the bag were dried in the oven at 105°C overnight and then weighed. The weight of the bag was subtracted off to get the final weight of the sample. The sample was then removed from the bag and lignin analysis and carbohydrate analysis by DNS assay were performed as described above. The pure lignin solid samples were washed from the inside of the vessel and collected by filtration

The sugars for the DNS Assay were calculated as a percent of the initial dry weight (Sun & Cheng, 2004) of the sample as follows:

$$\% \text{ Reducing Sugars} = \left(\frac{W_s}{W_1 \times 1000} \right) \times 100 \qquad (3.9)$$

Where: W_s = weight of sugars from DNS (mg)
W_1 = dry weight of sample (g)

The weight of sugars, W_s , in the samples were calculated by the following:

$$W_s = C_{DNS} \times V \qquad (3.10)$$

Where: C_{DNS} = Concentration of sugars found by DNS (mg/mL)
V = volume of liquid (mL)

3.4.7. Statistical Analysis

The temperature, holding time, particle size, lignin reduction and percent sugar values for each sample were entered into SAS (version 9.1.3, Cary, NC) where RSREG and GLM procedures were run. The RSREG procedure gave the coefficients and significance ($p \leq 0.05$) of the full quadratic model terms and the GLM procedure was used for Tukey comparisons. An enzymatic hydrolysis experiment was run using the three combinations with the highest lignin degradation and the three with the highest total percent sugar based on the Tukey rankings.

3.4.8. Enzymatic Hydrolysis

Three combinations each with highest lignin reduction and total sugar production were identified as optimum through statistical analysis and samples were hydrolyzed using cellulose and cellobioase enzymes. The optimum conditions for cellulase enzymatic hydrolysis have been found to be 50°C, pH 4.5, enzyme loading of 40 FPU/g substrate and substrate concentration of 2.5% (Krishna et al., 2000). Therefore, samples at 2% solids loading were placed in a shaking incubator at 50°C and 150 rpm for 72 hours with cellulase enzyme loading of 40 FPU/g of sample. Cellobiase was also added to supplement the cellulase at a ratio of 1:1.75 and tetracycline at a concentration of 40μg/mL to prevent contamination.

Chapter 4: Results and Discussion

4.1 Initial Cotton Stalk Composition

The composition of cotton stalks can vary by harvest time and location (Silverstein, 2004). The cotton stalks used in this project were obtained from Kinston, NC. The initial composition analysis results are in Table 4.1.

Table 4.1: Untreated Cotton Stalk Composition[1]

105C Moisture Content[2]	6.8	%
Ash Content[3]	6.0	%
Acid Soluble Lignin[3]	2.2	%
Acid Insoluble Lignin[3]	27.9	%
Extractables[3]	9.0	%
Glucose[3]	31.1	%
Xylose[3]	8.3	%
Arabinose[3]	1.3	%
Galactose[3]	1.1	%
DNS Reducing Sugars[2]	31.44	%

[1] Composition percentages are on a dry basis
[2] Values obtained from the current study
[3] Values obtained from Silverstein (2004)

The percent glucose of 31.1% is considered to be representative of the cellulose content while the sum of xylose, arabinose and galactose percentages (10.7%) is representative of the hemicellulose. The constituent sugar values were determined using HPLC carbohydrate analysis as outlined in Section 3.2.3.1. The DNS reducing sugars representing the total reducing sugars were determined as outlined in Section 3.2.3.2.

4.2. Chemical Pretreatments

Chemical pretreatments for 30, 60 and 90 minutes were performed with sodium hydroxide at 0, 4 and 8% w/v concentration and sodium percarbonate at 0, 0.5, 1, 2, 4 and 8% w/v concentration. The results are discussed in the following sections.

4.2.1 Effect of Sodium Hydroxide Pretreatments on Ground Cotton Stalks

Like other alkali pretreatments, sodium hydroxide works by saponification of intermolecular ester bonds and increases porosity through swelling of the biomass (Tarkov & Feist, 1969).

4.2.1.1 Composition of Pretreated Solids

The average compositions of sodium hydroxide pretreated samples and their standard deviations are shown in Table 4.2.

Table 4.2. Composition of NaOH Treated Cotton Stalks*

time	Conc %	LIGNIN %	GLUCOSE %	XYLOSE %	SOLIDS RECOVERY %
30	0	29.51 (4.45)	30.45 (1.85)	11.67 (0.35)	87.25 (1.95)
60	0	27.04 (10.45)	30.52 (3.21)	13.04 (0.96)	87.59 (0.81)
90	0	28.64 (4.00)	30.84 (5.48)	10.14 (1.79)	84.11 (0.98)
30	4	19.20 (0.88)	50.85 (3.78)	11.32 (0.53)	50.02 (1.29)
60	4	19.75 (2.08)	40.79 (0.85)	10.95 (0.32)	53.73 (0.49)
90	4	21.00 (0.14)	42.60 (0.47)	9.29 (0.22)	51.23 (2.13)
30	8	20.84 (0.65)	48.50 (8.45)	5.23 (1.09)	46.73 (0.42)
60	8	20.92 (2.01)	42.55 (2.48)	6.64 (0.25)	48.70 (1.09)
90	8	23.13 (2.73)	39.20 (10.61)	3.85 (1.35)	48.45 (1.23)

*Standard Deviation shown in parentheses

The percent lignin values ranged from 19.20% in the 30 minute 4% samples to 29.51% in the 30 minute 0% samples. The solids recovery percents ranged from 46.73% in the 8% 30 minute samples to 87.59% in the 0% 60 minute samples. The percent glucose availability ranged from 30.45% in the 0% 30 minute samples up to 50.85% in the 4% 30 minute samples. The concentration of sodium hydroxide had a significant effect ($p \leq 0.05$) on the percent of available glucose while time and the concentration-time interaction did not. There was a significant difference ($p \leq 0.05$) in glucose content at 0% and 4 and that at 8% concentrations. However, no difference was observed between 4 and 8%. The results suggested that using 8% sodium hydroxide pretreatments did not provide any significant ($p \leq 0.05$) benefit over pretreatment at 4%.

Silverstein (2004) performed similar pretreatments with .5, 1 and 2% sodium hydroxide at 121°C/15 psi on cotton stalks. The highest average glucan content for samples treated with 2% sodium hydroxide was 50.33% for the 60-minute samples. (Silverstein, 2004) Comparing with the averages obtained in the current study for 4 and 8%, which had a maximum of 50.85% glucan, the values are similar. This indicates that 2% sodium hydroxide pretreatment for 60 minutes may be sufficient to provide optimal glucose availability from cotton stalks.

The percent xylose values in the NaOH pretreated cotton stalks ranged from 3.85% in the 8% 90 minutes samples to13.04% in the 0% 60 minute samples. The concentration of sodium hydroxide and treatment time both significantly affected ($p \leq 0.05$) the percent xylose while the interaction between time and concentration did not. There was no significant difference ($p \leq 0.05$) between the percent xylose values obtained with 30 and 60-minute pretreatments. However, the xylose content in samples treated for 90 minutes was significantly lower than that in the 30 and 60-minute samples. This suggests that it would be advantageous to use 90-minute sample times to increase percent xylose.

4.2.1.2 Lignin Reduction

The lignin reduction resulting from sodium hydroxide pretreatment is presented in Figures 4.1.

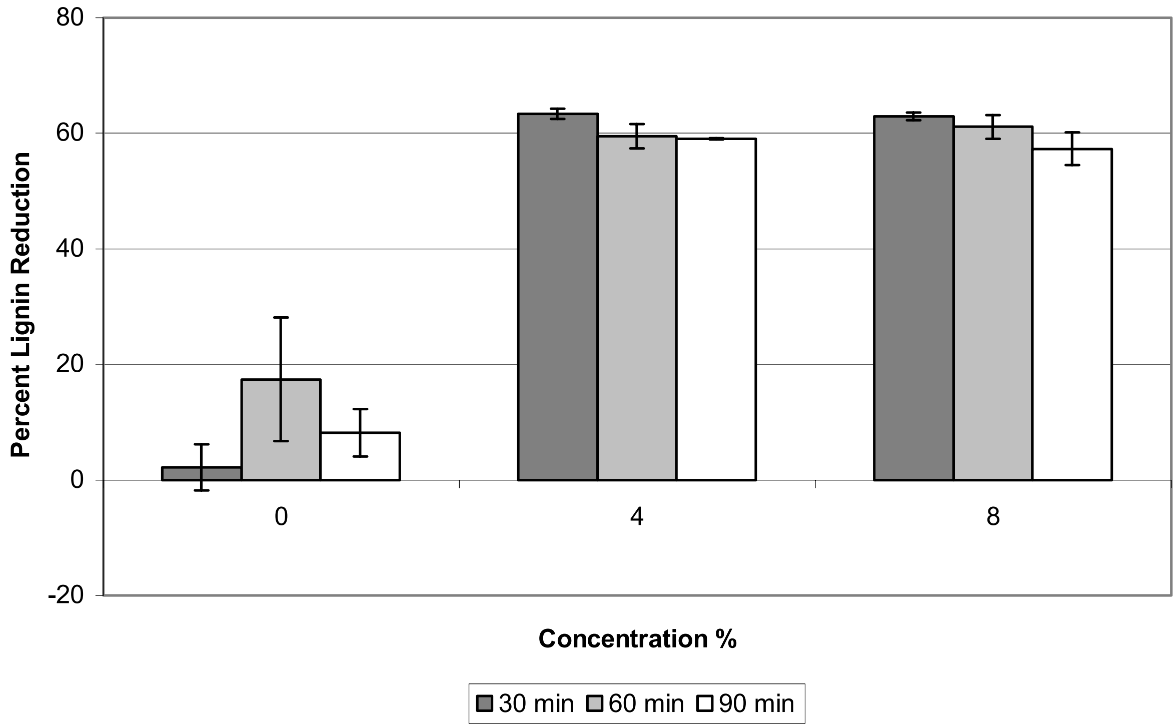

Figure 4.1. Percent Lignin Reduction in Sodium Hydroxide Treated Cotton Stalks

The average lignin reduction values ranged from 2.14% in the 0% 30 minute samples to 63.39% in the 4% 30 minutes samples. Chemical concentration and time-concentration interaction had a significant effect on the percent lignin reduction while time by itself did not. Lignin reduction at 0% was significantly lower ($p \leq 0.05$) than that at 4% and 8% concentration. However, there was no significant difference between the values at 4% and 8%. This suggests that increasing the concentration beyond 4% does not necessarily improve lignin reduction and therefore it would not be economical to use greater than 4% sodium hydroxide for lignin reduction purposes.

Through the pretreatment of cotton stalks with .5, 1 and 2% w/v sodium hydroxide Silverstein (2004) obtained the highest average lignin reduction of 65.63% in 2% sodium hydroxide 90-minute samples. Comparing the two studies, it is observed that the lignin reduction of 63.39% obtained with 4% NaOH is similar to the reduction of 65.63% at 2%.

This indicates that 2% sodium hydroxide may be sufficient to provide optimal lignin degradation in cotton stalks by this chemical.

4.2.1.3. Hemicellulose Solubilization

Hemicellulose solubilization values represent the amount of hemicellulose that was solubilized, or converted to xylan, during the treatment and are shown in Figure 4.2. This is measured by finding the amount of hemicellulose sugars released during hydrolysis. In this study, only xylose was accounted for in the pretreated samples because the arabinose and galactose concentrations were considered negligible (Arabinose average = 0.5% and Galactose average = 0.2%).

