ABSTRACT

SILVERSTEIN, REBECCA ANNE. A Comparison of Chemical Pretreatment Methods for Converting Cotton Stalks to Ethanol. (Under the direction of Ratna R. Sharma.)

The objective of this study was to investigate the effectiveness of sulfuric acid, sodium hydroxide, hydrogen peroxide, and ozone pretreatments for conversion of cotton stalks to ethanol. Sulfuric acid, sodium hydroxide, and hydrogen peroxide at concentrations of 0.5, 1, and 2% (w/v) were used to pretreat ground cotton stalk samples at a solid loading of 10% (w/v). Treatment temperatures of 90ºC and 121ºC at 15 psi were investigated for residence times of 30, 60, and 90 minutes. Ozone pretreatment was performed in the liquid phase at 4ºC with constant sparging. Lignin, carbohydrate, and moisture content analyses were performed on the pretreated solids. The pretreated solids from sulfuric acid, sodium hydroxide, and hydrogen peroxide pretreatment (at 2%, 60 min, 121ºC/15psi) showed significant lignin degradation and/or high sugar availability and hence were hydrolyzed by cellulases from *Trichoderma reesei* and β-glucosidase at 50ºC. The results showed that time, temperature and concentration were all significant ($p \leq 0.05$) factors in delignification for NaOH and xylan removal for H_2SO_4. Sulfuric acid pretreatment resulted in the highest xylan reduction (95.23% for 2% acid, 90 min, 121ºC/15psi) during pretreatment and the lowest cellulose to glucose conversion during hydrolysis (23.85%). Sodium hydroxide pretreatment resulted in the highest level of delignification (65.63% for 2% NaOH, 90 min, 121ºC/15psi) and the highest cellulose conversion (60.8%). Hydrogen peroxide pretreatment resulted in significantly lower ($p \leq 0.05$) delignification (maximum of 29.51% with 2%, 30 min, 121ºC/15psi) and cellulose conversion (49.8%) than sodium hydroxide pretreatment, but had a higher ($p \leq 0.05$) conversion than sulfuric acid pretreatment. Ozone pretreatment showed no

significant changes in lignin, xylan, or glucan contents with increasing time. Quadratic models using time, temperature, and concentration as numeric variables were developed to predict xylan reduction for H_2SO_4 pretreatment and lignin reduction for NaOH pretreatment. In addition, linear models relating a modified severity parameter (log M_o) combining the pretreatment parameters with xylan or lignin reduction were developed and resulted in R^2 values of 0.89 and 0.78, respectively.

A COMPARISON OF CHEMICAL PRETREATMENT METHODS FOR CONVERTING COTTON STALKS TO ETHANOL

by
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BIOGRAPHY

Rebecca Silverstein was born in Ramsey, NJ and is the second oldest of four children. She lived in New Jersey until graduating from Ramsey High School in June of 1998. She moved to Raleigh, NC to attend North Carolina State University and obtained a Bachelor of Science degree in Biological Engineering in May of 2002. She was a member of the NC State Women's Gymnastics team while attending college. Upon graduation, she went on a month-long cross-country road trip with a brewery theme entitled 'Mission: Self Discovery.' Although she had no life altering revelations, it was still a great experience which allowed her to explore her interest in photography as well as taste micro-brewed beers from across the country. After the road trip, she worked at the Olive Garden in Cary, NC with no end in sight. With a little push from her persistent professor, Dr. Michael Boyette, she finally decided to attend graduate school and pursue a Master of Science degree in Biological Engineering at North Carolina State University. Aside from her studies, Rebecca is an avid snowboarder and hopes to eventually learn how to surf, preferably in Costa Rica.

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Chapter 1: Introduction

1.1 Background

Fossil forms of biomass, such as petroleum and coal, currently supply the majority of energy needs for society (Ingram et al., 1995). Greenhouse gases such as carbon dioxide emitted from burning fossil fuels for energy have nevertheless contributed greatly to global climate changes and air pollution. Current and future policies and regulations designed to limit energy-related emissions of airborne pollutants, are likely to affect the composition and growth of global energy use (EIA, 2004). In addition, the oil reserves in the United States are limited and the future of oil imports from the volatile Middle East is uncertain due to constant depletion and concerns of national security. Therefore, due to a variety of issues including environmental, societal, political, and geological, development and use of alternative fuel sources is necessary.

Biomass, which includes animal and human waste, trees, shrubs, yard waste, wood products, grasses, and agricultural residues such as wheat straw, corn stover, rice straw, and cotton stalks, is a renewable resource that stores energy from sunlight in its chemical bonds (McKendry, 2002). It can be processed either chemically or biologically by breaking the chemical bonds and extracting the energy stored in those bonds. It releases carbon dioxide as it burns, and the CO_2 is then available to produce new biomass, thus creating a closed carbon cycle (McKendry, 2002). In order to maintain the high energy consumption lifestyles that people have grown accustomed to, the prospect of converting renewable biomass resources into biofuels such as ethanol, methanol, and biodiesel must thus be investigated.

Currently, corn is the primary raw material for ethanol production in the United States. Starch, which constitutes about 70% of the corn kernel is easily broken down into

glucose that is then fermented to ethanol. The corn to ethanol industry is quite mature with little possibility of process improvements, and the cost of the grain often exceeds the value of the fuel itself (Ingram et al., 1995). Alternatively, lignocellulosic feedstocks, which have the potential to reduce the cost of producing ethanol because they are less expensive than corn and available in large quantities, need to be explored.

Lignocellulosic biomass is a term used for organic material that stems from plants. It is produced by green plants converting sunlight into plant material through photosynthesis and includes all land and water-based vegetation including organic wastes. Over 70% of the materials placed in U.S. landfills are lignocellulosic consisting of paper, cardboard, wood products etc. Together with agricultural residues such as wheat straw, corn stover, rice straw, and cotton stalks, a large part of the 100 billion gallons of liquid fuel burned each year in the United States could be supplied as ethanol (Ingram et al., 1995) from lignocellulosic material. The technology for producing ethanol from lignocellulosics has been available since the early 1900s, but at a very high cost compared to the price of gasoline. Improvements in the processes for bioconversion of lignocellulosic biomass to ethanol offer the potential to increase efficiency and reduce the costs of fuel ethanol relative to petroleum (Ingram et al., 1995).

The conversion of lignocellulosic biomass to ethanol is more challenging than corn conversion due to the complex structure of the plant cell wall. This complex structural material in the cell wall, known as lignocellulose is a composite of cellulose fibers embedded in a cross-linked lignin-hemicellulose matrix (Brown, 2003). Most of the carbohydrate content is composed of structural polysaccharides that provide support, strength, and shape for the plant. The conversion of lignocellulosic materials to ethanol involves three main

processes: pretreatment, hydrolysis of the cellulose to fermentable sugars, and fermentation of the sugars to produce ethanol. In order to gain access to the cellulose core and convert it to fermentable glucose, the biomass must undergo pretreatment. The large number of pretreatments used for lignocellulosic materials can be classified as physical, physico-chemical, chemical, and biological processes. The step after pretreatment involves conversion of cellulose to glucose by acid or enzyme catalyzed hydrolysis. Then, through fermentation, the glucose and other hemicellulose sugars are converted to ethanol by yeasts (*Saccharomyces cerevisiae*) or bacteria such as *Zymomonas mobilis*.

The presence of large amounts of lignin and hemicellulose (up to 20-25% each) in the biomass makes access of cellulase enzyme to cellulose difficult, thus reducing the efficiency of hydrolysis. Pretreatment helps to increase the porosity, or accessible surface area, of lignocellulosic material, thus making the polysaccharides more susceptible to hydrolysis. In addition, pretreatment effectiveness and hydrolysis improvement has been correlated with removal of hemicellulose and lignin and the reduction of cellulose fiber crystallinity (McMillan, 1994). A successful pretreatment must thus (1) improve formation of sugars or the ability to subsequently form sugars by enzymatic hydrolysis; (2) avoid the degradation or loss of carbohydrates; (3) avoid the formation of byproducts inhibitory to the subsequent hydrolysis and fermentation processes; and (4) be cost effective (Sun, 2002).

Cotton (*Gossypium hirsutum*), which is one of the most abundant crops in the southern United States, and North Carolina in particular, is an important source of lignocellulosic biomass. Until 1919 the value of cotton exceeded all other commodities in the state of North Carolina. Cotton acreage reached a high of 1.8 million acres in 1926. In 1978, however, cotton acreage reduced to a low of 42,000 acres (NCDA, 2004). Insect

problems and the increase of synthetic fibers contributed to the decline of cotton production. The boll weevil was the major pest forcing the decline of cotton production. In 1987, the boll weevil was completely removed from North Carolina as the result of an extensive eradication effort. The elimination of the boll weevil and increased world demand for cotton helped renew interest in cotton production in North Carolina. In 2003, nearly 14 million acres of cotton were planted in the United States with almost 1 million acres planted in North Carolina and 1.1 million bales of cotton harvested (NCDA, 2004).

This increase in cotton planting and production is highly beneficial for North Carolina's economy, but it also raises concerns about the disposal of the cotton stalks (agricultural residue) left in the field after the cotton is picked. Removal of the stalks from the field is necessary to destroy feeding and fruiting sites that may be used by boll weevils and other insects to reproduce. Although the boll weevil is no longer a problem in North Carolina, other cotton growing states such as Texas (TBWEF, 2004) and Oklahoma (OBWEO, 2004) are still concerned with this pest. Early removal of the stalks from the field is thus important for controlling boll weevil populations and preventing their return following a successful eradication.

1.2 Objectives

There is a great opportunity to investigate the possibility of using efficiently pretreated cotton stalks as a feedstock for bioethanol production. Turning this agricultural waste into a value-added product would provide a method of disposal for the stalks and present farmers with the opportunity to earn a profit from their waste material. The objectives of this study were: 1) To investigate the effect of treatment time, temperature, and

treatment agent concentration during sulfuric acid, sodium hydroxide, hydrogen peroxide, and ozone pretreatments of cotton stalks. 2) Develop models to predict lignin degradation and xylan solubilization percentage during sulfuric acid and sodium hydroxide pretreatments. 3) To use data on the degrees of lignin degradation and hemicellulose solubilization during pretreatment to identify pretreatment(s) which provide the highest cellulose to glucose conversion during subsequent enzymatic hydrolysis.

Chapter 2: Literature Review

2.1 Defining the Resource

Biorenewable resources are usually classified as either wastes or dedicated energy crops. Categories of waste materials that qualify as biorenewable resources include agricultural residues, yard waste, municipal solid waste, food processing waste, and manure. Agricultural residues such as corn stover, rice hulls, wheat straw, cotton stalks, and bagasse, are the portion of the crop discarded after harvest. Municipal solid waste (MSW) is waste discarded as garbage, not all of which is suitable as biomass feedstock. In communities where yard waste is excluded, the important components of MSW are paper (50%), plastics and other fossil fuel derived materials (20%), food wastes (10%), and non-flammable materials including glass and metal (20%) (Brown, 2003). Food processing waste is the effluent from a variety of industries ranging from breakfast cereal manufacturers to alcohol breweries. One of the major benefits of using waste products for conversion to fuels and chemicals is their low cost. By definition, waste products have minimal economic value and can be acquired for little more than the cost of transporting the material from the point of origin to a processing plant. Sometimes, when a biorenewable resource processing plant is paid by a company to dispose of a waste stream, there is even a negative cost associated with the acquisition of the biomass (Brown, 2003).

Dedicated energy crops are the other classification of biorenewable resources. These crops are defined as plants specifically grown for applications other than food or feed. Numerous crops have been proposed or are being tested for commercial energy farming. Potential energy crops include woody crops and grasses/herbaceous plants, starch and sugar crops, and oilseeds. In general, the characteristics of the ideal energy crop are: high yield,

low energy input to produce, low cost, composition with least contaminants, and low nutrient requirements (McKendry, 2002).

2.2 Interest in Biomass and Biobased Products

In the past 10 years, there has been a renewed interest, world-wide, in biomass as an energy source (McKendry, 2002). Technological developments relating to crop production, conversion, etc. promise the coupling of biomass at lower cost with higher conversion than was previously possible. More advanced options to produce electricity are looking promising and allow a cost-effective use for energy crops in operations such as production of methanol and hydrogen by gasification processes (McKendry, 2002).

Air pollution is an important factor motivating interest in alternative fuels at the global level. Carbon dioxide is responsible for more than half of the projected anthropically-mediated climate change. Transportation fuels account for 27% of the 2.2 billion MT of carbon dioxide released annually in the United States from combustion of fossil fuels. Vehicles account for 4.7% of total worldwide carbon dioxide emissions, with U.S. vehicles accounting for 2.5% of total emissions (Ramamurthi et al., 2000). The use of biomass to produce energy has the potential to reduce the high emission levels of greenhouse gases. When produced by sustainable means, biomass emits roughly the same amount of carbon during conversion as is taken up during plant growth, so the use of biomass does not contribute to a buildup of carbon dioxide in the atmosphere (McKendry, 2002).

2.3 Fuel Ethanol

Ethanol is a high octane, water free alcohol produced from the fermentation of sugar or starch. It is used as a blending ingredient in gasoline or as a raw material to produce high octane fuel ether additives. The use of ethanol as an automobile fuel in the United States dates as far back as 1908, to the Ford Model 'T'. Henry Ford was a supporter of home-grown renewable fuels, and his Model T could be modified to run on either gasoline or pure alcohol (Ford Motor Company, 2004). Trillions of miles have been driven on ethanol-blended fuel since 1980 and ethanol blended fuels currently account for about 18% of automotive fuels sold in the United States (RFA, 2004). The Clean Air Act of 1990 and the National Energy Policy Act of 1992 created new market opportunities for alternative fuels by phasing in requirements for fleet vehicles to operate on cleaner fuels (NWICC, 2004).

2.3.1 Corn-based Ethanol Production

In the United States, corn is currently the most common feedstock used to produce ethanol. A bushel of corn can produce about 2.5 gallons of ethanol. Ethanol is produced from corn by using one of two standard processes to convert the starches in the kernel to fermentable sugars: wet-milling or dry-milling. Dry-milling plants cost less to build and produce higher yields of ethanol, but the value of co-products is less (NWICC, 2004). Approximately one billion bushels of corn were used for the record high 2.8 billion gallons of ethanol produced in 2003 (ARS, 2004).

2.3.2 Lignocellulose vs. Corn

Production of ethanol from lignocellulosic materials is a newer technology than corn-ethanol production. The biomass feedstock is less expensive than corn and is available in large quantities. Over 70% of the materials placed in U.S. landfills are lignocellulosic consisting of paper, cardboard, wood products etc. Together with agricultural residues such as wheat straw, corn stover, rice straw, and cotton stalk, a large part of the 100 billion gallons of liquid fuel burned each year in the United States could be supplied as ethanol (Ingram et al., 1995). However, the cost of producing ethanol from lignocellulosics is higher than that of corn. Three main steps are involved in producing ethanol from lignocellulosic materials: pretreatment, where lignin and hemicellulose are solubilized; hydrolysis, where cellulose is converted to glucose; and fermentation, where glucose is converted to ethanol. Compared to the corn-to ethanol process, the biomass-to-ethanol production requires processes such as bailing, bail washing, shredding and milling to small particles which increases the handling costs of the feedstock, while the high temperature pretreatment and longer residence time for saccharification and fermentation (6-7) days compared to two-day starch conversion requires more energy. The average price of corn-based ethanol over the past six years was $1.22/gallon, but increased with increasing gasoline prices up to $1.80 during the first week of May, 2004 (Egerstrom, 2004). The by-products from corn such as corn gluten feed and corn oil have a higher value than lignin from biomass-ethanol production, (DiPardo, 2000). In addition, the cost of cellulase enzymes is about $0.30 to $0.50/gallon of ethanol produced (DOE Biomass Program, 2004), which is more expensive than the $0.044/gallon of ethanol produced cost of α-amylase and glucoamylase used for corn (Sun, 2002). However, the U.S. Department of Energy's Biomass Program placed separate, parallel contracts in 2000 with

the world's two largest industrial enzyme manufacturers, Genecor International and Novozymes, with the goal of reducing cellulase costs for biomass conversion applications. As of early 2004, both companies reported over ten-fold decreases in the effective enzyme cost, and continuing work is expected to further reduce cellulase costs to about $0.10 per gallon of ethanol or less (DOE Biomass Program, 2004).

2.4 Stalk Composition

2.4.1 Cell Wall Organization

Most of the carbohydrate content of plants is structural polysaccharides that provide support, strength, and shape for the plant. This complex structural material in the cell wall, known as lignocellulose, is a composite of cellulose fibers embedded in a cross-linked lignin-hemicellulose matrix (Brown, 2003). The three main components of lignocellulosic materials are cellulose, hemicellulose, and lignin, with other minor components being ash, protein, and extractives. The distribution of cellulose, hemicellulose, and lignin in a typical plant cell wall is shown below in Fig 2.1. Lignin is most abundant in the middle lamella and decreases with increasing distance into the fiber cell wall, with percentages in the primary cell wall and S1 layer of the secondary cell wall higher than in the S2 and S3 sections of the secondary cell wall. Cellulose is most abundant in the secondary cell wall as seen in the diagram below. The cellulose microfibrils in the primary cell wall have no specific orientation, while the microfibrils in the secondary cell wall run parallel to each other, but at a different angle for each of the three layers S1, S2, and S3.

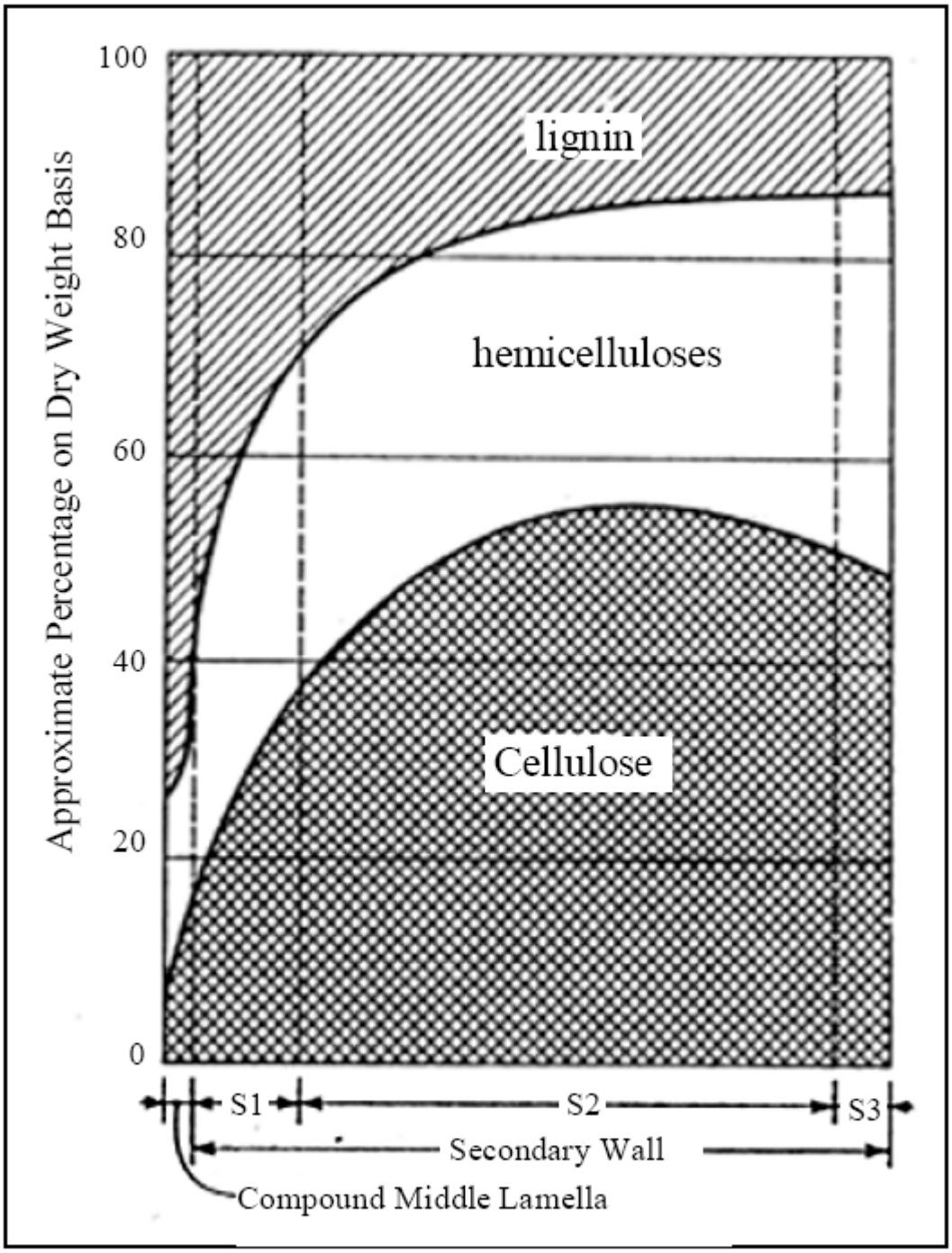

Figure 2.1 Distribution of cellulose, hemicellulose, and lignin in a typical plant cell wall (Panshin and Dezeeuw, 1980)

2.4.2 Cellulose

Cellulose is a linear polymer of anhydro D-glucose units connected by β-1,4 glycosidic bonds as shown below in Figure 2.2. Native cellulose exists in the form of microfibrils, which are paracrystalline assemblies of several dozen (1→ 4) β-D-glucan chains held together by intermolecular hydrogen bonds (Carpita and McCann, 2000). Intramolecular hydrogen bonds also form between two glucose units in the same chain (Fengel and Wegener, 1984). The combined bonding energies of the intermolecular and intramolecular hydrogen bonds increases the rigidity of cellulose and forms the crystalline structure that makes it highly insoluble and recalcitrant to most organic solvents. The cellulose microfibrils are imbedded in a matrix of noncellulosic polysaccharides, mainly hemicellulose and pectic substances (Sun, 2002), which complicates hydrolysis of cellulose to glucose even further. The cellulose in lignocellulosic biomass feedstocks provides the main source of glucose used during ethanol fermentation.

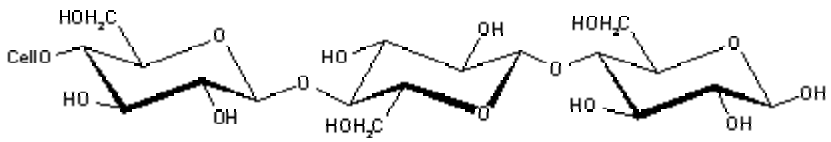

Figure 2.2 The structure of linear cellulose polymer (HUT, 2004)

2.4.3 Hemicellulose

Hemicelluloses are complex, highly branched polysaccharides that occur in association with cellulose in the cell walls (Klass, 1998). The monomers that comprise hemicellulose are hexoses (glucose, galactose, and mannose) and pentoses (arabinose and xylose). Hemicellulose can be classified into three groups, namely, xylans, mannans, and

galactans based on the polymer backbone that is very often homopolymeric with β-1,4 linkages (Brigham et al., 1996). In softwoods, the primary hemicellulose components are galactoglucomannans and arabinoglucuronoxylan, while the principal hemicelluloses in hardwoods are glucomannans and methylglucoronoxylans (Brigham et al., 1996). Xylan is the most important in terms of the percentage of total hemicellulose found in biomass. The structure of galactoglucomannan is shown in Figure 1.2. Galactoglucomannan consists of β-1,4-linked mannose and glucose units in a ratio of 3:1 to which O-acetyl groups and α-1,6-linked galactose side groups are attached (Puls and Schuseil, 1993).

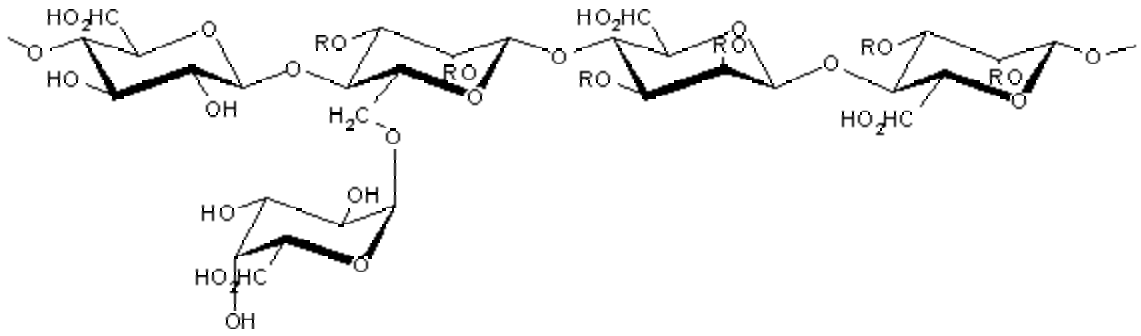

Figure 2.3 Structure of galactoglucomannan found typically in softwoods (HUT, 2004)

2.4.4 Lignin

Lignin is a three-dimensional phenylpropane polymer with phenylpropane units held together by ether and carbon-carbon bonds (Sun, 2002). It is constructed of three monomers: coniferyl alcohol, sinapyl alcohol, and coumaryl alcohol, each of which has an aromatic ring with different substituents (Brown, 2003). The dominant monomeric units in the polymers are benzene rings bearing methoxyl, hydroxyl, and propyl groups that can be attached to other units (Klass, 1998). When the plant is mature and the cell growth ceases, the middle lamella (the cement between the primary walls of adjacent cells) and the secondary wall

(inside of primary cell wall) have large amounts of lignin. Lignin strengthens the cell structures by stiffening and holding the fibers of polysaccharides together (Fan et al., 1987). The structure of a small section of a lignin polymer is shown below in Figure 1.3.

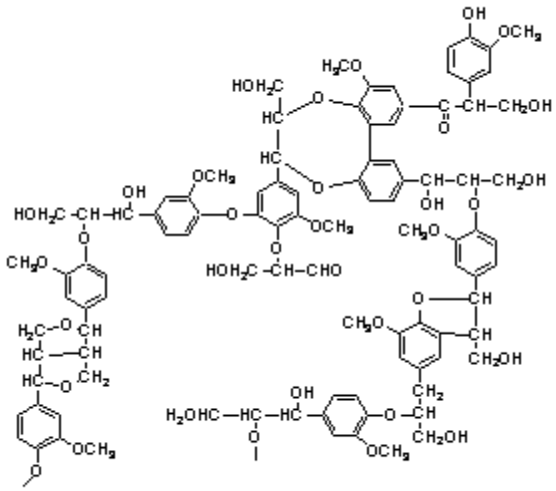

Figure 2.3 Structure of a section of a lignin polymer (Lignin Institute, 2002)

2.5 Preatreatment of Lignocellulosic Materials

Pretreatment is the first step required to fractionate lignocellulosic materials into its major plant components of lignin, cellulose and hemicellulose. The mechanisms by which pretreatments improve the digestibility of lignocellulose are however not well understood (Brown, 2003). An important goal of pretreatment is to increase the surface area of lignocellulosic material, making the polysaccharides more susceptible to hydrolysis. Along with an increase in surface area, pretreatment effectiveness and hydrolysis improvement has been correlated with removal of hemicellulose and lignin and the reduction of cellulose cyrstallinity (McMillan, 1994). A successful pretreatment must meet the following requirements: (1) improve formation of sugars or the ability to subsequently form sugars by hydrolysis; (2) avoid the degradation or loss of carbohydrate; (3) avoid the formation of

byproducts inhibitory to the subsequent hydrolysis and fermentation processes; and (4) be cost effective (Sun, 2002). The large number of pretreatments used for lignocellulosic materials can be classified into groups as physical, physico-chemical, chemical, and biological processes.

2.5.1 Physical Pretreatment

Since one of the main goals of pretreatment is to increase the surface area available to cellulase enzymes during hydrolysis, communition, or size reduction, is an integral part of pretreatment. Waste materials can be comminuted by a combination of chipping, grinding, and milling. The size of the materials is usually 10 to 30 mm after chipping and 0.2 to 2 mm after milling or grinding (Sun, 2002). The process has relatively low energy requirements, ranging from 24,000 kJ/dry ton for wheat straw to 200,000 kJ/dry ton for aspen wood. However, energy consumption increases exponentially with decreasing particle size (Brown, 2003). For enzymatic hydrolysis, particle size reduction is followed by additional pretreatment methods to further improve hydrolysis. Cellulase enzymes used during enzymatic hydrolysis are large proteins with molecular weights ranging from 30,000 to 60,000 and are thought to be ellipsoidal with major and minor dimensions of 30 and 200 Å. Typically, only 20% of the pore volume of plant tissue is accessible to these large molecules. Thus, without additional pretreatment beyond size reduction, sugar yields from enzymatic hydrolysis are less than 20% of theoretical, whereas further pretreatment can increase yields to 90% or higher (Brown, 2003).

2.5.2 Physico-Chemical Pretreatment

2.5.2.1 Steam Explosion (autohydrolysis)

Steam explosion is the most commonly used method for pretreatment of lignocellulosic materials (McMillan, 1994). Chipped biomass is treated with high-pressure saturated steam and then the pressure is quickly reduced, which makes the materials undergo an explosive decomposition. Steam explosion is initiated at a temperature of 160 to 260°C (corresponding pressure 0.69 to 4.83 Mpa) for several seconds to a few minutes before the material is exposed to atmospheric pressure (Sun, 2002). The process causes hemicellulose degradation and lignin transformation due to high temperature, thus increasing the potential of cellulose hydrolysis (Sun 2002).