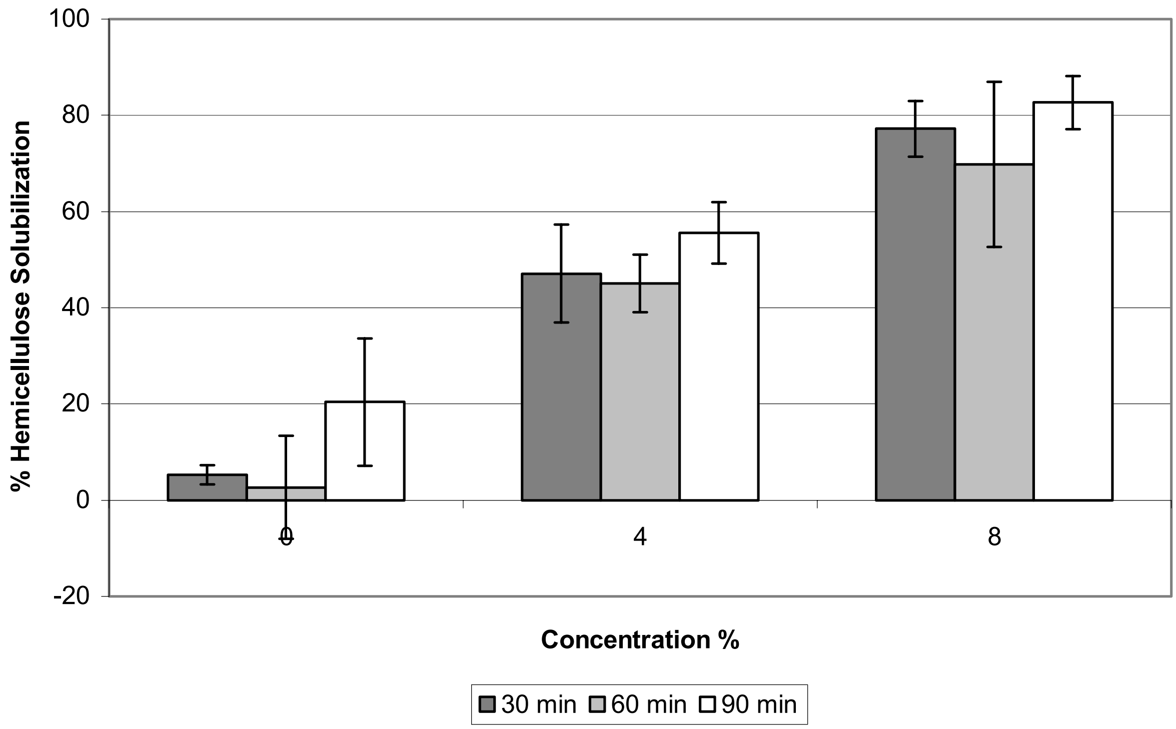

Figure 4.2. Hemicellulose % Solubilization in NaOH Treated Cotton Stalks

Hemicellulose solubilization as a result of NaOH pretreatment of cotton stalks ranged from 2.67% in the 0% 60-minute samples to 82.67% in the 8% 90-minute samples.

Concentration and time significantly ($p \leq 0.05$) affected the hemicellulose solubilization while time-concentration interaction did not. There was no significant difference ($p \leq 0.05$) between the 30 and 60-minute samples, but the 90-minute samples were significantly higher. All the concentrations were significantly different ($p \leq 0.05$) from each other with 8% being the most effective and 0% the least. This is expected since the higher concentrations are more likely to specify ester bonds and increase porosity more efficiently thus solubilizing more hemicellulose into xylan.

The hemicellulose solubilization obtained in the current study for 4 and 8% averaged 49.24 and 76.56% respectively and was higher than that reported by Silverstein (2004). In the pretreatment of cotton stalks with sodium hydroxide at 120°C, Silverstein (2004) obtained hemicellulose solubilization of 32.60% with 2% sodium hydroxide 90-minute pretreatment. The 4% and most definitely the 8% NaOH significantly differ from the 2% values indicating that using 8% sodium hydroxide may be effective in providing optimal hemicellulose solubilization with sodium hydroxide.

4.2.2. Effect of Sodium Percarbonate Pretreatments on Ground Cotton Stalks

The aqueous chemistry of sodium percarbonate is very similar to that of alkaline hydrogen peroxide. Any increase in reactivity from that of hydrogen peroxide is most likely due to the presence of the HCO_4- ion (McKillop & Sanderson, 1995).

4.2.2.1. Composition of Pretreated Solids

The average compositions of sodium percarbonate pretreated samples and their standard deviations are shown in Table 4.3.

Table 4.3. Composition of Sodium Percarbonate Treated Cotton Stalks*

time	Conc %	LIGNIN %		GLUCOSE %		XYLOSE %		SOLIDS RECOVERY %	
30	0	29.51	(0.77)	30.45	(1.85)	11.67	(0.35)	87.25	(1.95)
60	0	27.04	(1.39)	30.52	(3.21)	13.04	(0.96)	87.59	(0.81)
90	0	28.64	(1.41)	30.84	(5.48)	10.14	(1.79)	84.11	(0.98)
30	0.5	29.69	(1.06)	23.05	(4.55)	7.27	(1.34)	83.71	(0.51)
60	0.5	29.11	(0.42)	19.47	(2.13)	6.39	(0.78)	81.23	(0.78)
90	0.5	28.8	(0.28)	20.38	(2.59)	6.23	(0.80)	81.51	(0.45)
30	1	29.56	(0.56)	20.73	(2.12)	6.53	(0.79)	84.66	(1.76)
60	1	27.85	(1.54)	21.67	(6.67)	7.17	(2.28)	79.84	(0.99)
90	1	28.45	(0.35)	22.27	(0.68)	7.03	(0.70)	80.02	(0.92)
30	2	27.07	(0.40)	19.63	(1.18)	5.49	(0.27)	79.01	(1.26)
60	2	26.83	(0.24)	19.54	(2.32)	5.88	(0.55)	80.1	(1.23)
90	2	27.15	(0.84)	20.72	(2.78)	6.18	(0.82)	80.32	(2.28)
30	4	20.68	(5.46)	27.74	(5.28)	9.18	(1.59)	78.39	(0.12)
60	4	23.09	(0.49)	21.63	(0.97)	7.22	(0.33)	73.91	(0.61)
90	4	22.8	(0.47)	21	(0.53)	6.73	(0.21)	74.51	(0.67)
30	8	22.84	(0.42)	17.36	(2.18)	5.76	(0.69)	79.68	(1.79)
60	8	21.88	(0.17)	19.62	(0.71)	6.41	(0.13)	76.99	(1.93)
90	8	20.79	(0.15)	22.34	(1.09)	7.18	(0.32)	73.40	(2.65)

* Standard Deviations are shown in parentheses

The percent lignin in the pretreated cotton stalks ranged from 20.68% in the 4% 30 minute samples to 29.69% in the 0.5% 30 minute samples. The solids recovery percent values ranged from 73.40% in the 8% 90 minute samples to 87.59 in the 0% 60 minute samples. The values for percent glucose obtained from cotton stalks ranged from 19.47% in the 0.5% sodium percarbonate treated samples to 30.45% in the 0% samples. Concentration significantly ($p \leq 0.05$) impacted glucose availability while the time and concentration-time interaction did not. There was no significant difference between glucose content at any of the times but all the concentrations were significantly different from 0%, but not from each other. The sums of the percent glucose and xylose values for the 1% 30, 60 and 90 minutes samples were 27.26, 28.84 and 29.30% respectively. Most of these values were lower than those reported in a study on the treatment of corn fiber, distiller's dried grains, sugarcane bagasse and spent barley malt samples with 1.0% sodium percarbonate which resulted in 38.30, 25.60, 60.50 and 40.80% sugar release, respectively (Burdette et al., 2004). This could be explained by the fact that the current study measured only glucose and xylose while the other study includes all sugars. The discrepancy could also be due to differences in biomass composition and geographic conditions.

The above results suggest that using sodium percarbonate pretreatment may not be suitable for increasing percent glucose. A possible explanation for this phenomenon is that the dominant aqueous chemistry of sodium percarbonate is that of hydrogen peroxide (McKillop & Sanderson, 1995). Hydrogen peroxide has been shown to decompose soluble sugars during pretreatment (Kim & Hong, 2001). The glucose was most likely degraded during pretreatment with sodium percarbonate explaining the decrease in glucose with the addition of sodium percarbonate as opposed to pure water.

4.2.2.2. Lignin Reduction

Overall, the sodium percarbonate pretreatment was not as effective as the sodium hydroxide in lignin reduction. The values are shown in Figure 4.3.

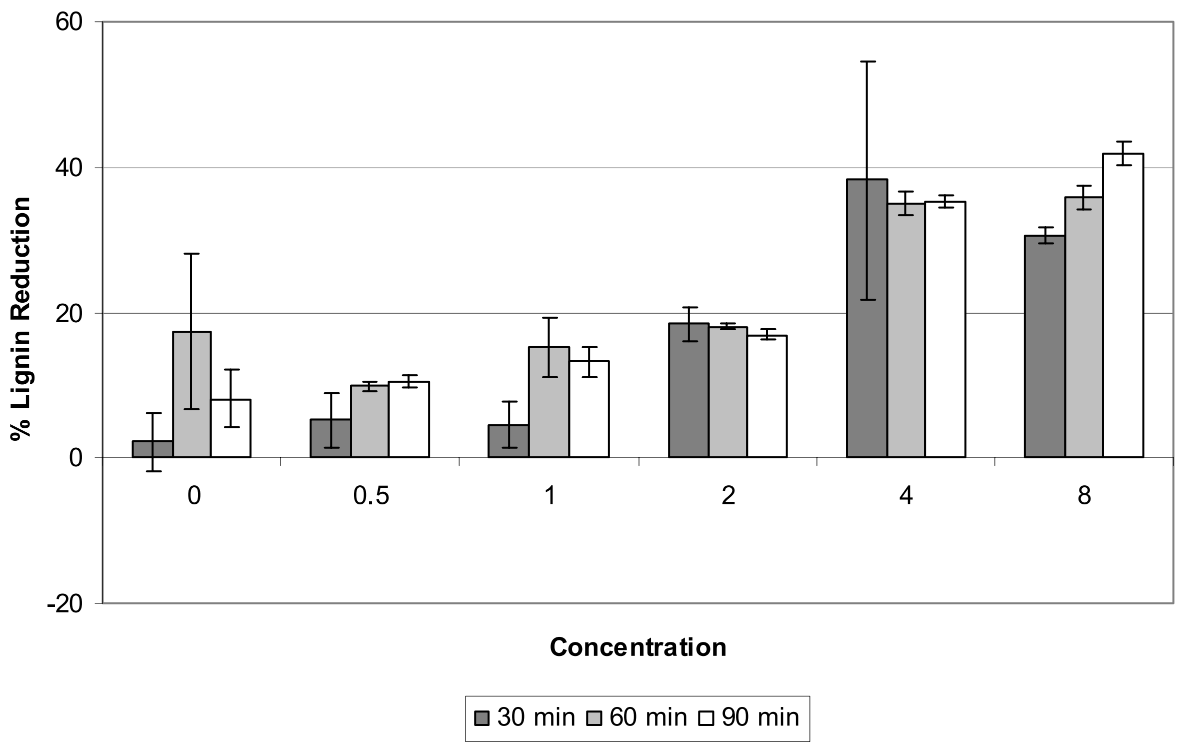

Figure 4.3. Lignin Reduction of Sodium Percarbonate Treated Cotton Stalk

The average lignin reduction values ranged from 2.14% reduction in the 0% 30 minute samples to 41.83% reduction in the 8% 90 minute samples. At a 5% significance level, the time and concentration both had an effect on lignin reduction while the time-concentration interaction did not. The delignification in 60 and 90 minute samples was significantly higher ($p \leq 0.05$) than that in the 30 minute samples, but there was no significant difference among them. This indicates that pretreatment of cotton stalks beyond 60 minutes does not effect lignin degradation. Relative to chemical concentration, there was no significant difference in lignin reduction between the 0, 0.5 and 1% pretreated samples or between the 1 and 2% samples. However, delignification was significantly lower ($p \leq 0.05$) with 0.5% compared to 2% samples. The 4 and 8% sample were significantly different from the rest, but not from each other. It may be inferred that 4% sodium percarbonate pretreatment of cotton stalks for 60 minutes would yield the maximum lignin reduction for this chemical.