Until recently, optimization strategies for the pretreatment of lignocellulosics have focused on the effects of temperature, residence time, and pH, but have not accounted for changes in severity by properties inherent to the starting feedstock (Cullis et al., 2004). Consequently, a study was conducted by Cullis et al. (2004) that evaluated the effects of chip properties, feedstock size (40-mesh, 1.5 x 1.5 cm, 5 x 5 cm), and moisture content (12% and 30%) on the overall bioconversion process, and more specifically on the efficacy of removal of recalcitrant lignin from the lignocellulosic substrates following steam explosion. The results indicated that both increased chip size and increased moisture content resulted in improved solids recovery and increased hemicellulose-derived sugar recovery as well as minimized condensation of lignin. Furthermore, a post steam-explosion refining step increased hemicellulose-derived sugar recovery and was most effectively delignified (to as low as 6.5%). The refined substrate could be enzymatically hydrolyzed to very high levels (98%) at relatively fast rates (1.23 g/L/h).

The addition of small amounts of mineral acids, usually sulfuric acid (H_2SO_4), improves hydrolysis at reduced temperatures. This process is known as acid-catalyzed steam explosion. Ground biomass is treated with 1 wt-% acid and incubated at 140°C for 30 minutes or at 160°C for as little as 10-15 minutes, to achieve complete hemicellulose removal, which increases enzymatic digestibility of the remaining cellulose to as high as 90% (Brown, 2003).

Addition of sulfur dioxide (SO_2) gas in steam explosion can also effectively increase sugar yields and improve enzymatic hydrolysis. The maximum sugar yields for steam explosion pretreatment of corn fiber were found when the material was pretreated at 190°C for 5 minutes after being exposed to 3% SO_2. Sequential SO_2-catalyzed steam explosion and enzymatic hydrolysis resulted in a conversion efficiency of 81% of the combined original hemicellulose and cellulose in the corn fiber to monomeric sugars (Bura et al., 2003).

2.5.2.2 Ammonia Fiber Explosion (AFEX)

Ammonia explosion or ammonia fiber explosion (AFEX) is a process in which ground, prewetted lignocellulosic material at a moisture content of 15-30% is placed in a pressure vessel with liquid ammonia (NH_3) at a loading of about 1-2 kg NH_3/kg dry biomass. Pressures exceeding 12 atm are required for operation at ambient temperature. The mixture is incubated from several minutes up to an hour to enable the ammonia to penetrate the lignocellulosic matrix. When the reaction is complete, a valve is opened to explosively release the pressure. AFEX pretreatment has been demonstrated to improve the saccharification rates of herbaceous crops and grasses. Materials pretreated using the AFEX process include alfalfa, corn stover, rice straw (Vlasenko et al., 1997), bagasse, coastal

Bermuda grass, newspaper (Holtzapple et al., 1990 b), kenaf newspaper (Holtzapple et al., 1992), switchgrass , wheat straw, barley straw, and municipal solid waste. The two mechanisms that AFEX pretreatment appears to act by are the increase in reactivity of cellulose due to exposure to liquid NH_3 and the increase in accessible surface area following AFEX treatment, probably as a combined effect of hemicellulose hydrolysis and explosive decompression (McMillan, 1994). AFEX pretreatment has not proven effective on hardwoods or softwoods, and results of a study (Holtzapple et al., 1990 b) on AFEX pretreatment of Bermuda grass (~ 5% lignin), bagasse (~ 20% lignin), and newspaper (~ 30% lignin) suggest decreasing AFEX effectiveness with increasing lignin content. Hydrolysis yields of over 90% were achieved for AFEX pretreated Bermuda grass and bagasse, while newspaper had hydrolysis yields of only about 40% (Holtzapple et al., 1990 b). Vlasenko et. al (1997) performed a study comparing acid-catalyzed steam explosion, dilute acid hydrolysis, and AFEX pretreatments for enzymatic hydrolysis of rice straw. The AFEX pretreatment was clearly different from the other pretreatments since it did not significantly solubilize hemicellulose.

2.5.3 Chemical Pretreatment

2.5.3.1 Acid Treatment

Acid pretreatment can utilize either dilute or concentrated acids to improve cellulose hydrolysis. At moderate temperatures, direct saccharification suffers from low yields due to sugar decomposition. However, prehydrolysis with dilute acid at temperatures higher than 121°C is very effective for increasing the enzymatic digestibility of cellulose (Grohmann et. al, 1986). There are primarily two types of dilute acid pretreatment processes: low solids

loading (5-10% [w/w]), high-temperature (T > 160°C), continuous-flow processes and high solids loading (10-40% [w/w], lower temperature (T < 160°C), batch processes (Grohmann et al., 1985). Dilute acid pretreatment (0.2-2.0% sulfuric acid, 121-220°C) of lignocellulose serves three important functions in the conversion process: 1) hydrolysis of the hemicellulose components to produce a syrup of monomeric sugars; 2) exposure of cellulose for enzymatic digestion by removal of hemicellulose and part of the lignin; and 3) solubilization of heavy metals which may be contaminating the feedstock (Ingram et al., 1997). In spite of these benefits, acid pretreatment presents potential problems such as the production of an acid waste stream that must be neutralized or reused, the formation of compounds such as acetic acid and furfural in the hydrolysate which are toxic to bacteria or yeasts during fermentation (Ingram et al., 1997), and the need for corrosion-resistant equipment (Brown, 2003).

The operating costs of pretreatment are highly contingent upon the consumption of steam that is needed to heat the biomass and acid to elevated temperatures. The simplest way to reduce steam consumption is by increasing the dry weight concentration of solids in the reactor. Grohmann et al. (1986) investigated dilute acid pretreatment of aspen wood and wheat straw at solids concentrations from 10 to 40%. A monomeric soluble sugar stream (mostly xylose) was produced with little sugar degradation and the cellulose remaining in the solids was highly digestible by enzymes thus proving that using higher solids concentrations is a feasible option for reducing the cost of steam.

Varga et al. (2002) used sulfuric acid and hydrochloric acid (HCl) for pretreatment of corn stover under mild conditions (121°C, 1 h). Pretreatment with 5% H_2SO_4 or HCl solubilized 85% of the hemicellulose fraction, but the enzymatic conversion increased only

two times compared to untreated stover. Much better results were obtained when acid pretreatment was combined with NaOH pretreatment as described below in Section 2.5.3.2.

2.5.3.2 Alkaline Treatment

Alkaline solutions can be used to pretreat lignocellulosic materials, and the effectiveness of pretreatment is dependent upon the lignin content of the material (McMillan, 1994). The mechanism of alkali pretreatment is believed to be saponification of intermolecular ester bonds crosslinking xylan hemicelluloses and other components such as lignin and hemicellulose. After alkali pretreatment, the porosity of the material is increased due to the extensive swelling facilitated by removal of the crosslinks (Tarkow and Feist, 1969).

Pretreatment of corn stover by Varga et al. (2002) with 10% sodium hydroxide (NaOH) for 60 minutes under pressure at 121°C in the autoclave decreased the lignin fraction by more than 95% and increased the enzymatic conversion more than four times to 79.4% as compared to untreated stover. In addition, by using dilute NaOH (0.5% w/w) and increasing the reaction time to 90 minutes, 80.1% enzymatic conversion was achieved (Varga et al., 2002).

Kim et al. (2003) pretreated corn stover with aqueous ammonia in a flow-through column reactor, in a process termed ammonia recycled percolation (ARP). This method delignified the biomass by 70-85% with 70% of the lignin removal occurring in the first 10 minutes of treatment. Subsequent enzymatic hydrolysis of corn stover treated for 90 minutes exhibited digestibility of 99% with 60 FPU/g glucan enzyme loading, and 92.5% with 10 FPU/g of glucan.

Alkali soaking can also be used in conjunction with other pretreatment methods such as peracetic acid (Teixeira et al., 1999), acid-catalyzed steam explosion (Schell et al., 1998), and hydrochloric acid and sulfuric acid (Varga et al., 2002). Teixeira et al. (1999) soaked hybrid poplar and sugar cane bagasse in NaOH or ammonium hydroxide (NH_4OH) for 24 hours prior to peracetic acid pretreatment. There was improved enzymatic conversion (95% for poplar) with 6% NaOH followed by acid treatment as compared to pretreatment with 9 or 15% peracetic acid alone. Varga et al. (2002) soaked corn stover in NaOH for 24 hours prior to H_2SO_4 pretreatment and achieved nearly theoretical maximum enzymatic conversion (95.7%). Pretreated Douglas fir produced by sulfuric acid-catalyzed steam explosion was treated with NaOH, NH_4OH, and lime to extract the lignin (Schell et al., 1998). NaOH was the most effective at removing the lignin (29% removed), however the susceptibility of the treated material to enzymatic hydrolysis was lower than the untreated control and decreased with increasing lignin removal. Furthermore, ethanol production by simultaneous saccharification and fermentation was similar for the control and the NaOH-treated material and lower for the other bases.

2.5.3.3 Oxidative delignification

The rate and extent of lignocellulose digestion by microorganisms present in the stomachs of ruminants are both greatly enhanced when the lignocellulose is first treated with an alkaline (pH 11.5) solution of hydrogen peroxide (H_2O_2). The increase in digestibility has been attributed not only to oxidative delignification but also to a possible decrease in cellulose crystallinity (Gould, 1985). Martel and Gould (1990) concluded from their study on wheat straw and kenaf that alkaline hydrogen peroxide (AHP) treatment loosened the

lignocellulosic matrix and caused a more open three-dimensional relationship between lignin, cellulose, and hemicellulose at the molecular level. They also observed that there was either no change or an increase in cellulose crystallinity after AHP treatment thus supporting the contention that the main effect of AHP treatment is that it detaches and makes soluble the lignin, thus increasing the amount of cellulose available for hydrolysis by enzymes (Martel and Gould, 1990), while it does not decrease cellulose crystallinity as previously hypothesized by Gould (1985).

Kim et al. (2001) examined the effect of pretreatment with ammonia, sulfuric acid, and water with and without hydrogen peroxide on the enzymatic digestibility of oak. In both acid and ammonia pretreatments, the addition of hydrogen peroxide improved enzyme hydrolysis, but decomposition of soluble sugars occurred. For water pretreatment, as the concentration of hydrogen peroxide increased from 0 to 0.8, 1.6, and 3.2%, hemicellulose recovery and delignification increased from 72 to 77, 89, and 92%, and from 24 to 37, 49, and 53%, respectively (Kim et al., 2001). For 1.6% H_2O_2, both hemicellulose recovery and enzymatic digestibility were about 90%, which was comparable to 0.2% sulfuric acid treatment, but with 23% higher delignification. It was also noted that glucose degraded significantly as hydrogen peroxide concentration increased, while hemicellulose was preserved. Optimization of the amount of hydrogen peroxide for the water-H_2O_2 pretreatment could provide better results than sulfuric acid pretreatment, and the neutralization step required for dilute-acid treatment could be avoided.

2.5.3.4 Ozone

Ozone has been used to degrade lignin and hemicellulose in lignocellulosic materials such as cotton stalks (Ben-Ghedalia et al., 1980; Ben-Ghedalia and Shefet, 1983; Yosef et al., 1994), corn stover (Quesada et al., 1999), wheat straw (Ben-Ghedalia and Miron, 1981), bagasse, and poplar sawdust. Some of the benefits of ozone pretreatment include the fact that no toxic residues are formed since ozone can be easily decomposed to oxygen using a catalytic bed or an increase in temperature thus eliminating the need for extensive down-stream processing and ozonation reactions take place at ambient temperature and pressure so energy and investment costs are minimized (Quesda et al., 1999). Ben-Ghedalia et al. (1980), pretreated cotton straw with ozone to examine the effect on the composition of the cell wall fractions and on *in vitro* organic matter digestibility. The most notable effects of ozone treatments were demonstrated by the 50% decrease in both lignin and hemicellulose (Ben-Ghedalia et al., 1980). The pH of ozone-treated cotton stalks was considerably more acidic and it was concluded that the low pH values were probably the result of the release of a mixture of formic, acetic, glyoxylic, or other acids from the oxidized lignin. Quesda et al. (1999) later confirmed this by showing the appearance of glycolic, oxalic, malonic, glyoxylic, glyceric, and malic acids in a chromatographic analysis of the aqueous extract of oxidized, extractive free corn stover, due to the generation of carboxylic acids from extensive lignin degradation. Yosef et al. (1994) showed through NMR analysis that lignin degradation by ozone is the result of ring cleavage directly evidenced by the decline in aromatic C from 13.0% in untreated cotton stalks to 7.40% in ozone-treated stalks. The rate of enzymatic hydrolysis increased by a factor of 5 following removal of 60% of the lignin from wheat straw during ozone pretreatment (Binder et. al, 1980). As the lignin content of

poplar sawdust decreased from 29 to 8% after ozonolysis, enzymatic conversion increased from 0 to 57% (Vidal and Molinier, 1988). The optimal moisture content of 60% was found to provide the highest degree of solubilization during ozone treatment of corn stover (Quesda et al., 1999). Results from the same study showed that lignin was the most affected polymer, followed by hemicelluloses and then cellulose.

2.5.4 Biological Pretreatment

Biological pretreatment involves microorganisms such as brown-, white- and soft-rot fungi that are used to degrade lignin and solubilize hemicellulose. White-rot fungi are the most effective basidiomycetes for biological pretreatment of lignocellulosic materials (Fan et al., 1987). Lignin degradation by white-rot fungi, specifically *Phanerochaete. chrysosporium*, *Pleurotus ostreatus*, *and Trametes versicolor,* is an oxidative process with lignin peroxidases (LiP), manganese peroxidases (MnP) and laccases acting as the key enzymes (Malherbe and Cloete, 2002).

Fungal pretreatment could potentially lower the severity requirements of chemical, temperature, and time resulting in less biomass degradation and lower inhibitory concentrations compared to conventional thermochemcial pretreatment (Keller et al., 2003). Sawada et al. (1995) investigated effects of fungal pretreatment and steam explosion pretreatment on enzymatic conversion of beech wood meal. They found that fungal pretreatment by *P. chrysosporium* for 28 days followed by steam explosion at 215°C for 6.5 minutes provided the maximum saccharification. Biodegradation of cotton stalks by the 'oyster mushroom' *Pleurotus ostreatus* was studied by Hadar et al. (1992). The study found that during four weeks of solid-state processing, lignin content decreased significantly and *in*

vitro rumen digestibility increased 2.2 times from 10% for untreated cotton stalks to 22% for fungal pretreated stalks. Preliminary results from a study performed by Keller et al. (2003) on fungal pretreatment of corn stover with *Cyathus stercoreus* showed a three to five-fold increase in enzyme digestibility from 1.2–12.1% to 8.3–35.7% of theoretical (cellulase loadings of 15, 25, 60 FPU/g cellulose). The advantages of biological pretreatment include low energy requirements and mild environmental conditions, while a disadvantage is the long time period required for lignin degradation.

2.6 Hydrolysis

2.6.1 Acid Hydrolysis

Both concentrated-acid and dilute-acid hydrolysis can be used to hydrolyze polysaccharides to fermentable sugar monomers. The use of concentrated acid (72% H_2SO_4) at room temperature provides high sugar yields (nearly 100% of theoretical hexose yields), but the large volume of acid (about equal to the weight of the sugars produced) needed during the process requires recovery and reuse of the acid. Recovery of sulfuric acid is complicated by its high boiling point, while HCl is more expensive and corrosive, but can possibly be recovered by distillation (Brown, 2003). Dilute acid hydrolysis (about 1% by weight) requires less acid, but the elevated temperatures at which it is performed greatly reduce sugar yields to only 55-60% of theoretical due to decomposition of released oligosaccharides. In addition, decomposition products include microbial toxins such as acetic acid and furfural that inhibit fermentation (Brown, 2003).

2.6.2 Enzymatic Hydrolysis

Enzymatic hydrolysis provides a method to convert cellulose to glucose at high yields without sugar product degradation. Enzymatic hydrolysis of cellulose proceeds in several steps to break glycosidic bonds by the use of cellulase enzymes. Factors effecting hydrolysis of cellulose include type of substrate, cellulase loading, reaction conditions such as temperature and pH, and end-product inhibitors.

2.6.2.1 Cellulases

Cellulases are synthesized by fungi, bacteria, and plants, with most research focused on fungal and bacterial cellulases produced both aerobically and anaerobically. The aerobic mesophilic fungus, *Trichoderma reesei* QM 6a and its mutants have been the most intensely studied sources of cellulases (Philippidis, 1996). Cellulase is not a single enzyme, but is made up of a family of at least three groups of enzymes: 1,4- β-D-glucan glucanohydrolases (endoglucanases) (EC 3.2.1.21), 1,4- β-D-glucan cellobiohydrolases and 1,4- β-D-glucan glucohydrolases (exoglucanases) (EC 3.2.1.91), and β-D-glucoside glucohydrolases (β-glucosidases) (EC 3.2.1.21) (Xiao et al., 2004; Philippidis, 1994). A collaborative effort among these enzymes is required to break down cellulose. Enzymatic hydrolysis typically involves three steps: adsorption of endoglucanases and exoglucanases onto the surface of cellulose, biodegradation of cellulose to glucose, and desorption of cellulases. Native cellulose is hydrolyzed by the cellobiohydrolases to yield cellodextrins and cellobiose. The cellodextrins are further hydrolyzed to cellobiose, a disaccharide of glucose, by endoglucanases, and then β-glucosidase hydrolyzes cellobiose to glucose (Brown, 2003). Cellulases from *T. reesei* have the advantage of having all three groups of enzymes, being

more resistant to chemical inhibitors, and exhibiting better stability at 50°C than other fungal cellulases. Unfortunately, they are sensitive to product inhibition and activate slowly even at their optimum temperature (Philippidis, 1994).

Increasing the cellulase loading can enhance the yield and rate of hydrolysis, but would increase the cost significantly. Cellulase enzyme loading in hydrolysis vary from 7 to 33 FPU/g substrate depending on the type and concentration of the substrate (Sun, 2002). It has been reported that the addition of non-ionic surfactants, particularly Tween species, effectively improves cellulase activity as well as preserves them for recycle (Helle et al., 1993; Kaya et al., 1995). Experiments were conducted by Kaar and Holtzapple (1998) using lime pretreated corn stover to determine the optimal Tween 20 and Tween 80 loadings for improving enzyme efficiency. Tween 20 proved to be slightly more effective with the recommended loading of 0.15g Tween/g dry biomass. The 72-h enzymatic hydrolysis of pretreated corn stover with 5 FPU cellulase/g dry biomass at 50°C with Tween 20 improved cellulose, xylan, and total polysaccharide conversion by 42, 40, and 42%, respectively. They concluded that Tween improves corn stover hydrolysis through three effects: acting as an enzyme stabilizer by protecting the enzymes from thermal deactivation, disrupting the lignocellulose matrix thus making more substrate available to the enzymes, and by acting as an enzyme effector that helps the enzyme maintain affinity for the substrate as well as promoting the availability of reaction sites.

2.6.2.2 End-Product Inhibition

A rapid release of glucose is usually observed in the first 24 hours of hydrolysis, with the remaining cellulose hydrolysis taking as long as 2 more days to complete (Gregg and

Sadler, 1996). End product inhibition has been shown to play a significant role in slowing the hydrolysis rate (Tengborg et al., 2001), and glucose, cellobiose, and ethanol have demonstrated inhibitory effects on the activity of both β-glucosidase and cellulase (Holtzapple et al., 1990 a). Xiao et al. (2004) quantified the degree of inhibition of both β-glucosidase and cellulase mixtures by glucose and cellobiose as well as the inhibitory effects of mannose, galactose, and xylose on the enzymes. They found that high glucose content (10 g/L) in the hydrolysate resulted in a dramatic increase in the degrees of inhibition of β-glucosidase and cellulase activities, while supplementation with mannose, xylose, and galactose only inhibited cellulase activity (Xiao et al. 2004). Several methods have been developed to reduce inhibition including the use of high concentrations of enzymes, supplementation of β-glucosidase during hydrolysis, and the removal of sugars during hydrolysis by ultra filtration or simultaneous saccharification and fermentation (SSF) (Sun, 2002).

2.7 Fermentation

Ethanol fermentation has become one of the most challenging biotechnological processes of our time (Tripetchkul et al., 1998). Research aimed at optimizing the production process has focused on four main approaches including physiological, biological, genetic, and engineering (Lawford, 1988). The physiological approach recognizes that the process parameters involved in productivity are affected by environmental factors including pH and temperature, the chemical composition of the fermentation medium, and the concentration of essential nutrients or inhibitory compounds. The biological approach replaces the more traditional alcohol producing microorganism, yeast, with more efficient

and productive species. The genetic approach aims to improve the metabolic characteristics of the microorganism by attempting to correct known weaknesses or deficiencies such as broadening the range of substrates the organism can use as carbon sources. Finally, the engineering approach aims to increase productivity by using fermentors that operate in continuous mode rather than batch mode (Lawford, 1988). There are three main ethanol fermentation processes that are used: separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), and direct microbial conversion (DMC).

2.7.1 Separate Hydrolysis and Fermentation (SHF)

The SHF process uses separate pretreatment, enzymatic hydrolysis and fermentation steps. The primary advantage of this approach is that by separating these steps, undesirable interactions are avoided. Using separate reactors allows each step to be carried out at its optimum temperature: 40-50°C for enzymatic hydrolysis and 30°C for fermentation (Philippidis, 1996; Brown, 2003). The disadvantage of this method is the inhibition of cellulase and β-glucosidase enzymes by glucose released during hydrolysis, which calls for lower solids loadings and higher enzyme loadings to achieve reasonable yields (Philippidis, 1996; Brown, 2003). Lower sugar yields result in lower ethanol concentrations and thus increase the cost of fermentation and ethanol recovery.

2.7.2 Simultaneous Saccharification and Fermentation (SSF)

Extensive research has shown that simultaneous saccharifcation and fermentation (SSF) is a promising way to biochemically convert cellulose into ethanol (Philippidis, 1996)

and is generally accepted as the most effective and economical way to convert cellulose to ethanol (Wyman, 1996). The process combines the enzymatic hydrolysis of cellulose to glucose by cellulolytic enzymes with the catabolism of glucose to ethanol by fermentative microorganisms. By combining cellulose and glucose in the same reactor, glucose is rapidly removed before it can inhibit the cellulase enzymes during hydrolysis. The optimum temperature for the reaction (37-38°C) is a compromise between the optimum temperatures for the enzymes in hydrolysis and the yeast in fermentation.

2.7.3 Direct Microbial Conversion (DMC)

Direct microbial conversion combines cellulase production, cellulose hydrolysis and glucose fermentation into a single step. The process is attractive in that it reduces the number of reactors, simplifies operation, and reduces the cost of chemicals (Brown, 2003). However, the ethanol yields are low, several metabolic byproducts are produced, and the organisms usually have a low tolerance to ethanol (Philippidis, 1996). The organism most investigated for DMC of cellulose is *Clostridium thermocellum* (Johnson et al., 1982). Studies on this microorganism have shown ethanol tolerance in the range of 2.9 to 3.6% ethanol, while the typical tolerance of ethanologenic yeast ranges from 8-10% ethanol. In addition, a large fraction of the catabolized carbon goes into acetic and lactic acid during DMC, which reduces ethanol yield and increases the cost of production (Klapatch et al., 1994).

2.7.4 Fermentation Improvement

2.7.4.1 Physiological Approach

Improvements to fermentation productivity can be made by understanding that the parameters involved in productivity, namely, the specific rate of ethanol production, and the growth yield coefficient ($Y_{x/s}$) are affected by environmental factors. Finding the ideal chemical/nutrient composition of the fermentation medium and the optimal temperature and pH can improve product yield. Lawford (1988) manipulated and controlled the chemical environment to increase the kinetic performance of *Zymomonas mobilis*. Energetically uncoupled phenotypes were generated under conditions of nutrient limitation of nitrogen, phosphate, or potassium in steady-state continuous culture, which increased specific rates of ethanol production. The effect of changes in pH affected the maintenance coefficient (m_e) rather than the max growth yield coefficient ($Y_{x/s}^{max}$) and ethanol production was maximized in the pH range of 4.0-4.5, whereas the optimal pH for growth of *Z. mobilis* on a complex medium was 6.0-6.5 (Lawford, 1988).

2.7.4.2 Yeast vs. Bacteria

Yeasts such as *Saccharomyces cerevisiae* have been traditionally used to ferment glucose to ethanol. *S. cerevisiae* ferments glucose through the Embden-Meyerhoff-Parnas pathway (Picataggio et al., 1994). This yeast is a facultative anaerobe that prefers aerobic growth, but is capable of growing in anaerobic environments. The ability of the yeast to ferment sugars at a low pH provides protection against bacterial contamination during cultivation. The high indigenous levels of glucose-inducible pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) help insure high fermentation rates and specific

productivities, while providing resistance to glucose catabolite repression during fermentation of mixed sugar hydrolysates (Picataggio et al., 1994). The gram negative bacterium, *Zymomonas mobilis* has attracted considerable attention due to its superior kinetic and yield characteristics, and unlike *S. cerevisiae*, there are no oxygen requirements for lipid synthesis. *Z. mobilis* ferments glucose through the Entner-Douderoff pathway (Picataggio et al., 1994). Like yeasts, *Zymomonas* is acid tolerant and is resistant to bacterial contamination. In addition, the bacterium is able to grow at high sugar concentrations (> 25% glucose) and to produce and tolerate ethanol at concentrations up to 13% (w/v) (Rogers et al., 1979).

The main disadvantage for these microorganisms is their limited substrate utilization ranges. Their inability to ferment xylose, the primary pentose present in hemicellulose, as well as all other monosaccharides in lignocellulosic materials makes producing ethanol from lignocellulose less efficient and therefore less attractive from an economic perspective. Current research, however, aims to use genetic engineering to modify these organisms, as well as others, to increase ethanol yields.

2.7.4.3 Genetically Modified Organisms

One important requirement for improving ethanol production from lignocellulose is use of an efficient microorganism that is able to ferment both pentoses and hexoses as well as tolerate stress conditions (Zaldivar et al., 2001). Through metabolic engineering, bacterial and yeast strains have been constructed which have desirable traits for producing ethanol from lignocellulose. Essential traits include broad substrate utilization range, high ethanol yields, minimal byproduct formation, high ethanol tolerance, increased tolerance to

inhibitors, and tolerance to sudden changes in environmental conditions. Some other traits that are desirable, but not required are: simultaneous sugar utilization, hemicellulose and cellulose hydrolysis, GRAS (Generally Regarded as Safe) status, recyclable, minimal nutrient supplementation, and tolerance to low pH and high temperature (Zaldivar et al., 2001). The three main microorganisms that have been investigated are *Saccharomyces cerevisiae*, *Zymomonas mobilis*, and *Escherichia coli*.

2.7.4.4 Continuous vs. Batch Fermentation

Batch processes are closed systems where nothing is added after inoculation except possibly acid or alkali for pH control or air for aerobic fermentations. Continuous culture, on the other hand, is an open system where fresh medium is continuously added and product is removed at the same rate, thus resulting in a constant volume system. Continuous fermentation with cell recycle involves separating the yeast or bacteria cells from the effluent and recycling them back to the fermentor thus minimizing cell removal from the reactor. One of the first steps taken to improve ethanol productivity from yeast was switching from batch mode to operating in continuous mode. This change increased productivity by three times from about 2 to 6 g EtOH/L/h (Cysewski and Wilke, 1977). In addition, operating continuously at higher cell densities using cell recycle reactors was another effective way to increase productivity. A single-stage continuous stirred-tank reactor (CSTR) operating at high biomass loadings (50-80 g yeast/L) has an ethanol productivity of 30-40 g EtOH/L/h (Cysewski and Wilke, 1978).

2.8 Conclusion

Evidently a large amount of research has focused on improving every aspect of ethanol production from lignocellulosic materials. Optimization of pretreatment, hydrolysis, and fermentation processes is being investigated to provide an economically feasible renewable fuel source that is an alternative to fossil fuels. Examination of substrates, such as cotton stalks, that have not been widely studied provides the opportunity to utilize an abundant and inexpensive agricultural residue for fuel production. Extensive investigation of the composition of the stalks and their reaction to different pretreatment methods known to have varying effects on the substrate (lignin degradation, hemicellulose solubilization, etc.) will provide evidence as to whether or not cotton stalks are a competitive alternative to more widely investigated and traditional materials such as corn stover and sugar cane bagasse.

Chapter 3: Materials and Methods

3.1 Biomass

The cotton stalks, harvested in early October 2003, were obtained from Cunningham Research Station in Kinston, NC. The stalks were shredded and bailed in the field soon after the cotton was picked, and then transported to North Carolina State University in Raleigh, NC. The biomass primarily consisted of stalks, leaves, and cotton residue.