4.2.2.3. Hemicellulose Solubilization

The average hemicellulose solubilization in cotton stalks pretreated with sodium percarbonate is presented in Figure 4.4. Only the xylose solubilization was accounted for in the pretreated samples because the arabinose and galactose concentrations were considered negligible. (Arabinose average = 1.86 mg/l and Galactose average = .83mg/l)

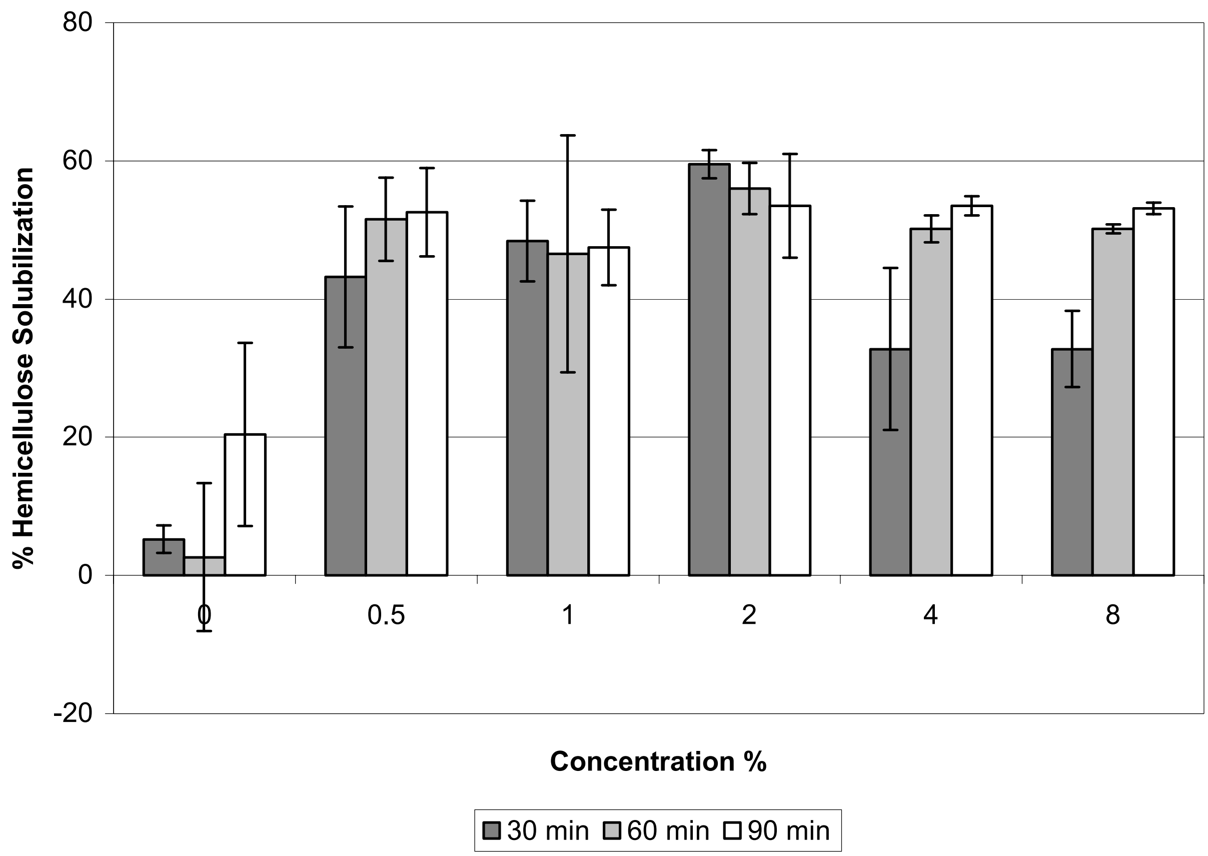

Figure 4.4. Hemicellulose Solubilization in Sodium Percarbonate Treated Cotton Stalks

Concentration and the time-concentration interaction significantly ($p \leq 0.05$) affected hemicellulose solubilization while time did not. The hemicellulose solubilizations at the various pretreatment times were not significantly different from each other. The solubilization values ranged from 2.67% in the 0% 60-minute samples to 59.54% in the 2% 30 minute samples. There was no significant difference ($p \leq 0.05$) between the 0.5, 1, 2, 4 and 8% samples but 0% was different from all the others. This suggests that using a 0.5% sodium percarbonate pretreatment of cotton stalk for 30 minutes may be sufficient to achieve the maximum xylose solubilization while minimizing time but more intense treatments may be necessary to obtain other desirable pretreatment effects such as lignin reduction.

4.2.3. Chemical Pretreatment Conclusions

The chemical pretreatments demonstrated the ability to reduce lignin and increase sugar availability. Sodium hydroxide degraded significantly more lignin in the cotton stalks than sodium percarbonate and resulted in significantly higher hemicellulose solubilization. However, further economic analysis and scale-up studies are needed to determine if the cost of the additional concentration of sodium hydroxide can be justified by the additional sugars released during pretreatment.

4.3. Subcritical Water Pretreatment

The major reaction pathways for cellulose in subcritical water are hydrolysis, retro-aldol condensation, keto-enol tautomerism and dehydration (Sasaki et al., 2004). The hydrolysis reaction is important in the production of monomeric sugars, as the glucose and xylose monomers hydrolyzed from cellulose and hemicellulose, respectively, are critical for high ethanol yields (Sasaki et al., 2004). All percent lignin reduction values for the subcritical water pretreatments were calculated using an initial lignin percentage of 28.8%.

4.3.1. Effect on Lignocellulosic Biomass Components in Pure Form

Pretreatment experiments were conducted on pure lignin and cellulose to study the effect of subcritical water on the individual components of lignocellulosic feedstocks. This would help to understand their behavior in the pure form as well as the interaction in the biomass. Organosolv lignin and cotton balls were used to represent the lignin and cellulose components, respectively.

4.3.1.1. Pure Lignin

The data for percent acid soluble lignin in the pretreatment liquid and the percent reduction in acid insoluble lignin of the solid pretreated pure organosolv lignin sample is shown in Figures 4.5A and B, respectively.

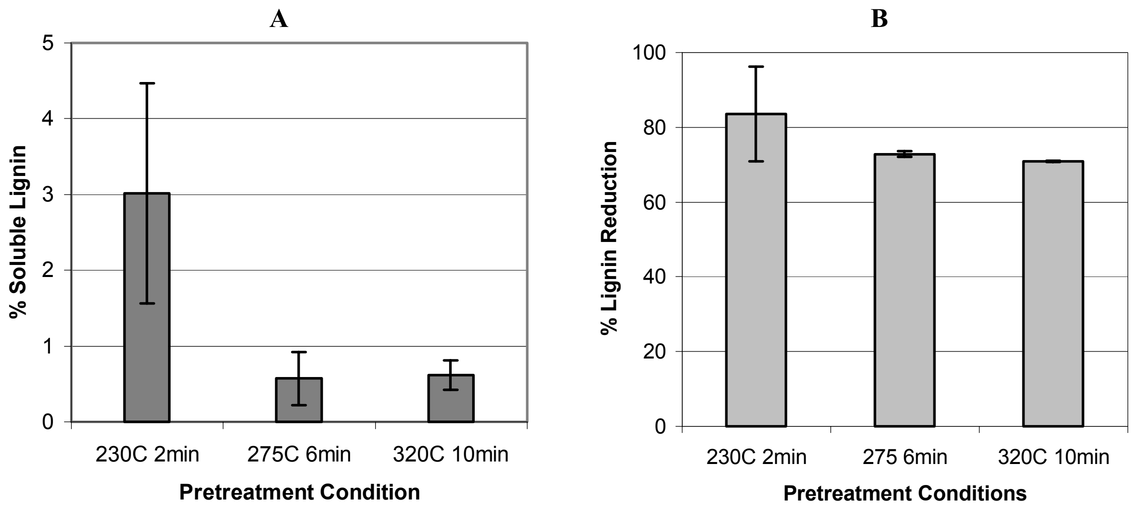

Figure 4.5. Lignin Percentages for Pure Lignin Samples

The percent lignin reduction at the three pretreatment conditions was not significantly different ($p \leq 0.05$) from each other. However, the percent soluble lignin in 230°C 2 minute samples was significantly higher than the two more severe combinations. It is possible that the 320°C 10 minute and 275°C 6 minute completely degraded the lignin into molecules that no longer absorbs light at 205 nm.

4.3.1.2. Pure Cellulose

The percent sugars in the pretreated solids and pretreatment liquid of the pure cellulose samples are presented in Figure 4.6.

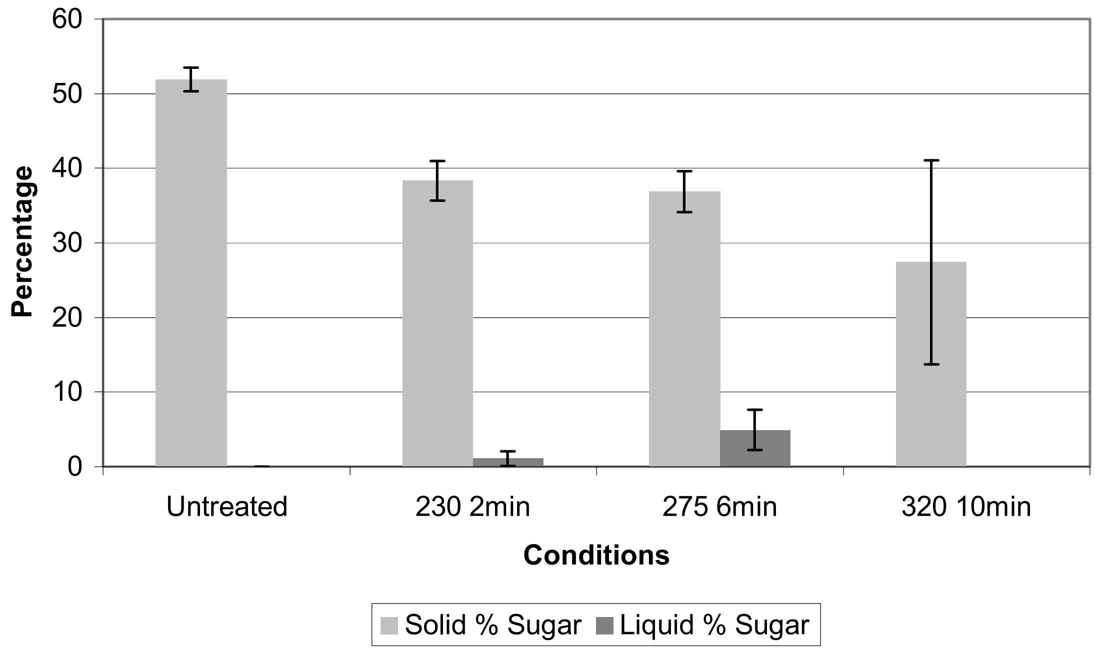

Figure 4.6. Percent Sugars in Pure Cellulose Samples

The total percent sugars (solid + liquid) obtained from pure cellulose after pretreatment at 230°C/2minute, 275°C/6minute and 320°C/10minute were not significantly different ($p \leq 0.05$) from each other. These percentages are lower than expected for pure cellulose. The untreated cotton balls, which were assumed to be 100% sugars, only produced 51.91 total percent sugars. One explanation could be that cotton balls contain impurities that reduce pure cellulose content, although literature suggests that composition of cotton balls is very close to 100% cellulose (Gao & Jangala, 2003). Another explanation could be that the sugars are degraded or transformed by the acid during analysis.

4.3.2. Effect on Whole and Smashed Cotton Stalks

In addition to grinding to different particles sizes, the cotton stalks were also smashed with a sheet metal roller and left as is (whole) for a set of pretreatments.

4.3.2.1. Sugar Availability

The percent sugar in the liquid (SugarsL), solid (SugarsS) and total sugars (Sugars) for subcritical water pretreated whole (W) and smashed (S) cotton stalks are shown in Table 4.4.

Table 4.4. Whole and Smashed Percent Sugars

Temp (C)	Time (min)	Size (in)	% SugarsL		% SugarsS		% Sugars	
230	2	S	1.70	*A*	20.78	*BCD*	22.49	*BCD*
230	2	W	2.85	*A*	20.05	*BCD*	22.90	*BCD*
230	6	S	2.67	*A*	12.12	*BCDE*	14.79	*BCDE*
230	6	W	2.45	*A*	22.16	*BC*	24.61	*BC*
230	10	S	3.50	*A*	9.84	*BCDE*	13.34	*BCDE*
230	10	W	1.32	*A*	43.45	*A*	44.77	*A*
275	2	S	2.30	*A*	19.63	*BCD*	21.92	*BCD*
275	2	W	3.57	*A*	20.14	*BCD*	23.71	*BCD*
275	6	S	1.93	*A*	12.18	*BCDE*	14.10	*BCDE*
275	6	W	2.04	*A*	15.63	*BCDE*	17.67	*BCDE*
275	10	S	2.14	*A*	11.37	*BCDE*	13.50	*BCDE*
275	10	W	0.74	*A*	25.58	*AB*	26.32	*B*
320	2	S	2.57	*A*	20.04	*BCD*	22.61	*BCD*
320	2	W	1.76	*A*	1.32	*DE*	3.09	*E*
320	6	S	3.19	*A*	7.86	*BCDE*	11.05	*BCDE*
320	6	W	1.58	*A*	-0.54	*E*	1.04	*E*
320	10	S	2.00	*A*	6.05	*BCDE*	8.05	*CDE*
320	10	W	1.88	*A*	2.84	*CDE*	4.72	*DE*

* Averages in the same column followed by the same letter are not significantly different

The maximum total percent sugar for the whole cotton stalks was 44.77% for samples pretreated with the 230°C, 10 minutes combination. The highest percent total sugar for the smashed samples was 22.61% for the 320°C, 2 minutes pretreatment combination.