3.2 Composition Analysis

Prior to beginning work on the pretreatments, it was necessary to analyze the initial composition of the cotton stalks. The stalks were ground to a 40-mesh particle size prior to analysis. The percentages of total solids, ash, acid-insoluble and acid-soluble lignin, extractives, holocellulose, hemicellulose sugars, and glucose were determined. All experiments were performed in triplicate.

3.2.1 Determination of Total Solids

The total solids content was determined by following the Laboratory Analytical Procedure No. 001 (LAP-001) from the National Renewable Energy Laboratory (NREL), (Ehrman, 1994). The biomass was placed in a convection oven at 105°C overnight or until constant weight was achieved (±0.1% change in moisture after one hour of reheating). The moisture content was then calculated on a 105°C dry weight basis using the following:

$$\% \text{ Total Solids } (T_{final}) = \left[\left(\frac{W_2 - W}{W_1} \right) * 100 \right] \quad (3.1)$$

Where: W = dry dish weight, g
W_1=initial sample weight, g
W_2=sample weight plus dish weight after drying, g

3.2.2 Determination of Ash Content

The ash content was determined by placing moisture free biomass in a muffle furnace at 500°C overnight to burn off the carbon (Han and Rowell, 1997). The ash content was then calculated as a percentage of moisture free solids as follows:

$$\% \text{ ash content} = \left(\frac{W_2 - W}{W_1} \right) * 100 \quad (3.2)$$

Where: W = ignited dish weight, g
W_1=initial moisture-free sample weight, g
W_2=sample weight plus dish weight after removal from furnace, g

3.2.3 Determination of Lignin

The percent of acid-insoluble lignin, which is defined as the residue, corrected for acid-insoluble ash, retained on a medium porosity filter crucible after primary (72%) and secondary (4%) sulfuric acid hydrolysis, was determined by following the summative analysis procedure outlined in LAP-003 (Templeton and Ehrman, 1995). The biomass (0.3 g) was hydrolyzed in 10 mL beakers with 3 mL of 72% sulfuric acid at room temperature for 2 hours. The hydrolyzate was then transferred to serum bottles and diluted to 4% sulfuric acid by adding 84 ± 0.04 mL of deionized (DI) water. The bottles were crimp sealed and autoclaved in a liquid vent cycle for one hour at 121°C and 15 psi. The solution was then vacuum filtered through a medium porosity filtering crucible. The filtrate was saved for use

in the acid-soluble lignin analysis. The filtered residue was washed free of acid with hot DI water. The crucibles and contents were dried at 105°C for 2 hours, weighed, and then placed in a muffle furnace at 500°C overnight. The percentage of acid-insoluble lignin in the biomass was calculated on a 105°C dry weight basis using the following equation:

$$\%\ acid\text{-}insoluble\ lignin = \left(\frac{W_2 - W_3}{W_1 * \frac{T_{final}}{100\%}} \right) * 100 \qquad (3.3)$$

Where: W_1 = initial sample weight
W_2 = weight of crucible, acid-insoluble lignin, and acid-insoluble ash after drying in oven
W_3 = weight of crucible and acid-insoluble ash after removal from furnace
T_{final} = % total solids content of shredded sample on a 105°C dry-weight basis

The acid-soluble lignin, which is defined as the portion of the lignin that dissolves during 72% sulfuric acid hydrolysis, was determined using LAP-004 (Ehrman, 1996). The filtrate collected during the filtration of the acid-insoluble lignin was used for this procedure. The filtrate was analyzed within 6 hours of hydrolysis in compliance with this procedure. A 4% sulfuric acid (H_2SO_4) reference blank was prepared and the spectrophotometer (PharmaSpec UV-1700, Shimadzu) was set up and calibrated. The absorbance of the hydrolyzate was measured at 205nm, using a 1-cm light path quartz cuvette. The absorbance exceeded 1.0, hence the filtrate was diluted ten-fold with DI water so the absorbance would fall below 1.0. The percentage of acid-soluble lignin in the biomass was then calculated on a 105°C dry weight basis using the following equation:

$$\%\ acid\text{-}soluble\ lignin = \left(\frac{\frac{A}{b*a} * df * V * \frac{L}{1000mL}}{\frac{W * T_{final}}{100}} \right) * 100 \qquad (3.4)$$

Where: A = absorbance at 205 nm
df = dilution factor
b = cell path length, 1 cm
a = absorptivity, equal to 110 L/g-cm
V = filtrate volume, 87 mL
W = initial biomass sample weight
T_{final} = % total solids content of biomass sample

3.2.4 Extractives Determination

Extractives are a group of cell wall chemicals mainly consisting of fats, fatty acids, fatty alcohols, phenols, terpenes, steroids, resin acids, rosin, waxes, etc (Han and Rowell, 1997). These chemicals were removed from a 10 g cotton stalk sample by a Soxhlet extraction procedure using a 225 mL mixture of toluene and ethanol in a ratio of 2:1. The extraction was carried out in a well-ventilated fume hood for 24 hours making sure the liquid boiled so that it flushed at least four times per hour. A picture of the Soxhlet extraction setup is shown below in Figure 3.1. After 24 hours, the sample was washed five times with ethanol and placed in a vacuum oven at 45°C for 24 hours. Extraction is usually complete at this stage, but a 28-hour extraction was also performed for comparison purposes. The following equation was used to calculate the % extractives on a dry-weight basis:

$$\%\ extractives = \left(\frac{W_1 - W_2}{W_1} \right) * 100 \qquad (3.5)$$

Where: W_1 = initial dry sample weight
W_2 = final dry sample weight

Figure 3.1 Soxhlet extraction setup used for toluene ethanol extractives determination

3.2.5 Carbohydrate Analysis

The initial carbohydrate content of the cotton stalks was determined by measuring the hemicellulose (galactose, arabinose, and xylose) and cellulose (glucose) sugar contents of the untreated biomass. The LAP-002 carbohydrate analysis procedure from NREL was modified for use with a Dionex DX-300 Chromatography System (Dionex Corporation, Sunnyvale, CA) equipped with a CarboPac™ PA10 (4x250mm) anion exchange column, CarboPac™ PA10 (4x250mm) guard column, gradient pump, automated sampler and pulsed amperometric detector with a gold working electrode. Voltage was applied to the electrochemical cell by a potentiostat in a series of three potentials: E1= 0.05 V, E2 = 0.6 V, E3 = -0.6V, with three pulse durations: t1 = 480ms, t2 = 120 ms, t3 = 60 ms. The separation was achieved by eluting the column with 100% 200 mM NaOH solution and 0% 10 mM NaOH solution at a flow rate of 1 mL/min for 14 minutes and creating a linear gradient to 0% 200 mM NaOH and 100% 10 mM NaOH between 14 and 15 minutes. The concentration

was maintained at 10% NaOH for the next 15 minutes to equilibrate the column before the next injection. The sugar content of the filtrate from the lignin analysis was measured during this procedure. The filtrate was stored at -20°C until HPLC analysis was performed. A 16 mL sample of filtrate was first neutralized with 2.3g of barium hydroxide to adjust the pH between 4 -5. Aside from neutralization, the barium hydroxide also served the purpose of precipitation of sulfates from the sulfuric acid that would be harmful to the HPLC column. The samples were vortexed on high to ensure proper mixing and dissolution of the barium hydroxide. The mixtures were centrifuged for 10 minutes at 3500 rpm and then filtered using Millipore 0.45µm nylon syringe filters. A fucose internal standard was added, the samples were diluted 10 fold with deionized water and then run through the HPLC. Concentrations of the sugars (mg/L) were calculated based on a sugar standard curve generated using the software provided by Dionex. The standard curve, the monosaccharide residence times, and an example chromatograph are included in Appendix 7.1. The percentage of each sugar fraction was calculated using the following equation:

$$\% \text{ sugar} = \left(\frac{C * df * \frac{1g}{1000mg} * V_f}{W_1 * \frac{\%T_{final}}{100}} \right) * 100 \qquad (3.6)$$

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

3.2.6 Determination of Holocellulose

Holocellulose is the water-insoluble carbohydrate fraction of plant materials, which is made up of polymers of simple sugars, mainly glucose, mannose, galactose, xylose, arabinose, and glucuronic acid (Han and Rowell, 1997). The holocellulose content, which is the combination of hemicellulose and cellulose, was determined in order to find the total amount of hemicellulose and cellulose in the biomass. For holocellulose determination, an extractive and moisture-free sample was used and the procedure outlined in Han and Rowell was followed (1997). A flask containing a mixture of 80 mL hot deionized water, 0.5 mL acetic acid, and 1 g sodium chlorite, and 2.5 g biomass was heated in a water bath at 70°C for one hour. 0.5 mL of acetic acid and 1 g of sodium chlorite were added each hour for the next 5 hours, thus making a total of six additions of the two chemicals and six hours of chloriting. The samples were left in the water bath overnight, and at the end of 24 hours of reaction time, the samples were cooled and the holocellulose was filtered. The holocellulose was washed with acetone and then placed in a vacuum oven at 105°C for 24 hours. The percentage of hollocelulose in the cotton stalks was calculated as follows:

$$\% \text{ holocellulose} = \left(\frac{W_2 - W}{W_1} \right) * 100 \tag{3.7}$$

Where: W = dry weight of crucible, g
W_1 = initial sample weight, g
W_2 = sample weight plus crucible weight after drying, g

3.3 Pretreatment

3.3.1 Preparation of Cotton Stalks

The cotton stalks were initially shredded in the field, but prior to pretreatment, they were ground finer using a 3 mm sieve on a Thomas Wiley Laboratory Mill (Model No. 4). Once ground, the biomass was stored in a sealed plastic bag at room temperature until pretreatment.

3.3.2 Sulfuric Acid Pretreatment

Sulfuric acid (H_2SO_4) at concentrations of 0.5, 1, and 2% (w/v) was used to pretreat 10 g ground cotton stalk samples at a solid loading of 10% (w/v). Treatments were performed at 90°C and in the autoclave at 121°C with 15 psi pressure for residence times of 30, 60, and 90 minutes. The collected solids were washed with 750 mL of hot deionized water. Portions of the solid residues were used for determination of total residual weight and lignin, carbohydrate, and moisture content analyses prior to storing at 4°C for enzymatic hydrolysis. The filtrates from the lignin content analyses were collected and an HPLC carbohydrate analysis similar to that for the initial composition analysis was performed (Section 3.2.5). The reduction in lignin following pretreatment was calculated based on the initial dry-weight of lignin in the untreated sample (LU) and the dry-weight of lignin in the remaining solids after pretreatment (LP). In addition, the percentage of solids recovered was calculated on an oven-dry basis as follows:

$$\% \text{ solids recovered} = \left(\frac{W_2}{W_1}\right) * 100 \quad (3.8)$$

Where: W_1 = dry sample weight of whole biomass before pretreatment (g)
W_2 = dry sample weight after pretreatment (g)

LU and LP were calculated as follows:

$$\text{LU} = \frac{\%LU}{100} * W \quad (3.9)$$

Where: %LU = percent acid-insoluble lignin in untreated sample (reference equation 3.3)
W = dry sample weight (g)

The percentage of lignin reduction was calculated with the following equation:

$$\% \text{ lignin reduction} = \left(\frac{LU - LP}{LU}\right) * 100 \quad (3.10)$$

Where: LP = dry-weight lignin in pretreated sample (reference equation 3.3)
LU = dry-weight lignin in untreated whole biomass sample (reference equation 3.3)

The solubilization of xylan and glucan from the cotton stalks during pretreatment was calculated in the same manner by substituting the appropriate percentages for xylan and glucan.

3.3.3 Sodium Hydroxide Pretreatment

Sodium hydroxide (NaOH) at concentrations of 0.5, 1, and 2% (w/v) was used to pretreat 10 g ground cotton stalk samples at a solid loading of 10% (w/v). Pretreatment temperatures and times were the same as those used for sulfuric acid pretreatment. The analyses performed were also similar to those for sulfuric acid.

3.3.4 Hydrogen Peroxide Pretreatment

Hydrogen peroxide (H_2O_2) at concentrations of 0.5, 1, and 2% (w/v) was used to pretreat 10g ground cotton stalk samples at a solid loading of 10% (w/v). Pretreatment temperatures, times, and subsequent analyses were the same as those used for sulfuric acid pretreatment.

3.3.5 Ozone Pretreatment

Ozone gas was generated by passing 5 L/min of oxygen through an AOS-1M/MS series ozonator obtained from Applied Ozone Systems, CA. The ozone was continuously sparged for 30, 60, and 90 minutes through a 10% (w/v) mixture of cotton stalks and deionized water in flask with inlet and outlet ports, placed in a water bath at 4°C. A picture of the setup for ozone generation and sparging is below in Figure 3.2. The solids and filtrate were collected for analyses similar to those described for sulfuric acid. Ozone concentrations in pure deionized water sparged with ozone for 30, 60, and 90 minutes were determined by measuring the absorbance using a Shimadzu PharmaSpec UV-1700 spectrophotometer at 258 nm. Ozone concentrations for water containing cotton stalks could not be determined due to particle interference with UV light. The following equation was used to calculate ozone in pure water (in ppm):

$$c = A / b\varepsilon \tag{3.11}$$

Where: c = the concentration of ozone in water (ppm)
A = absorbance at 258 nm
b = length of path of light, which equals width of quartz cuvette, cm
ε = absorptivity equal to 6.042×10^{-2} L/mg-cm

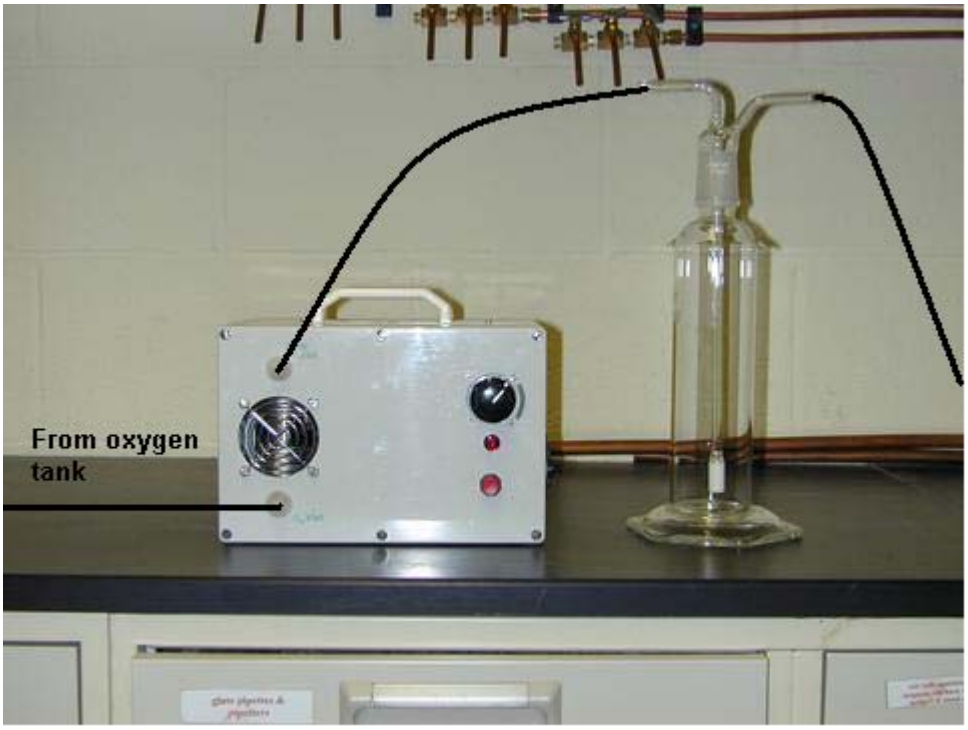

Figure 3.2 Setup used for ozone generation and sparging during ozone pretreatment

3.4 Enzymatic Hydrolysis

3.4.1 Enzymes

Cellulase from *Trichoderma reesei* (Celluclast 1.8L, Sigma Co., St. Louis, MO), with an activity of 96.1 FPU/mL, supplemented with cellobiase (-glucosidase) from *Aspergillus niger* (Novozyme 188, EC No. 232-589-7, Sigma Co., St. Louis, MO) at a ratio of 1:1.75 was used for hydrolysis experiments. Enzymatic treatments were performed at a cellulase activity of 40 FPU/g cellulose. The Filter Paper Unit (FPU) is used to define enzyme activity. The quantity 0.1875 FPU, as defined in LAP-006, is the enzyme activity that will produce reducing sugar equivalent to 2.0 mg of glucose (Adney and Baker, 1996).