The only combinations that show a significant difference between the whole and smashed total sugars are the 230°C, 10 minutes and the 320°C 2 minutes. For the 320°C, 2 minute samples the percent total sugar in smashed samples was significantly higher than the whole. For the 230°C, 10-minute comparison, the whole samples had significantly higher ($p \leq 0.05$) sugars than the smashed. These differences may be explained by variation in the cotton stalk composition as well as other factors not accounted for in the experiment such as the presence of acetyl groups and uronic acids in the hemicellulose and variations in surface and substrate availability (Garrote et al., 1999).

4.3.2.2. Lignin Reduction

The percent lignin reduction for the pretreated smashed and whole cotton stalks is shown in Table 4.5.

Table 4.5. Whole and Smashed Lignin Reduction and Solids Recovery

Temp (C)	Time (min)	Size (in)	% Lignin Red*		% Solids Recovery	
230	2	S	12.04	*B*	68.53	(10.68)
230	2	W	14.50	*B*	59.22	(5.03)
230	6	S	13.34	*B*	61.33	(14.72)
230	6	W	19.02	*AB*	58.26	(6.04)
230	10	S	21.83	*AB*	56.12	(0.95)
230	10	W	14.14	*B*	56.94	(4.06)
275	2	S	15.05	*B*	59.21	(5.87)
275	2	W	21.60	*AB*	50.00	(3.72)
275	6	S	24.15	*AB*	50.04	(5.03)
275	6	W	6.55	*B*	48.84	(16.89)
275	10	S	18.51	*AB*	55.01	(6.58)
275	10	W	7.06	*B*	59.22	(11.77)
320	2	S	18.08	*B*	51.97	(2.01)
320	2	W	26.92	*AB*	26.85	(4.25)
320	6	S	27.60	*AB*	33.97	(1.46)
320	6	W	59.41	*A*	14.89	(11.06)
320	10	S	29.58	*AB*	23.81	(0.30)
320	10	W	15.88	*B*	35.82	(21.50)

* Averages in the same column followed by the same letter are not significantly different

The maximum lignin reduction for the whole stalks was 59.41% with the 320°C, 6 minute pretreatment as shown in Table 4.5. There was no significant difference in the percent lignin reduction between smashed and whole samples for any pretreatment combination. There was no significant difference in the percent lignin reduction between the corresponding combinations when the whole and smashed were compared to the ground samples.

4.3.3. Effect on Ground Cotton Stalks

The effect of subcritical water pretreatment on ground cotton stalks was also evaluated for particle sizes 1/8, 3/16 and 1/4 inches.

4.3.3.1. Sugar Availability

The percent sugars found in subcritical water pretreated ground cotton stalk samples are shown in Table 4.6.

Table 4.6. Percent Sugars from Subcritical Water Pretreatment of Ground Cotton Stalks

Temp (deg C)	time (min)	size (in)	% Sugars-Liquid[1]		% Sugars-Solid[1]		% Total Sugars[2]	
230	2	3/16	4.12	(1.32)	42.12	(2.78)	46.24	A
230	6	1/4	2.21	(0.41)	25.44	(9.30)	27.65	ABC
230	6	1/8	4.58	(1.76)	28.79	(3.11)	33.37	AB
230	10	3/16	5.05	(0.15)	41.28	(4.68)	46.33	A
275	2	1/4	0.74	(0.12)	23.75	(1.24)	24.49	BCD
275	2	1/8	1.04	(0.29)	23.17	(2.63)	24.21	BCD
275	6	3/16	4.44	(0.91)	41.16	(15.85)	45.61	A
275	6	3/16	5.53	(1.18)	24.61	(3.84)	30.14	ABC
275	6	3/16	3.14	(0.28)	22.16	(1.03)	25.3	BCD
275	6	3/16	4.01	(2.50)	21.13	(7.76)	25.14	BCD
275	6	3/16	2.31	(0.69)	18.18	(1.15)	20.48	BCD
275	10	1/4	2.95	(0.09)	16.07	(3.30)	19.02	BCD
275	10	1/8	5.32	(1.27)	30.58	(6.61)	35.91	AB
320	2	3/16	8.08	(0.57)	27.01	(10.30)	35.09	AB
320	6	1/4	2.16	(1.93)	7.91	(8.68)	10.08	CD
320	6	1/8	5.56	(0.61)	11.78	(0.83)	17.35	BCD
320	10	3/16	1.67	(1.15)	5.63	(4.23)	7.296	D

[1] Standard Deviations are shown in parenthesis
[2] Averages with the same letter are not significantly different

The percent total sugars were calculated by dividing the milligrams of sugars obtained after pretreatment by the milligrams of initial cotton stalk sample and include all reducing sugars (glucose, xylose, etc). The highest percent sugar of 8.08% in the pretreatment liquid sample was obtained for 320°C, 2 minutes and 3/16 inch particle size, however it appears that the began to degrade after 2 minutes. The highest percent sugar of 42.12% in the solid sample was found for 230°C, 2 minutes and 3/16 inch particle size. The highest percent total sugar of 46.33% was found for 230°C, 10 minutes and 3/16 inches.

As shown in Table 4.6 the samples treated with 230°C water had the highest percent total sugars and were not significantly different from each other. From this information it can be inferred that lower temperatures generate the significantly higher ($p \leq 0.05$) percent total sugars and at lower temperatures time and particle size did not have a large impact. However, at higher temperatures holding time had a significant impact. The percent total sugars at 320°C and 3/16 inch particle size for 2 and 10 minutes were 35.09 and 7.30% respectively and were significantly different ($p \leq 0.05$) from each other. This may be due to the breakdown of sugar molecules in both the solid and liquid factions at high temperature and time combinations. The findings in this study are comparable to those of (Sasaki et al., 2004) who found that longer reaction times at lower temperatures could yield results similar to those with shorter reaction times and higher temperatures. Hong and Spritzer (2002) have reported complete gasification of lignocellulosic biomass at supercritical temperatures so it was expected that some degradation would occur at subcritical temperatures.

There was significant variation in the results from repeated experiments at the midpoint 275°C, 6 minute, 3/16 inch. As shown in Table 4.8, the percent total sugar values for the midpoints ranged from 45.61 to 20.48% of which to values were significantly higher ($p \leq 0.05$) than the other three. As mentioned above, this can be explained by heterogeneity of the cotton stalks as well as other factors not accounted for in the experiment such as the presence of acetyl groups and uronic acids in the hemicellulose and variations in surface and substrate availability (Garrote et al., 1999).

4.3.3.2. Lignin Reduction

The percent lignin reduction and solids recovery are shown in Table 4.7.

Table 4.7. Lignin Reduction and Solids Recovery After Subcritical Water Pretreatment of Ground Cotton Stalks

Temp (deg C)	time (min)	size (in)	% Lignin Reduction[1]		% Solids Recovery[2]	
230	2	3/16	14.30	C	65.60	(1.84)
230	6	1/4	17.84	BC	66.31	(1.23)
230	6	1/8	20.72	ABC	61.70	(4.91)
230	10	3/16	21.00	ABC	60.28	(7.24)
275	2	1/4	22.41	ABC	65.60	(6.47)
275	2	1/8	21.06	ABC	62.41	(3.25)
275	6	3/16	17.68	BC	59.22	(3.25)
275	6	3/16	25.14	ABC	57.09	(6.50)
275	6	3/16	25.53	ABC	48.94	(5.03)
275	6	3/16	23.28	ABC	65.60	(3.74)
275	6	3/16	25.06	ABC	55.32	(12.69)
275	10	1/4	20.71	ABC	49.65	(5.46)
275	10	1/8	27.52	ABC	45.39	(12.90)
320	2	3/16	36.70	A	32.27	(2.46)
320	6	1/4	28.03	ABC	25.18	(0.61)
320	6	1/8	30.82	AB	36.17	(4.88)
320	10	3/16	34.99	A	25.53	(1.63)

[1] Averages with the same letter are not significantly different
[2] Standard Deviations shown in parenthesis

The highest percent lignin reduction of 36.70% was found in 3/16 inch cotton stalks pretreated at 320°C/ 2 minutes. This value was however not significantly different ($p \leq 0.05$) from values in the range of 20.72% to 28.03% lignin reduction. Generally higher times and temperatures yielded higher percent lignin reductions. Song et al. (2004) in a study on the pretreatment of corn stover obtained maximum lignin reduction of 25% to be at 600K (327°C) from a range of temperatures between 300 and 850K. This suggests that pretreatments at temperatures beyond those in the current study (230-320°C) may not improve lignin reduction. The highest percent lignin reduction corresponds to the highest percent liquid sugars, but there does not appear to be any correlations between other percent lignin reduction and percent sugars in pretreatment liquid.

4.3.3.3. Tukey Comparisons Between Whole, Smashed and Ground Samples

The Tukey comparisons for the smashed and whole data analyzed separately are shown in Table 4.8. This means that the data sets were only compared within themselves and not to each other.

Table 4.8. Tukey Comparisons for Whole and Smashed Separately*

Temp (C)	Time (min)	% Sugars Liquid		% Sugars Solid		% Total Sugars		% Lignin Reduction	
Whole									
230	2	2.85	A	20.05	BC	22.90	BCD	14.50	AB
230	6	2.45	A	22.16	ABC	24.61	ABC	19.02	AB
230	10	1.32	A	43.45	A	44.77	A	14.14	AB
275	2	3.57	A	20.14	BC	23.71	BCD	21.60	AB
275	6	2.04	A	15.63	BC	17.67	BCDE	6.55	B
275	10	0.74	A	25.58	AB	26.32	AB	7.06	B
320	2	1.76	A	1.32	C	3.09	DE	26.92	AB
320	6	1.58	A	-0.54	C	1.04	E	59.41	A
320	10	1.88	A	2.84	BC	4.72	CDE	15.88	AB
Smashed									
230	2	1.70	*A*	20.78	*AB*	22.49	*A*	12.04	*A*
230	6	2.67	*A*	12.12	*ABC*	14.79	*AB*	13.34	*A*
230	10	3.50	*A*	9.84	*ABC*	13.34	*AB*	21.83	*A*
275	2	2.30	*A*	19.63	*AB*	21.92	*A*	15.05	*A*
275	6	1.93	*A*	12.17	*ABC*	14.10	*AB*	24.15	*A*
275	10	2.14	*A*	11.37	*ABC*	13.50	*AB*	18.51	*A*
320	2	2.57	*A*	20.04	*A*	22.61	*A*	18.08	*A*
320	6	3.19	*A*	7.86	*BC*	11.05	*AB*	27.60	*A*
320	10	2.00	*A*	6.05	*C*	8.05	*B*	29.58	*A*

* Values for each treatment separately (whole or smashed) in the same column with the same letter are not significantly different.

As shown in Table 4.8, the maximum of total sugars value for the whole stalks (230°C, 10 minutes) was not significantly different from 230°C, 6 minutes and 275°C 10 minute values. The maximum percent total sugar in the smashed samples (320°C, 2 minutes) was not significantly higher ($p \leq 0.05$) than all but one other pretreatment: 320°C 10 minutes. This means that this pretreatment combination should yield significantly less total sugars than the top combination. The maximum percent lignin reduction for the whole cotton stalks was only significantly higher than all but two of the data averages: 275°C, 6 and 10 minutes as shown in Table 4.6.

Tukey comparisons were conducted between ground, whole and smashed cotton stalk samples. There were no significant differences in percent lignin reduction. The results for percent total sugars are shown in Table 4.9.