3.4.2 Hydrolysis

Cotton stalks pretreated in the autoclave at 121°C/15 psi for 60 min with 2% (w/v) sulfuric acid, sodium hydroxide, or hydrogen peroxide were subjected to enzyme hydrolysis. Pretreated samples at 5% solids concentration (grams dry weight per 100 mL) in 50 mM acetate buffer (pH 4.8) containing 40 µg/mL tetracycline (an antibiotic added to avoid microbial contamination) were preincubated in flasks in a water bath shaker at 50°C and 150 rpm for 10 minutes. 2.18 and 3.82 mL of cellulase and cellobiase, respectively, were added to start hydrolysis after temperature acclimation. Aliquots of 2.0 mL were taken at 72 hours, immediately chilled on ice, and centrifuged at 5000*g* for 10 min. The supernatant was stored at -20°C until HPLC sugar analyses for glucose and xylose were performed (Yang and Wyman, 2004). The sugar analysis results were used to determine the percent cellulose conversion based on the percent of glucose in the supernatant. The conversion of xylan to xylose was also determined. The percent cellulose conversion was calculated as follows:

$$\% \text{ cellulose conversion} = \frac{\%GH}{\%GP} * 100 \qquad (3.12)$$

Where: %GH = dry-weight percentage of glucose in enzyme hydrolysis supernatant
%GP = dry-weight percentage glucose in pretreated sample

3.5 Data Analysis and Modeling

A statistical analysis was performed on the data using Proc GLM in SAS (SAS Institute, Cary, NC). The effects of pretreatment time, concentration, and temperature on the mean lignin, glucan, and xylan contents in the pretreated samples were analyzed using a Tukey-Kramer pairwise t-test ($p \leq 0.05$). In addition, PROC GLM in SAS was used to develop equations to detect significant effects of time, temperature, and concentration on

lignin reduction and xylan and glucan solubilization for sulfuric acid, sodium hydroxide, hydrogen peroxide, and ozone pretreatments. The equation development involved using concentration, time, and temperature as class variables, which distinguishes between levels of each factor, but does not treat time, for example, as a numeric variable. This form of the equation makes predictions for each level of a treatment factor, but cannot predict what happens between levels, for example, at 1.5% concentration. Time, temperature, concentration, and the two and the three-way interactions were included in the models. Significant main treatment effects were determined by looking at the p-values in the Type III Sum of Squares ANOVA tables (Tables 7.2-7.10 in Appendix 7.2) and simple treatment effects were examined by looking at significant ($p \leq 0.05$) differences between levels of each factor. Use of the least squares means (ls means) statement with the slice command in SAS allowed for the examination of simple treatment effects of one parameter (time, temperature, or concentration) while holding the other two treatment parameters constant. The residual plots for lignin reductioin and xylan and glucan solubilization were examined for each treatment and did not indicate inhomogeneity of variance or asymmetrically distributed errors, thus meeting the assumptions made for use of a general linear model.

Models predicting percent lignin reduction for sodium hydroxide pretreatment and xylan solubilization for sulfuric acid pretreatment were developed using the experimental data. The same data set was used for both model development and validation, thus a bias may have been introduced in the predictive ability of the models. Since the data set was relatively small to quantify the predictability of the models, the focus was on model development rather than assessment of predictive ability of the models. Empirical quadratic

models were developed using SAS with time, temperature, and concentration treated as continuous numeric variables.

In addition, modeling based on combining the effects of time, temperature, and concentration into one single parameter was used to develop a linear model expressing the relationship between pretreatment severity and lignin reduction or xylan solubilization. Overend and Chornet (1987) initially defined this severity parameter to relate temperature and time for steam explosion pretreatment. This was accomplished by defining a severity parameter based on the assumption that xylan solubilization and lignin reduction follow first-order kinetics and obey the Arrhenius equation:

$$k = A * \exp\left(-\frac{E_a}{RT}\right) \tag{3.13}$$

Where: k = rate constant
A = Arrhenius frequency factor
E_a = activation energy (kJ/kg mol)
R = universal gas constant (8.314 kJ/kg mol K)
T = absolute temperature (K)

Using this relationship allowed them to define a reaction ordinate:

$$R_o = \int_0^t \exp\left[\frac{Tr - Tb}{14.75}\right] dt \tag{3.14}$$

Where: R_o = Reaction Ordinate
t = residence time (min)
Tr = reaction temperature ($^{\circ}$C)
Tb = base temperature (100 $^{\circ}$C)
(14.75 is the conventional energy of activation assuming the overall reaction is hydrolytic and the overall conversion is first order)

So,

$$R_o = t * \exp\left[\frac{(Tr - Tb)}{14.75}\right] \tag{3.15}$$

The log value of the reaction ordinate is used to define the severity during steam explosion pretreatment:

$$\text{Severity} = \log_{10}(R_o) \tag{3.16}$$

A modified severity parameter was later developed by Chum et. al (1988) for use with sulfuric acid pretreatment:

$$M_o = t * C^n * \exp\left[\frac{(Tr - Tb)}{14.75}\right] \tag{3.17}$$

Where: C = acid concentration (wt-%)
n = an arbitrary constant

This equation was further adapted to apply to sodium hydroxide pretreatment by substituting the alkaline concentration for the acid concentration and calculating a different n-value.

Chapter 4: Results and Discussion

4.1 Composition of Cotton Stalks

The chemical composition of cotton stalks and other agricultural byproducts varies depending on the growing location, season, and harvesting methods (Agblevor et al., 2003). The composition of the cotton stalks used in this study from Kinston, NC is shown in Table 4.1.

Table 4.1 Summative composition of untreated cotton stalks[1]

Component	Percentage
Holocellulose	41.8
Glucan	31.1
Xylan	8.3
Arabinan	1.3
Galactan	1.1
Acid-insoluble lignin	27.9
Acid-soluble lignin	2.2
Extractives	9.0
Ash	6.0
Other	13.1

[1] Composition percentages are on a dry-weight basis

Based on the HPLC carbohydrate analysis as outlined in Section 3.2.5, percent sugar fractions from holocellulose, i.e. the summation of cellulose and hemicellulose, was 41.8% of the dry biomass. Of the total sugars, 10.7% were obtained from the hemicellulose portion. Glucan, which is derived from both the cotton fiber and the plant cell wall, was the major component, followed by acid-insoluble lignin and xylan. Glucan is the portion that is hydrolyzed into glucose monomers and later fermented into ethanol, and the fact that it

makes up the highest percentage of the initial composition is important in terms of its potential for ethanol production. However, the glucan content of 31.1 % was lower than the 40-50% that has been reported for other lignocellulosic materials (Philippidis, 1994). The xylan portion (8.3%), which is usually the major hemicellulose component of lignocellulosic materials, was also lower than the expected range of 15-35% found in other agricultural residues and hardwoods (Milne et al., 1992), although it was still the highest hemicellulose constituent. Arabinan and galactan accounted for only a small portion of the biomass composition, while mannan was not detected in the biomass.

The holocellulose fraction, determined by the procedure in Section 3.2.6, was 51.1% of the total composition. The discrepancy between holocellulose content and percent total sugars in cotton stalks is probably due to sugar degradation during the intense hydrolysis with 72% sulfuric acid used for the carbohydrate analysis procedure. It may be possible to test this hypothesis by performing the hydrolysis steps on a polysaccharide solution with known monomeric sugar concentrations. A comparison of the resulting monomeric sugar concentrations from HPLC analysis and the actual concentrations could be used to calculate a correction factor to account for the difference in holocellulose percentages.

The acid-insoluble material content of the cotton stalks (27.9% dry-weight basis) was higher than expected. The fraction of the sample that was insoluble in 72% sulfuric acid was comparable to that found in hardwoods (18-25%), rather than falling in the 10-20% range, which is the expected acid-insoluble material content of herbaceous species and agricultural residues (McMillan, 1994). The acid-insoluble material from woody biomass is normally classified as lignin, however, it would be incorrect to classify all of the insoluble material from cotton stalks as lignin. Cotton stalks are a complex mixture of organic and inorganic

materials, and there could be other sources of acid-insoluble material aside from lignin. A possible non-lignin acid-insoluble material is the cottonseed. The cottonseed is composed of 32% hull, 23% protein, 12% fibers, 20% oil, and 14% carbohydrates. Upon analysis of the cottonseed from the Emporia gin in Virginia, Agblevor et al. (2003) discovered that the cottonseed contained 34% acid-insoluble material. The hull, which is lignocellulosic, and thus the only source of lignin, makes up only 32% of the cottonseed. Thus, the acid-insoluble material is expected to be composed of lignin and other condensable compounds. Since it is known that proteins condense and become insoluble in concentrated sulfuric acid (Agblebvor, 1994), it could be surmised that the high acid-insoluble material content of the cottonseed, and in turn, the cotton stalks, is a combination of lignin and condensed proteins from the cottonseed (Agbelvor, 2003). However, since the majority of the acid insoluble material is lignin, it has been referred to as such in this study in order to limit confusion.

A study performed by Ververis et al. (2004), found 42.2% cellulose, 15.5% acid insoluble lignin, and 3.5% ash in cotton stalk samples grown in Greece. The higher cellulose and considerably lower lignin and ash contents, which would have been desirable findings for this experiment, could be attributed to differences in cultivation and harvesting methods and analysis procedures. A composition similar to that of the cotton stalks used in this experiment was, however, found in a study that examined the composition of cotton gin residue (immature bolls, cottonseed, hulls, sticks, leaves, and dirt) sampled two to three times on different days from five different cotton gins throughout Virginia (Agblevor et al., 2003). The composition (dry-weight basis) of the cotton gin residue varied depending on the discharge date and the gin location, with approximate ranges of each component being 21-

38% glucan, 3-12% xylan, 0.5-3% each of mannan, galactan, and arabinan, 5-13% extractives, 18-26% acid-insoluble lignin, and 7-14% ash.

4.2 Effect of Pretreatment

4.2.1 Sulfuric Acid

Dilute acid pretreatment of lignocellulosic biomass is one of the more effective pretreatment methods, and has been extensively researched for decades. High temperatures result in hemicellulose hydrolysis during pretreatment, thus releasing monomeric sugars from the cell wall matrix into the hydrolyzate. Hemicellulose removal increases porosity and improves enzymatic digestibility, with maximum enzymatic digestibility usually coinciding with complete hemicellulose removal (McMillan, 1994). The effect of sulfuric acid pretreatment on lignin degradation is minimal, and is not considered to be a substantial contributor in terms of improving enzymatic digestibility.

After dilute sulfuric acid pretreatment of the cotton stalks, the solids were analyzed for lignin, glucan and xylan contents. The lignin, insoluble in 72% sulfuric acid, remaining after acid pretreatment varied from 28.72% (1%, 30 min, 90°C) to 40.68% (2%, 60 min, 121°C /15 psi). A summary of these results, as well as those for the amount of xylan and glucan in the acid pretreated solids, is presented in Table 4.2. The reduction in lignin based on a comparison between the weight of lignin in the initial 10g (dry-weight) sample before pretreatment and the weight of lignin in the solids remaining after pretreatment showed reductions in lignin ranging on average from 2.27% to 24.16% as shown in Figure 4.1. Temperature, concentration, and time had significant ($p \leq 0.05$) effects on percent lignin reduction during sulfuric acid pretreatment. Concentration had a significant ($p \leq 0.05$) effect

on delignification for treatments at 90°C for 90 minutes and 121°C /15psi for 60 and 90 minutes. Increasing the temperature from 90°C to 121°C /15psi significantly increased delignification for 60 minutes at 2% H_2SO_4 and 90 minutes at 0.5, 1, and 2% H_2SO_4.[rs1]

Table 4.2 Composition of sulfuric acid pretreated cotton stalks[1]

Time (min), Concentration (%), Temperature (C)	Lignin %	Xylan %	Glucan %	Solids Recovered %
30, 0.5, 90	30.20 (0.33)[2]	10.24 (0.58)	35.75 (1.00)	80.27 (3.86)
30, 1.0, 90	28.72 (1.27)	10.05 (0.80)	34.85 (2.93)	85.05 (2.81)
30, 2.0, 90	30.25 (0.67)	9.98 (0.56)	33.74 (3.30)	81.18 (4.24)
60, 0.5, 90	30.59 (1.16)	9.82 (1.20)	35.41 (4.57)	83.32 (2.61)
60, 1.0, 90	31.36 (0.78)	9.74 (0.60)	34.00 (0.88)	83.56 (0.36)
60, 2.0, 90	34.33 (0.74)	10.14 (0.37)	36.98 (0.54)	73.42 (3.28)
90, 0.5, 90	32.24 (2.08)	9.45 (0.38)	34.22 (3.58)	83.83 (1.61)
90, 1.0, 90	35.14 (1.23)	9.05 (1.51)	35.41 (3.83)	77.87 (4.96)
90, 2.0, 90	33.75 (1.83)	8.33 (0.35)	37.20 (2.27)	75.02 (2.56)
30, 0.5, 121/15psi	33.65 (1.34)	7.62 (0.49)	37.73 (1.70)	75.45 (1.12)
30, 1.0, 121/15psi	34.91 (1.08)	4.64 (0.32)	39.17 (0.84)	67.71 (1.97)
30, 2.0, 121/15psi	38.59 (1.06)	2.79 (0.44)	41.41 (1.20)	62.16 (0.41)
60, 0.5, 121/15psi	35.60 (0.41)	7.15 (0.30)	40.63 (1.60)	73.07 (0.73)
60, 1.0, 121/15psi	39.30 (0.74)	3.63 (0.22)	41.55 (1.51)	64.44 (1.88)
60, 2.0, 121/15psi	40.68 (1.44)	0.00 (0.00)[rs2]	46.30 (2.81)	56.93 (3.04)
90, 0.5, 121/15psi	34.21 (0.97)	5.01 (0.35)	38.79 (2.43)	68.23 (3.04)
90, 1.0, 121/15psi	37.34 (2.00)	2.69 (0.68)	44.26 (2.20)	60.56 (1.98)
90, 2.0, 121/15psi	37.24 (1.85)	0.81 (1.41)	44.12 (2.20)	56.95 (0.99)