Table 4.9. Subcritical Water Pretreated Whole and Smashed Compared to Ground Cotton Stalks*

Temp	time	size (ground)	Sig Diff between		% Sugars (ground)
			Smashed?	Whole?	
230	2	3/16	**Y**	**Y**	46.237
230	6	1/8	N	N	33.371
230	6	1/4	N	N	27.653
230	10	3/16	**Y**	N	46.330
275	2	1/8	N	N	24.210
275	2	1/4	N	N	24.493
275	6	3/16	N	N	25.298
275	6	3/16	N	N	20.482
275	6	3/16	**Y**	**Y**	45.605
275	6	3/16	N	N	25.138
275	6	3/16	N	N	30.144
275	10	1/8	**Y**	N	35.905
275	10	1/4	N	N	19.019
320	2	3/16	N	N	35.089
320	6	1/8	N	N	17.345
320	6	1/4	N	N	10.075
320	10	3/16	N	N	7.296
* Significant Differences are indicated by a **Y**					

Significant differences (p≤0.05) between smashed and ground pretreated cotton stalks were found for four of the ground sample combinations. The ground samples were higher in percent total sugars than smashed samples at the time temperature combinations of 230°C 2 minute 3/16 inch, 230°C 10 minute 3/16 inch, 275°C 6 minute 3/16 inch and 275°C 10 minute 1/8 inch ground samples. There were two pretreatment combinations with significantly different percent total sugar values for whole and ground cotton stalks. The percent total sugars were significantly higher in the ground than the corresponding whole time temperature combination for the 230°C 2 minute 3/16 inch and 275°C 6 minute 3/16 inch pretreatments.

4.3.3.4. Response Surface Modeling

A response surface model of the form in Equation 4.1 was developed using the RSREG procedure in SAS. The corresponding coefficients calculated by SAS are shown in Table 4.10. The R-squared values for the percent total sugars and percent lignin reduction models were .6048 and .5112, respectively.

$$\text{\% Predicted Variable} = A + A_1 t + A_2 T + A_3 s + A_4 t^2 + A_5 tT + A_6 T^2 + A_7 st + A_8 sT + A_9 s^2 \quad (4.1)$$

Table 4.10. RSM Coefficients

Term	% Sugars	% Lignin Reduction
Intercept (A)	-29.0229	34.3418
Time (A_1)	10.1238	4.3056
Temp (A_2)	-0.0572	-0.4313
Size (A_3)	804.3590	158.8633
Time*Time (A_4)	0.2563	0.0618
Time*Temp (A_5)	-0.0387	-0.0117
Temp*Temp (A_6)	0.0002	0.0012
Size*Time (A_7)	-17.1692	-8.1722
Size*Temp (A_8)	-0.1379	0.0088
Size*Size (A_9)	-1926.9188	-358.9235

The model predicted values were calculated in MS Excel (MS Office, 2000) and are shown in Table 4.11.

Table 4.11. RSM Predicted Values

Temp (deg C)	Time (min)	size (in)	Predicted % Sugars	Predicted % Lignin Red
230	2	3/16	39.91	15.81
230	6	¼	29.26	22.58
230	6	1/8	35.88	22.45
230	10	3/16	48.48	18.16
275	2	¼	29.16	26.83
275	2	1/8	27.97	22.58
275	6	3/16	29.31	15.32
275	6	3/16	29.31	22.58
275	6	3/16	29.31	33.70
275	6	3/16	29.31	21.60
275	6	3/16	29.31	20.31
275	10	¼	15.21	19.95
275	10	1/8	31.19	22.58
320	2	3/16	32.89	31.80
320	6	¼	7.52	29.05
320	6	1/8	15.69	22.58
320	10	3/16	13.58	31.93

The maximum experimental and predicted percent total sugars were 46.33% and 48.48% at 230°C, 10 minutes and 3/16 inch particle size. The experimental and predicted values for maximum percent lignin reduction, 36.70% and 31.80% respectively, coincided at 320°C, 2 minutes and 3/16 particle size. The residuals for the percent sugar and lignin reduction models are plotted in Figures 4.7. and 4.8.

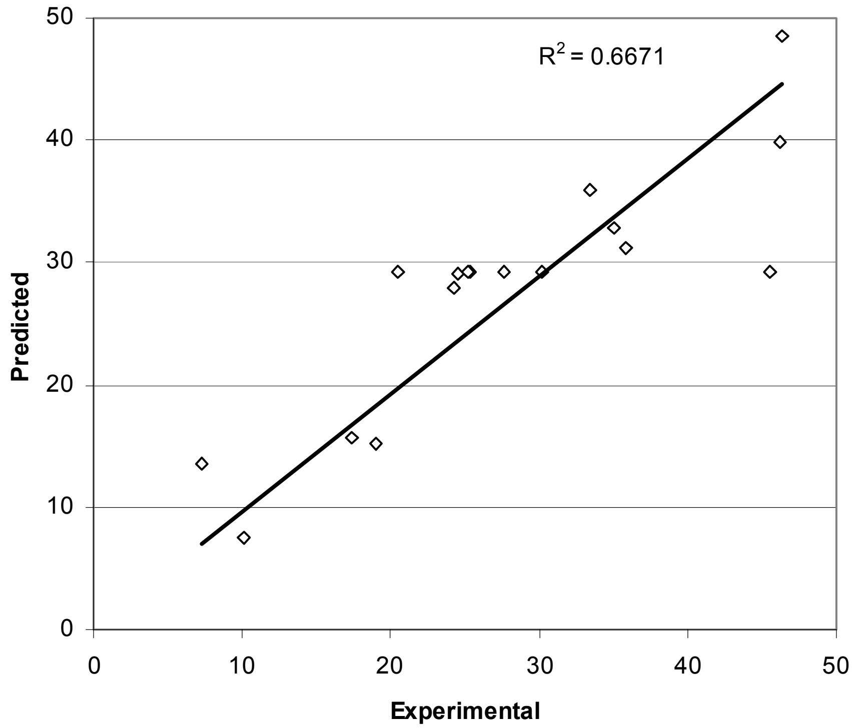

Figure 4.7. Response Surface Model for Percent Total Sugars

The R-squared value for the linear regression through the plot between predicted and experimental percent total sugars was .6671. The R-squared value for the linear regression between predicted and experimental percent lignin reduction values was 0.851. For both the percent total sugars and lignin reduction, the most discrepancy between predicted and experimental data was observed at the midpoints (275°C, 6 minutes, 3/16 inches). The low predictive ability of the model may be attributed to factors such as diffusion of subcritical water, heterogeneity of cotton stalks and solids loading that were not investigated in this study.

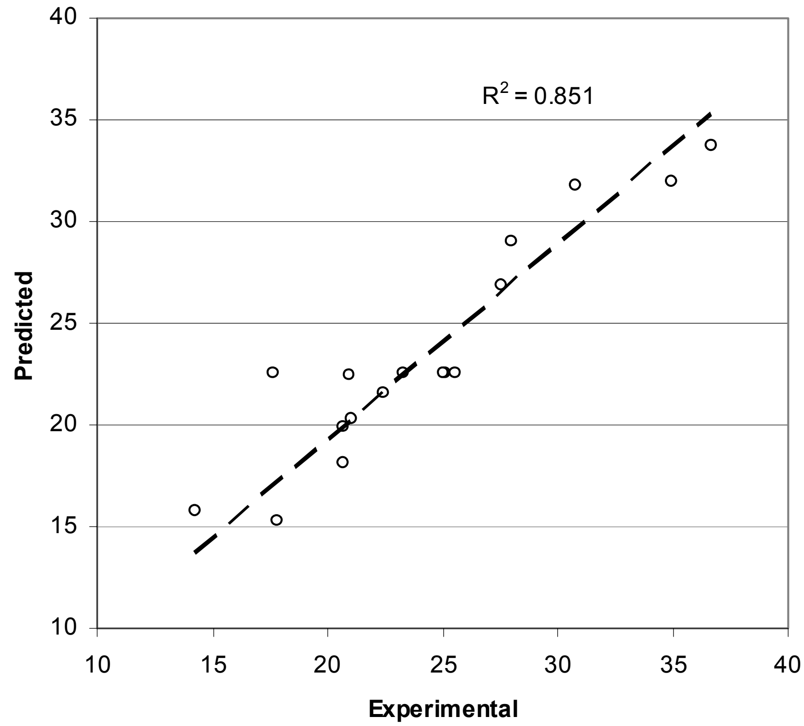

Figure 4.8. Response Surface Model for Percent Lignin Reduction

Figures 4.9 – 4.11 present experimental data superimposed on the response surface plots of the model predicted values over the entire range of variable investigated. Two plots for each independent variable are shown here; the rest are included in the Appendix.

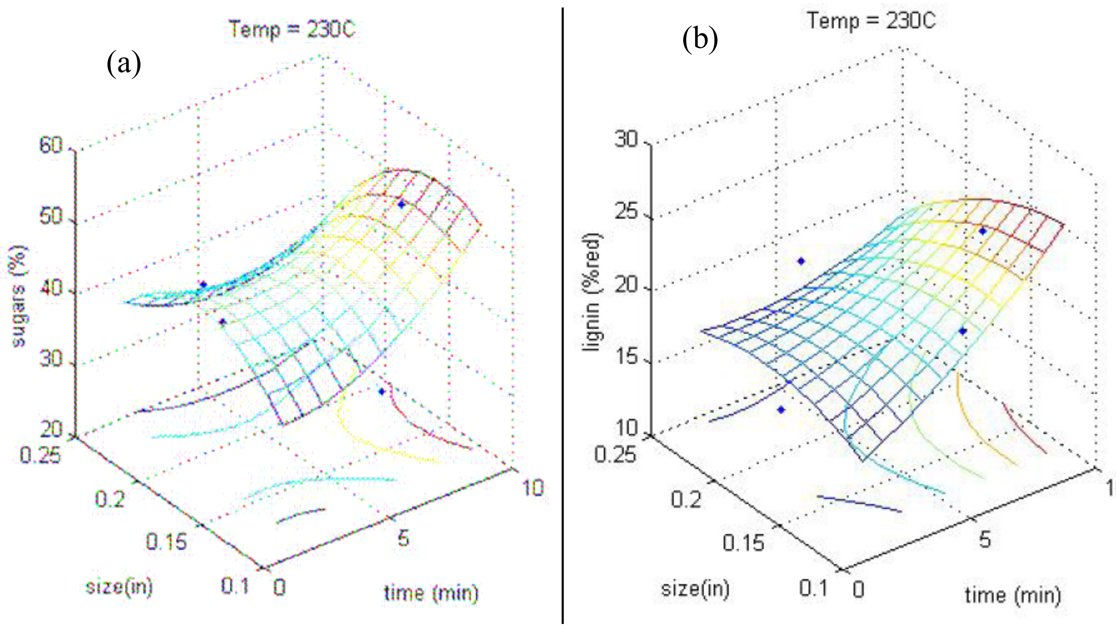

Figure 4.9. Response Surface Model Temperature Plots
(a) % Total Sugars (b) % Lignin Reduction

Figure 4.9 (a) shows the predicted and experimental percent total sugars data for the lowest temperature, 230°C. The percent total sugars model values were higher for longer time intervals at medium particle size and correspond with the experimental data. Figure 4.9. (b) shows the RSM percent lignin reduction and experimental data points for the lowest temperature, 230°C. The percent lignin reduction model values were high for longer holding time and lower particle size values.