[1] Composition percentages on are on a dry-weight basis
[2] Standard deviations of three replicates in parentheses

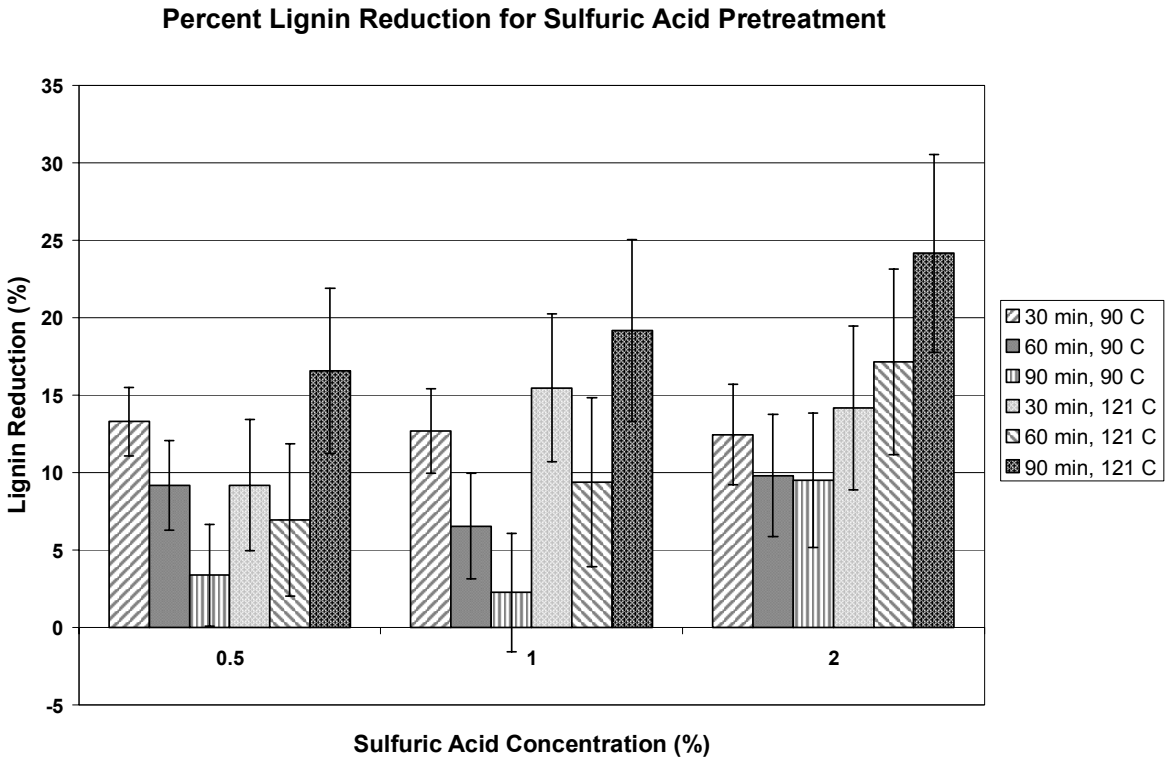

Figure 4.1 Percent lignin reduction for sulfuric acid pretreatment

The filtrate from the lignin analysis was used for the carbohydrate analysis. The xylan content, which makes up the largest portion of hemicellulose in the cotton stalks, is the most important indicator of pretreatment effectiveness for acid pretreatment. Arabinan and galactan, although making up 1.3 and 1.1%, respectively of the untreated sample, were below the HPLC detection limit after dilution of the carbohydrate samples, and therefore xylan is the only hemicellulose sugar discussed hereafter. The xylan content of the sulfuric acid pretreated samples (Table 4.2) ranged from 0% (2%, 60 min, 121°C /15psi) to 10.24% (0.5%, 30 min, 90°C). It is important to mention that the HPLC analysis for all three replicates at 2% acid, 60 minutes, and 121°C /15psi did not detect any xylan in the samples, thus indicating the possibility of complete solubilization of xylan during pretreatment. In order to

reconfirm these results, three new samples were pretreated and analyzed. Similar results were obtained, which supported the original data and eliminated the possibility of experimental or equipment error. In addition, the results obtained for 90 minutes at 2% with the same temperature were similar in that two of the three samples had no detectable levels of xylan, while the third sample had only about 2.44%. A reasonable explanation for this would be that the amount of xylan remaining in the 0.3 g (0.066 g dry weight) sample used for analysis was low enough that it was below the detection limit for the HPLC analysis. It may be assumed that there was solubilization of at least 75% of the xylan since this was the amount of solubilization by 1% acid pretreatment for 60 min at 121°C/15 psi. Sulfuric acid pretreatment effectively solubilzed 14.57% of the xylan for the least severe pretreatment (0.5%, 30 minutes,90°C) and an average of 95.2% for the most severe treatment (2%, 90 minutes, 121°C /15psi) (Fig 4.2). [rs3]

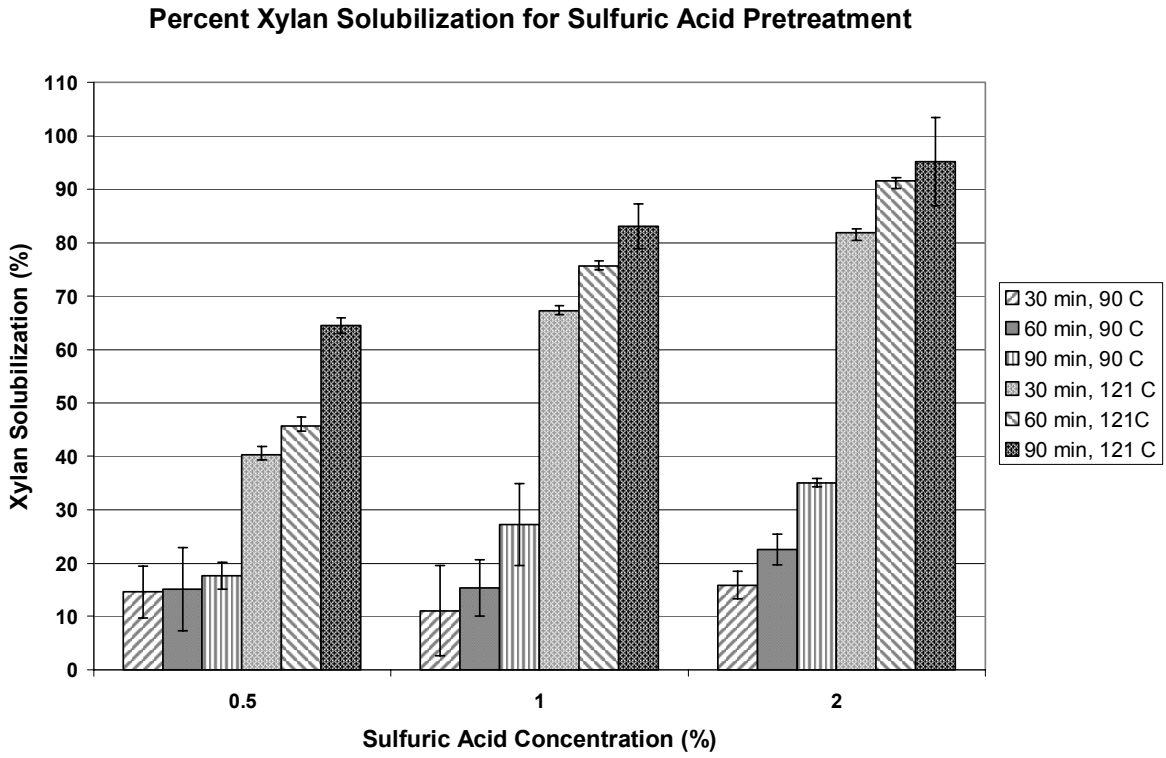

Figure 4.2 Percent xylan solubilization for sulfuric acid pretreatment

Results from this study are comparable to those obtained by Varga et al. (2002) where they observed 85% percent solubilization of hemicellulose at 121°C /15psi for 1 hour with 5% sulfuric acid. The solubilization of xylan increased with increasing time, temperature, and concentration. Increasing the temperature played the most significant role in increasing xylan solubilization. In addition, there was a more pronounced concentration effect for the pretreatments performed at the higher temperature (121°C).

When the simple treatment effects were determined in SAS with the ls means/slice statement, there was no significant concentration effect ($p>0.05$) on xylan solubilization at 90°C for 30 or 60 minutes. This indicates that the amount of xylan solubilization did not significantly increase with increasing concentration for the two lowest combinations of time and temperature. Therefore, increasing the concentration only increases the amount of xylan

solubilization when the time is sufficient (90 minutes at 90°C) or the temperature is at least 121°C. In addition, there was no significant time effect at 90°C, 0.5% acid, which indicates that the severity of the treatment at the lowest concentration and temperature does not show any significant improvement with an increase in time from 30 to 90 minutes. The temperature effect was significant for all combinations of time and concentration, showing that 121°C had a more significant effect on xylan solubilization than 90°C.

Glucan constituted between 33.74% (2%, 30 min, 90°C) and 46.3% (2%, 60 min, 121°C/15 psi) of the pretreated solids (Table 4.2). The main effects of temperature and concentration were significant ($p \leq 0.05$) for glucan solubilization during sulfuric acid pretreatment. The percentage of glucan solubilization during pretreatment increased with increasing concentration and averages among the three replicates were between 10.00% (0.5%, 60 min, 121°C/15 psi) and 23.88% (2%, 90 min, 121°C/15 psi) as shown in Figure 4.3. This means that between 76.12 and 90% of the glucan remained in the solid residue. It is desirable for the cellulose portion of the biomass to be virtually unaffected by the pretreatment, but because the loose cotton fiber is about 95% α-cellulose and is not imbedded in lignin and hemicellulose, the acid has direct access to the cellulose and causes more glucan degradation than is usually the case with other feedstocks.

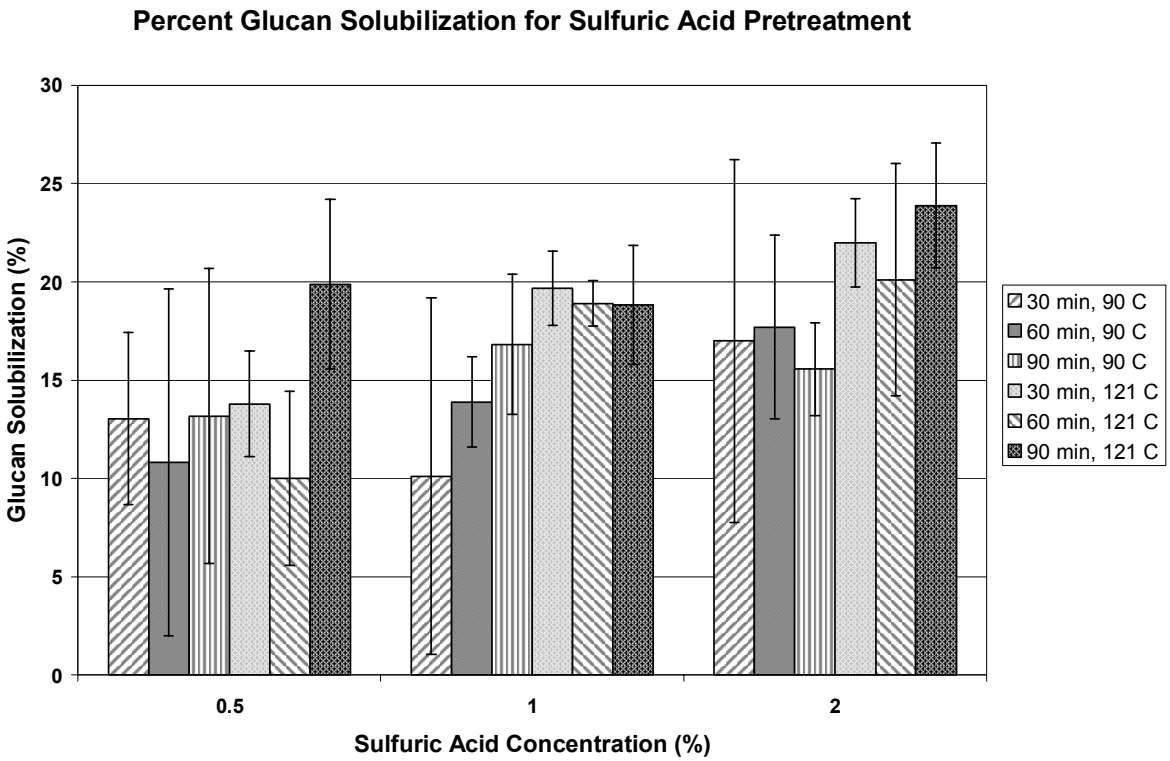

Figure 4.3 Percent glucan solubilization for sulfuric acid pretreatment

4.2.2 Sodium Hydroxide

Using sodium hydroxide to pretreat lignocellulosic materials is an alternative to sulfuric acid pretreatment. Its main effect on lignocellulosic biomass is delignification by breaking the ester bonds cross-linking lignin and xylan, thus increasing the porosity due to swelling allowed by breaking of the cross-links. The amount of lignin in the solids after NaOH pretreatment ranged from 23.31% (30 min, 90°C) to 25.22% (30 min, 121°C/15psi) for 0.5% NaOH, 19.46% (60 min, 121°C/15psi) to 21.90% (30 min, 90°C) for 1% NaOH, and 17.64% (90 min, 121°C/15psi) to 20.94% (30 min, 121°C/15psi) for 2% NaOH, with changes in concentration causing the most significant ($p \leq 0.05$) decrease in the lignin contents (Table 4.3).