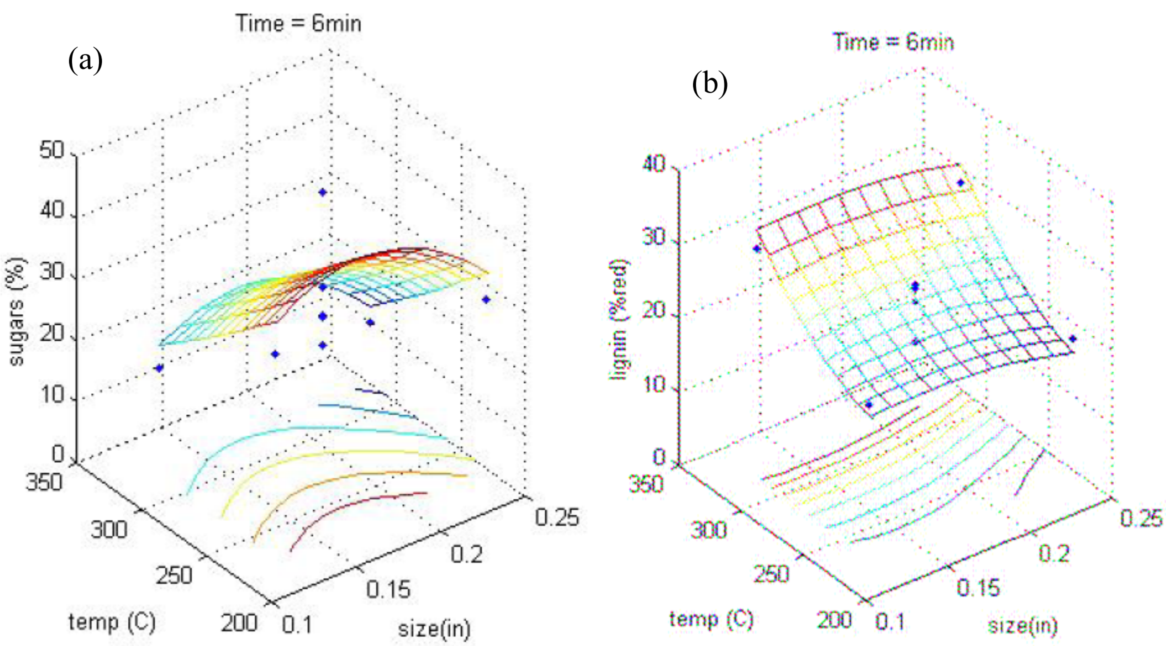

Figure 4.10. Response Surface Model Time Plots
(a) % Total Sugars (b) % Lignin Reduction

Figure 4.10 (a) shows the percent total sugars for the intermediate time value, 6 minutes. The model shows high percent total sugars at low temperatures and medium particle size. Figure 4.10 (b) shows the percent lignin reduction for the six-minute treatment and indicates higher percent lignin reduction at higher temperatures. The superimposed experimental data confirms the trend presented by the RSM plot.

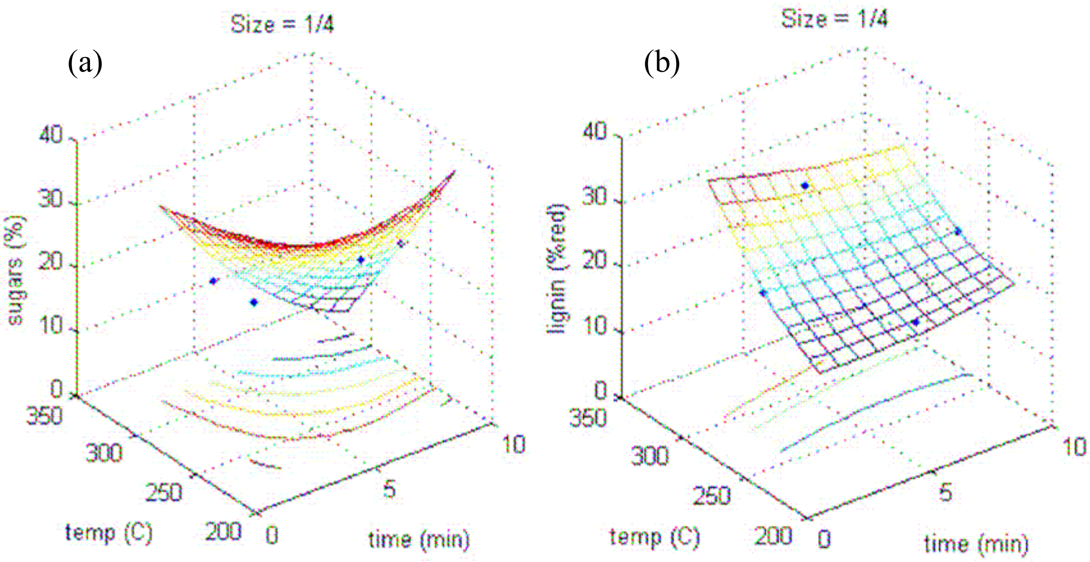

Figure 4.11. Response Surface Model Size Plots
(a) % Total Sugars (b) % Lignin Reduction

Figures 4.11 (a) and (b) show the modeled and corresponding experimental data for percent total sugars and percent lignin reduction at 1/4 inch particle size. Higher percent total sugars were obtained at low temperatures and longer times as well as at high temperature and shorter times. The plots correlate with the trend of the experimental data. The RSM plot of percent lignin reduction shows the highest reduction at high temperatures and low time values.

Overall, the models were successful at revealing trends in the experimental data, but did not give an optimum as expected. The RSREG analysis with SAS version 9.1.3 (Cary, NC) determined that both the percent total sugars and lignin reduction prediction models generated saddle points implying that the optimum was not within the experimental range, but on one end of the range.

4.3.3.5. Enzymatic Hydrolysis

The enzymatic hydrolysis results are listed in Table 4.12.

Table 4.12. Enzymatic and Acid Hydrolysis Sugars*

Temp (C)	Time (min)	Size (in)	% Sugars (Hydrolysis)	% Sugars (Pretreatment)
230	2	3/16	23.75 A	42.11 B
230	10	3/16	29.49 A	41.28 B
275	6	3/16	28.48 A	41.16 B
320	2	3/16	22.97 A	23.45 A
320	6	1/8	23.61 A	16.31 A
320	10	3/16	12.10 A	5.63 A

* Values in the same row with the same letter are not significantly different

The maximum sugar released during enzymatic hydrolysis was 29.489% for the 230°C, 10 minute, 3/16 inch samples. The sugars in the 320°C, 10 minute, 3/16 inch samples were significantly lower ($p \leq 0.05$) than all other samples except the 320°C 6 minutes, 3/16 inch samples. There were no other significant differences among the enzymatic hydrolysis samples. Percent cellulose reduction calculations were not made since individual sugars values were not known.

The enzymatic hydrolysis sugars from the top three percent total sugars combinations were all higher than the hydrolysis sugars from the top three percent lignin reduction combinations. This suggests that lignin reduction may not be as critical for sugar release during subcritical water pretreatment as reported for other pretreatment methods. Besides, samples with the highest lignin reductions corresponded with the highest temperature treatments wherein the sugars may have been degraded and were not detected by the DNS assay. One way to check for this would be to analyzed the pretreatment liquid for sugars degradation products such as glyceraldehydes and 5-HMF (Sasaki et al., 1998)

All the samples had higher pretreatment sugars (analyzed using procedures in 3.2.1 and 3.2.3.2) than the enzymatically hydrolyzed sugars except the 320°C, 10 minute, 3/16 inch and the 320°C, 6 minute, 1/8 inch samples. However, the higher temperature samples had no significant difference between the enzymatic and acid hydrolysis sugar yields. The only significant differences in percent sugar between enzymatically hydrolyzed and pretreated samples were at 230 and 275°C. The differences in sugar levels between enzymatic and acid hydrolyzed samples at various conditions is probably because enzymes are highly specific and release mostly glucose from cellulose (Sun & Cheng, 2002) while acid is not specific and releases all types of sugars from cellulose and hemicellulose (Lee et al., 1999). The degradation of cellulose and hemicellulose during pretreatment may have been affected by pretreatment conditions.

4.3.4. Subcritical Water Pretreatment Conclusions

It was found that lower temperatures produce more total sugars and higher temperatures produce higher lignin degradation. Similar to the ground up samples, the lower temperatures produced more total sugars from the whole stalks. Except in one case, smashing the stalks did not increase the percent total sugars and the lignin reduction in any cases when compared to whole stalks. Also, the ground stalks did not show any significant increase in lignin degradation but increased the percent total sugars for two of the whole sample sets and four of the smashed sample sets. The interaction of the components in the lignocellulosic biomass also play a role in the effectiveness of the pretreatment as pure lignin samples produced significantly higher lignin reduction and cellulose samples produced significantly higher percent total sugars in some cases. The percent lignin reduction for the pure lignin samples was significantly higher than all of the ground cotton stalk samples. This may be due to the easy access of subcritical water in the absence of lignin interaction with the other components in lignocellulosic feedstocks. While the percent total sugars from the pure cellulose samples treated for 10 minutes at 320°C were significantly higher ($p \leq 0.05$) than the corresponding ground cotton stalk samples, there was no significant difference in sugar yield from pure cellulose and stalk samples pretreated at other time-temperature combinations. This suggests that the inhibitory effects of interaction between cellulose and other components in the lignocellulosic biomass is more pronounced for treatments at higher temperature and longer times. Overall, low temperatures and large particle size appeared to produce the most total sugars while high temperatures produced high lignin reduction. Sample comminution did not seem to play as an important role for this pretreatment method as expected.

Chapter 5: Conclusions

5.1. Summary

This study investigated the potential of sodium hydroxide, sodium percarbonate and subcritical water as pretreatments of cotton stalks. For the sodium hydroxide pretreated cotton stalk samples, the percent glucose values ranged from 30.45% in the 0% NaOH, 30 minute samples to 50.85% in the 4%NaOH samples treated for 30 minutes. The percent xylose values ranged from 3.85% in the 8% 90 minutes samples to13.04% in the 0% 60 minute samples. The average lignin reduction values ranged from 2.14% reduction in the 0% 30 minute samples to 63.39% reduction in the 4% 30 minutes samples. The hemicellulose solubilization values ranged from 2.66% in the 0% 60-minute samples to 82.67% in the 8% 90-minute samples. When comparing to the study Silverstein(2004) it can be concluded that using 8% sodium hydroxide for 90 minute may be effective in providing optimal hemicellulose solubilization with sodium hydroxide.

For the sodium percarbonate treated samples, values for percent glucose ranged from 19.47% in the 60 minute 0.5% sodium percarbonate samples to 30.83% in the 0% 90 minute samples. The average lignin reduction ranged from 2.14% reduction in the 0% 30 minute samples to 41.83% in the 8% 90 minute samples. However, statistical analysis suggests that 4% sodium percarbonate pretreatment for 60 minutes will yield the statistically similar percent lignin reduction to the maximum value for this chemical. The hemicellulose solubilization values range from 2.67% in the 0% 60-minute samples to 59.54% in the 2% 30 minute samples. Statistical analysis suggests that using 0.5% for 30 minutes will give statistically similar percent xylose solubilization to the maximum while minimizing time and chemical usage.

For the subcritical water pretreated samples, the highest percent sugar in the pretreatment liquid sample (8.08%) was measured for 320°C, 2 minutes and 3/16 inch particle size while the highest percent sugar in the solid sample (42.12%) was obtained with 230°C, 2 minutes and 3/16 inch particle size. The highest percent total sugar measured, 46.33%, was for 230°C, 10 minutes and 3/16 inches. The highest percent lignin reduction of 36.70% was recorded for 320°C, 2 minutes and 3/16 inch particle size.

5.2. Conclusions

Overall, both pretreatment methods revealed success in reducing lignin and increasing sugar availability. Subcritical water was more successful at reducing lignin than both chemical pretreatments. Comparison between sugar availability and delignification with subcritical water and chemical pretreatments is difficult since two different designs of experiments were used for the two studies.

5.2.1. Comparison to Target

As mentioned earlier, potential pretreatment methods for lignocellulosic biomass can be compared to values from the commonly used dilute acid pretreatment of corn stover. The maximum percent xylose solubilization achieved with chemical pretreatments in the current study was 82.67% in the 8% 90-minute sodium hydroxide treated samples treated at 120°C/15psi**.** Dilute acid pretreatment using 1 weight % sulfuric acid has been found to solubilize over 90% xylose at a temperature of 190°C (Tucker et al., 2003). The xylose solubilization values for the chemical pretreatments in this study were lower than this target value perhaps because of the lower temperatures in the current study (121°C vs. 190°C). Using 1.16 (w/w) % sulfuric acid on corn stover for 6 minutes at 179°C has been shown to yield 68% xylose solubilization (Schell et al., 2003). The xylose solubilization values for the current study were higher than this target perhaps because of the longer treatment time (90 vs. 6 minutes). Hence it is essential to determine a time-temperature combination that can provide desirable results.

Enzymatic hydrolysis of the subcritical water pretreated samples resulted in a maximum sugar release of 29.49% for the 230°C, 10 minute, 3/16 inch pretreated samples. Saha and Bothast (1999) found that 703 mg sugars/g of corn fiber, which consists of corn hulls and residual starck, or 70.30% total sugars were released during enzymatic hydrolysis at 45°C and pH 5.0 for 72 hours from samples pretreated with 0.5% sulfuric acid at 121°C for 1 hour. The enzymatic hydrolysis sugar yields are higher from the acid pretreated samples than the subcritical water pretreated samples. This is most likely due to the fact that the corn fiber has higher starch content. However, the supercritical water pretreatment may still be more economical if the cost of the acid and its removal and/or neutralization is more than the profit decrease caused by lower sugar yields in subcritical water pretreatment. Further economic analysis is necessary to determine conclusively which is more cost-effective

5.2.2. Interaction of Components

In the chemical pretreated samples, samples with high lignin reduction generally correlated to high percent glucose values. This is expected since the presence of lignin hinders the release of sugars (Zaldivar et al., 2001). However, in the subcritical water pretreatments, the samples with high lignin reduction had lower total percent sugars. This could be explained by the phenomena that cellulose sometimes decomposes into other molecules such as ketones and aldehydes at higher temperatures (Sasaki, et al., 1998). The sugars values for the whole stalks were also sometimes higher than the ground and smashed stalks implying that the interaction of the components did not have an effect. However, the sugars in the whole stalks could have been better protected from the pretreatment and therefore not degraded as quickly as in the ground and smashed samples.

5.3. Future Work

This study provides critical information needed in the development of a feasible method for producing ethanol from lignocellulosic biomass. Future work to build on this study might include working with lower temperatures and longer times in the subcritical water pretreatment to find an optimum as the temperatures and times appeared to miss the optimum in the current study. Investigations into the degradation products present in the pretreatment liquid would also be useful in assessing whether and how much the sugars were degraded for each run. This could be done using GC or HPLC mass spectrometry. This could explain the low sugar numbers in the high temperature samples as well as the high sugars numbers in the whole stalk sample sugar data. The sugars in the subcritical water treated samples could also be also be measured using high performance liquid chromatography to gain insight on what particular reducing sugars are released during pretreatment and hydrolysis. Combining the chemical and subcritical water pretreatment methods may also hold potential. The methods may be combined using sodium hydroxide or sodium percarbonate as a cosolvent in the subcritical water pretreatment.

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APPENDICES

7.1. Enzymatic Hydrolysis Details

Table 7.1. Enzymatic Hydrolysis Details

	Temp (C)	Time (min)	Size (in)	sample (g)	rounded sample (g)	mL buffer	rounded buffer (mL)	FPU	cellulase (ml)	cellulase (ul)	cellobiase (ul)
Optimal Lignin	320	10	3/16	0.0056	0.01	0.28	0.3	0.4	0.004657	4.7	8.1
	320	10	3/16	0.01214	0.01	0.607	0.6	0.4	0.004657	4.7	8.1
	320	10	3/16	0.02702	0.03	1.351	1.4	1.2	0.01397	14	24.4
	320	2	3/16	0.01698	0.02	0.849	0.8	0.8	0.009313	9.3	16.3
	320	2	3/16	0.01486	0.01	0.743	0.7	0.4	0.004657	4.7	8.1
	320	6	1/8	0.0156	0.02	0.78	0.8	0.8	0.009313	9.3	16.3
	320	6	1/8	0.10382	0.1	5.191	5.2	4	0.046566	46.6	81.5
Optimal Sugars	230	10	3/16	0.2009	0.2	10.045	10	8	0.093132	93.1	163
	230	10	3/16	0.20191	0.2	10.0955	10.1	8	0.093132	93.1	163
	230	10	3/16	0.20014	0.2	10.007	10	8	0.093132	93.1	163
	230	2	3/16	0.20075	0.2	10.0375	10	8	0.093132	93.1	163
	230	2	3/16	0.20103	0.2	10.0515	10.1	8	0.093132	93.1	163
	230	2	3/16	0.20111	0.2	10.0555	10.1	8	0.093132	93.1	163
	275	6	3/16	0.20054	0.2	10.027	10	8	0.093132	93.1	163
	275	6	3/16	0.20134	0.2	10.067	10.1	8	0.093132	93.1	163
	275	6	3/16	0.16752	0.17	8.376	8.4	6.8	0.079162	79.2	138.5

7.2. Response Surface Model Plots

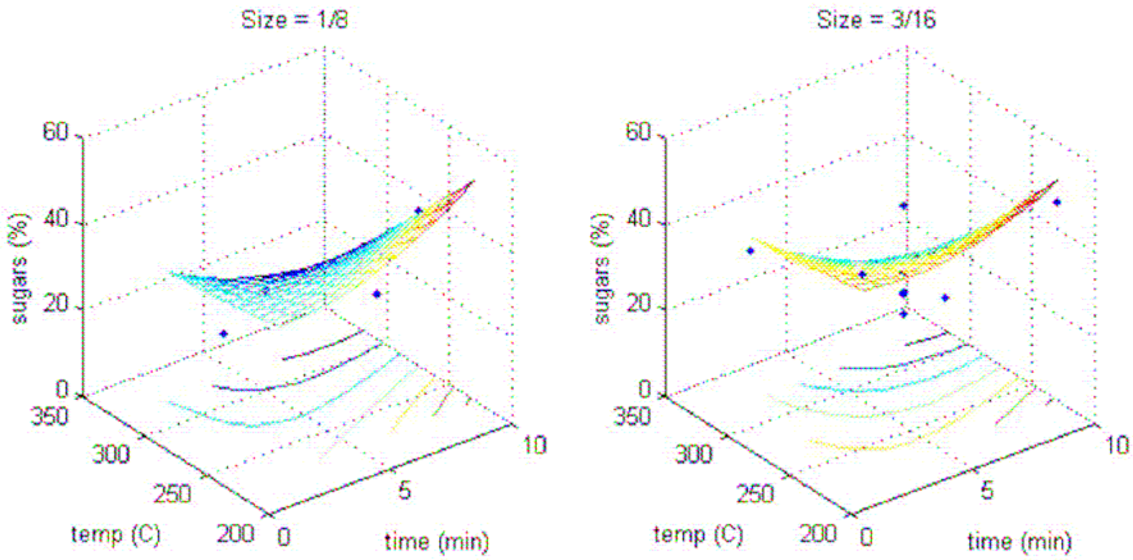

Figure 7.1. Percent Total Sugar RSM Plots for Size

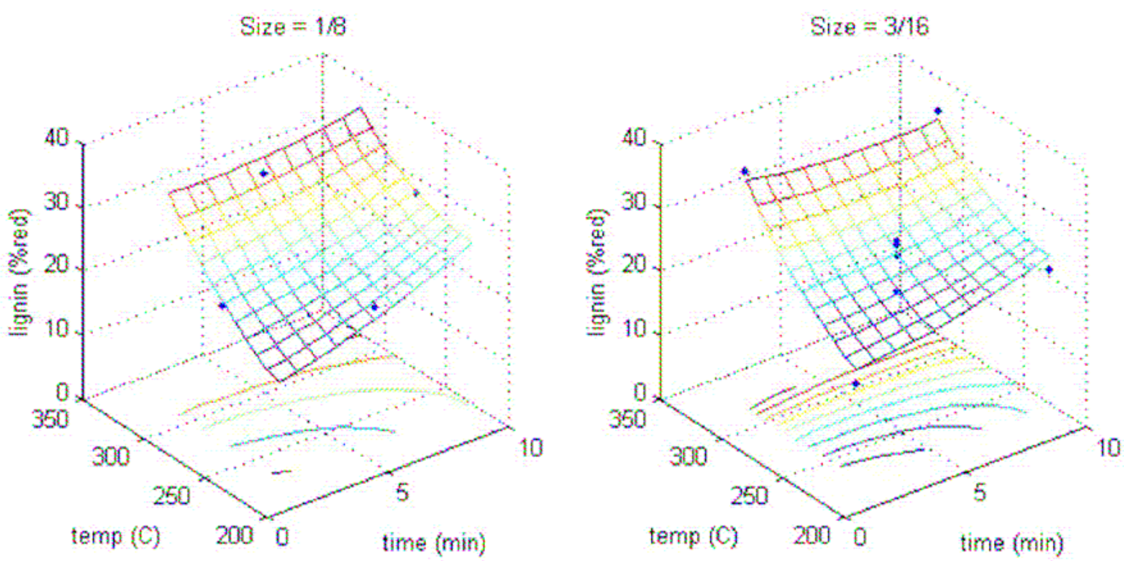

Figure 7.2. Percent Lignin Reduction RSM Plots for Size

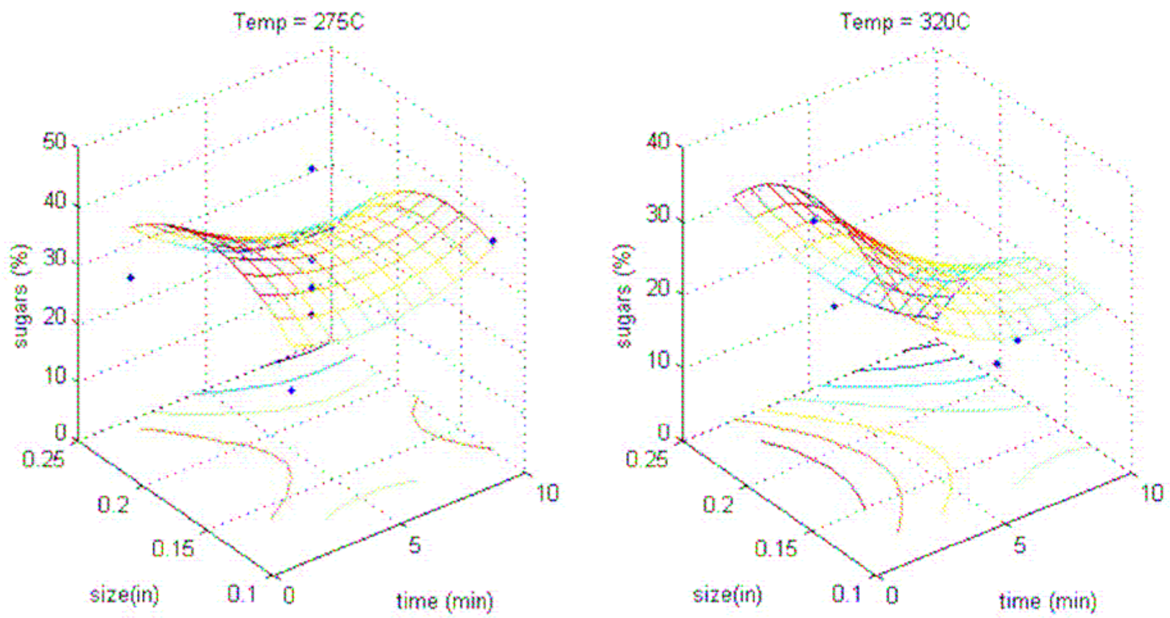

Figure 7.3. Percent Total Sugar RSM Plots for Temperature

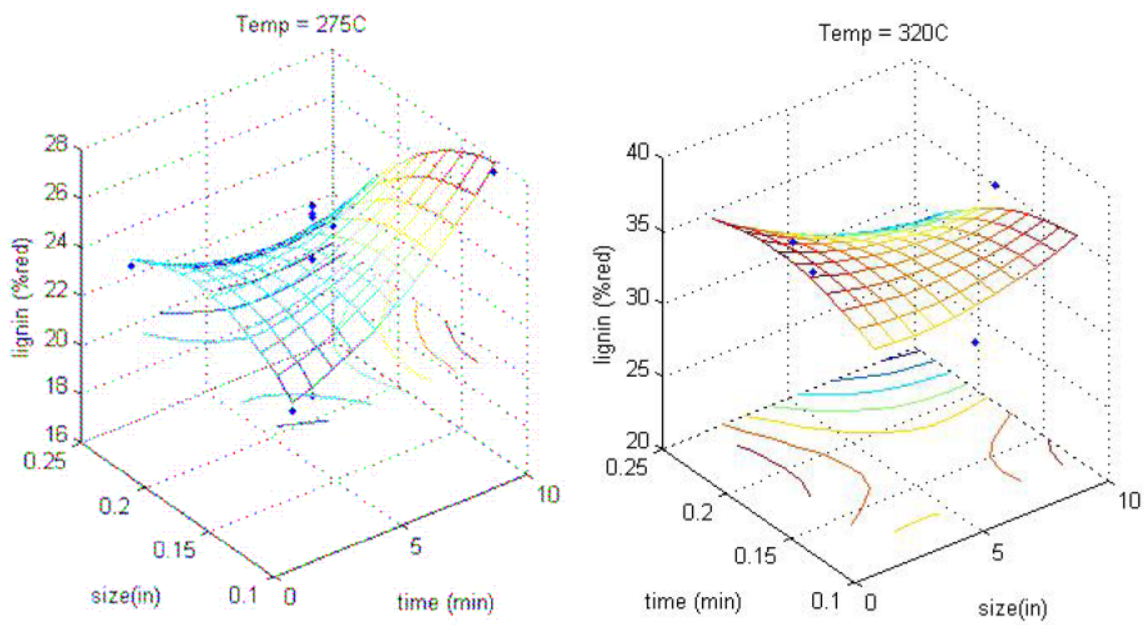

Figure 7.4. Percent Lignin Reduction RSM Plots for Temperature

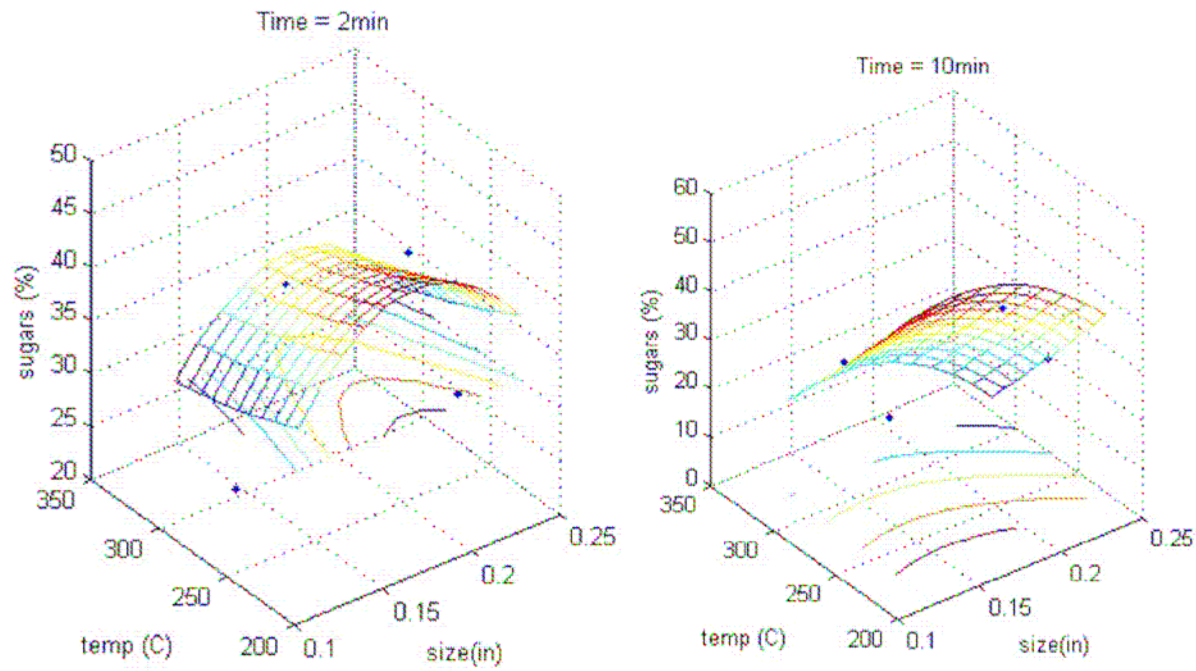

Figure 7.5. Percent Total Sugar RSM Plots for Time

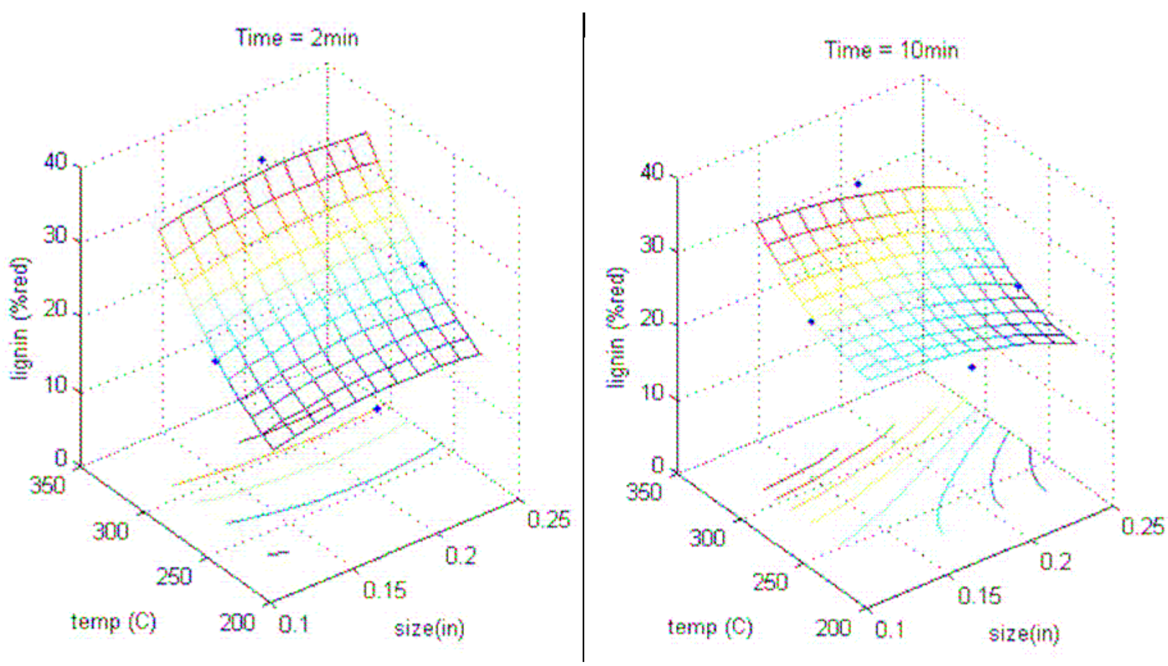

Figure 7.6. Percent Lignin Reduction RSM Plots for Time

7.3. Statistical Tables

Table 7.2. RSREG Procedure Results for Subcritical Water Pretreatment Total Sugars Model

Response Surface for Variable sugars

Response Mean	27.864159
Root MSE	8.773278
R-Square	0.6048
Coefficient of Variation	31.4859

Regression	DF	Type I Sum of Squares	R-Square	F Value	Pr > F
Linear	3	3133.831433	0.3924	13.57	<.0001
Quadratic	3	889.605410	0.1114	3.85	0.0162
Crossproduct	3	806.126656	0.1010	3.49	0.0240
Total Model	9	4829.563498	0.6048	6.97	<.0001

Residual	DF	Sum of Squares	Mean Square
Total Error	41	3155.787022	76.970415

Parameter	DF	Estimate	Standard Error	t Value	Pr > |t|	Parameter Estimate from Coded Data
Intercept	1	-29.022920	107.130299	-0.27	0.7878	29.333539
time	1	10.123837	4.712477	2.15	0.0376	-2.685088
temp	1	-0.057168	0.697661	-0.08	0.9351	-10.473079
size	1	804.358977	349.279464	2.30	0.0264	-3.698816
time*time	1	0.256261	0.154281	1.66	0.1043	4.100181
temp*time	1	-0.038731	0.014070	-2.75	0.0088	-6.971571
temp*temp	1	0.000150	0.001219	0.12	0.9025	0.304412
size*time	1	-17.169248	10.130509	-1.69	0.0977	-4.292312
size*temp	1	-0.137927	0.900490	-0.15	0.8790	-0.387920
size*size	1	-1926.918834	631.935946	-3.05	0.0040	-7.527027

Table 7.3. RSREG Procedure Results for Subcritical Water Pretreatment Lignin Reduction Model

Response Surface for Variable lignin

Response Mean	24.281586
Root MSE	5.667848
R-Square	0.5112
Coefficient of Variation	23.3422

Regression	DF	Type I Sum of Squares	R-Square	F Value	Pr > F
Linear	3	376.890838	0.1399	3.91	0.0152
Quadratic	3	461.326431	0.1712	4.79	0.0060
Crossproduct	3	539.064625	0.2001	5.59	0.0026
Total Model	9	1377.281894	0.5112	4.76	0.0002

Residual	DF	Sum of Squares	Mean Square
Total Error	41	1317.104759	32.124506

Parameter	DF	Estimate	Standard Error	t Value	Pr > |t|	Parameter Estimate from Coded Data
Intercept	1	165.717702	69.209966	2.39	0.0213	25.057205
time	1	-6.809681	3.044427	-2.24	0.0308	-2.620855
temp	1	-1.032552	0.450714	-2.29	0.0272	-2.648947
size	1	317.671032	225.646899	1.41	0.1667	1.348326
time*time	1	-0.301892	0.099671	-3.03	0.0042	-4.830278
temp*time	1	0.025948	0.009090	2.85	0.0067	4.670699
temp*temp	1	0.001884	0.000788	2.39	0.0214	3.814739
size*time	1	14.087388	6.544668	2.15	0.0373	3.521847
size*temp	1	-1.163227	0.581748	-2.00	0.0522	-3.271577
size*size	1	-161.959061	408.252992	-0.40	0.6936	-0.632653

Table 7.4. ANOVA for Sodium Hydroxide Pretreatment Lignin Reduction Model Using class Statement

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	8	16236.05939	2029.50742	111.03	<.0001
Error	18	329.01393	18.27855		
Corrected Total	26	16565.07332			

R-Square	Coeff Var	Root MSE	Lignin Mean
0.980138	9.841335	4.275342	43.44270

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Time	2	97.66812	48.83406	2.67	0.0964
Conc	2	15798.38032	7899.19016	432.16	<.0001
Time*Conc	4	340.01095	85.00274	4.65	0.0094

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Time	2	97.66812	48.83406	2.67	0.0964
Conc	2	15798.38032	7899.19016	432.16	<.0001
Time*Conc	4	340.01095	85.00274	4.65	0.0094

Table 7.5. ANOVA for Sodium Hydroxide Pretreatment Xylan Solubilization Model Using class Statement

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	8	21501.13854	2687.64232	65.67	<.0001
Error	18	736.62585	40.92366		
Corrected Total	26	22237.76439			

R-Square	Coeff Var	Root MSE	xylsol Mean
0.966875	14.19150	6.397160	45.07741

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Conc	2	20512.47526	10256.23763	250.62	<.0001
Time	2	894.51789	447.25895	10.93	0.0008
Time*Conc	4	94.14538	23.53635	0.58	0.6843

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Conc	2	20512.47526	10256.23763	250.62	<.0001
Time	2	894.51789	447.25895	10.93	0.0008
Time*Conc	4	94.14538	23.53635	0.58	0.6843

Table 7.6. ANOVA for Sodium Percarbonate Pretreatment Lignin Reduction Model Using class Statement

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	8468.225420	498.130907	18.91	<.0001
Error	36	948.075044	26.335418		
Corrected Total	53	9416.300464			

R-Square	Coeff Var	Root MSE	Lignin Mean
0.899316	25.93175	5.131805	19.78966

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Time	2	294.319130	147.159565	5.59	0.0077
Conc	5	7659.040539	1531.808108	58.17	<.0001
Time*Conc	10	514.865750	51.486575	1.96	0.0691

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Time	2	294.319130	147.159565	5.59	0.0077
Conc	5	7659.040539	1531.808108	58.17	<.0001
Time*Conc	10	514.865750	51.486575	1.96	0.0691

Table 7.7. ANOVA for Sodium Percarbonate Pretreatment Xylan Solubilization Model Using class Statement

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	14927.75932	878.10349	14.61	<.0001
Error	36	2164.12193	60.11450		
Corrected Total	53	17091.88124			

R-Square	Coeff Var	Root MSE	XylSol Mean
0.873383	17.77810	7.753354	43.61181

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Conc	5	13369.67126	2673.93425	44.48	<.0001
Time	2	251.41466	125.70733	2.09	0.1383
Time*Conc	10	1306.67339	130.66734	2.17	0.0432

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Conc	5	13369.67126	2673.93425	44.48	<.0001
Time	2	251.41466	125.70733	2.09	0.1383
Time*Conc	10	1306.67339	130.66734	2.17	0.0432