Table 4.3 Composition of sodium hydroxide pretreated cotton stalks[1]

Time (min), Concentration (%), Temperature (C)	Lignin %	Xylan %	Glucan %	Solids Recovered %
30, 0.5, 90	23.31 (0.67)[2]	7.91 (3.55)	35.54 (0.83)	75.47 (3.92)
30, 1.0, 90	21.90 (0.80)	10.26 (0.71)	36.81 (2.80)	70.42 (1.28)
30, 2.0, 90	20.82 (1.27)	9.49 (1.53)	37.30 (4.90)	62.22 (3.30)
60, 0.5, 90	24.57 (1.97)	8.15 (5.07)	35.39 (5.60)	70.79 (4.58)
60, 1.0, 90	20.42 (0.72)	11.35 (0.71)	40.71 (2.06)	60.64 (2.50)
60, 2.0, 90	20.76 (0.90)	11.98 (0.80)	43.19 (1.66)	53.40 (1.69)
90, 0.5, 90	24.70 (0.90)	11.03 (0.76)	37.89 (0.54)	74.17 (1.44)
90, 1.0, 90	21.14 (0.89)	10.92 (1.15)	41.37 (0.92)	63.57 (1.38)
90, 2.0, 90	19.85 (0.82)	10.53 (2.66)	43.32 (6.16)	55.39 (3.98)
30, 0.5, 121/15psi	25.22 (3.66)	11.26 (1.83)	38.19 (4.42)	71.56 (7.97)
30, 1.0, 121/15psi	21.90 (0.94)	11.70 (0.56)	42.61 (2.46)	68.24 (4.87)
30, 2.0, 121/15psi	20.94 (1.77)	11.66 (0.90)	45.44 (1.80)	60.02 (2.03)
60, 0.5, 121/15psi	24.55 (1.55)	11.05 (0.10)	41.61 (1.01)	69.16 (0.83)
60, 1.0, 121/15psi	19.46 (1.40)	11.62 (1.26)	44.24 (4.84)	59.07 (1.49)
60, 2.0, 121/15psi	18.40 (0.16)	12.13 (0.40)	50.33 (1.84)	55.14 (1.29)
90, 0.5, 121/15psi	24.20 (1.33)	10.37 (0.41)	37.59 (1.20)	72.95 (1.32)
90, 1.0, 121/15psi	20.44 (1.33)	13.00 (1.35)	49.49 (4.73)	58.11 (1.82)
90, 2.0, 121/15psi	17.64 (0.61)	11.89 (0.56)	49.05 (1.68)	54.50 (1.02)

[1] Composition percentages on a dry-weight basis
[2] Standard deviations of three replicates in parentheses

The results of lignin reduction due to NaOH pretreatments are presented in Figure 4.4. The maximum reduction in lignin was 65.63% for 2% NaOH for 90 min at 121°C/15psi. Pretreatment of corn stover with 10% NaOH for one hour in the autoclave resulted in a 95% reduction in lignin (Varga et al., 2002). The high reduction level may be attributed to a higher NaOH concentration of 10%, which in this study was limited to 2%.

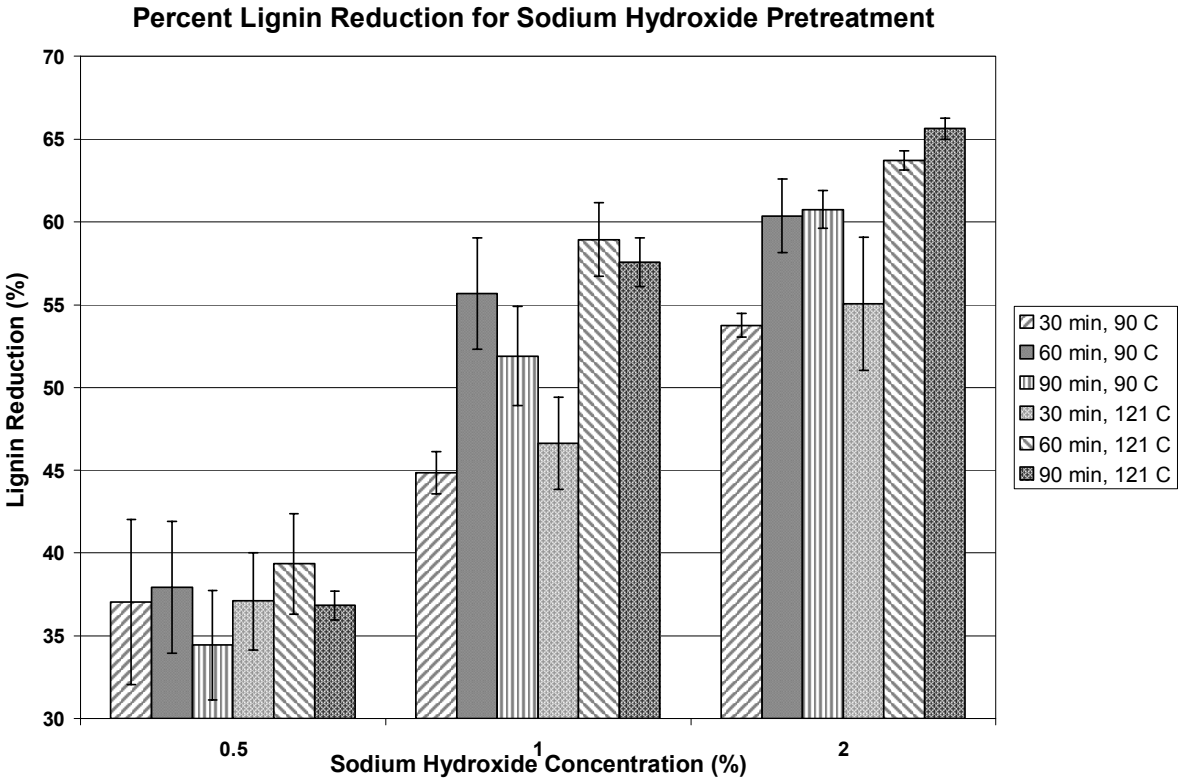

Figure 4.4 Percent lignin reduction for sodium hydroxide pretreatment

When comparing the treatment means for lignin reduction, the simple concentration effect was significant ($p \leq 0.05$) at all combinations of temperature and time, showing that increasing the concentration improved delignification for all treatments. There was no significant ($p > 0.05$) time effect observed for 0.5% NaOH at either temperature, thus indicating that this concentration of sodium hydroxide is too low to have a significant effect on delignification for treatment times up to 90 minutes and temperatures up to 121°C in the autoclave. The effect of temperature for sodium hydroxide pretreatment was significant ($p \leq 0.05$) only when the residence time was 90 minutes at 1 and 2% NaOH. This indicates that increasing the temperature only improved the amount of lignin removal for longer times and higher concentrations. Thus the extra energy expenditure associated with increasing the

temperature from 90 to 121°C when the treatment time is 30 or 60 minutes at any concentration or 0.5% for 90 minutes is not an economical option because there is no significant improvement in lignin reduction. However, this does not necessarily mean that raising the temperature higher than 121°C would not result in marked improvements at lower concentrations and times.

Sodium hydroxide pretreatment also had an effect on the xylan content of the pretreated solids. Though the reduction in xylan content was lower than it was for sulfuric acid, the solubilization of xylan in conjunction with substantial lignin reduction is expected to significantly improve enzymatic hydrolysis. The xylan content of the pretreated solids ranged from 7.91% (0.5%, 30 min, 90°C) to 13.00% (1%, 90 min, 121°C/15 psi) (Table 4.3). The solubilization of xylan ranged from 13.90% (0.5%, 90 min, 90°C) to 40.02% (2%, 90 min, 90°C) (Figure 4.5). Concentration, time, and temperature did not cause significant ($p > 0.05$) differences in percent xylan solubilization means for any of the treatment combinations.

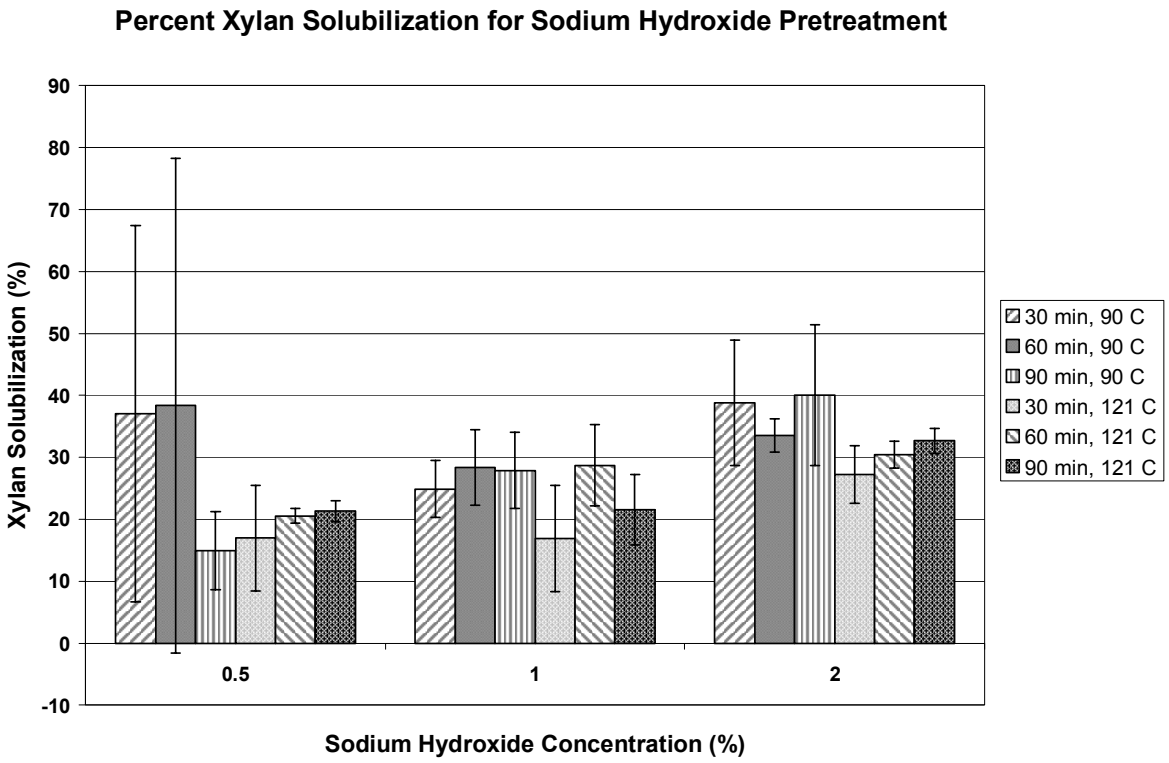

Figure 4.5 Percent xylan solubilization for sodium hydroxide pretreatment

The glucan content of the NaOH pretreated solids ranged from 35.54% (0.5%, 30 min, 90°C) to 50.33 % (2%, 60 min, 121°C/15psi). The solubilization of glucan during pretreatment was more substantial than expected, with averages of about 12.82% (1%, 30 min, 121°C/15psi) to 30.14% (2%, 60 min, 90°C) of glucan solubilization as illustrated in Figure 4.6. Temperature and concentration both had significant ($p \leq 0.05$) effects on glucan solubilization in the overall model. Percent glucan solubilization significantly increased with increasing concentration for 90°C at 90 min and the temperature effect was significant for 0.5% NaOH for 60 min and 2% NaOH for 30 and 60 minutes. The standard deviations among some replicates were rather large. This could be attributed to the heterogeneous nature of cotton stalks and the fact that amount of free cotton fiber could vary from one

sample to the other. Therefore, the amount of cellulose directly exposed to NaOH could vary, thus subjecting samples to variable degradation.

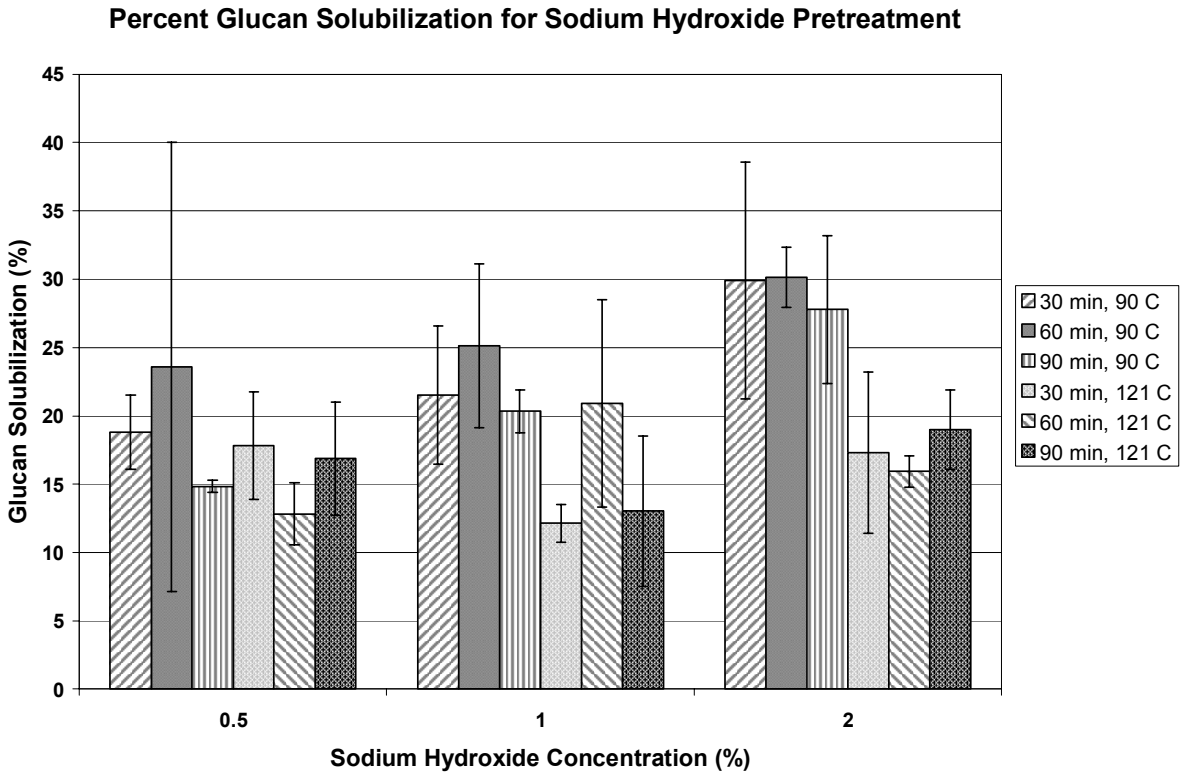

Figure 4.6 Percent glucan solubilization for sodium hydroxide pretreatment

4.2.3 Hydrogen Peroxide

Hydrogen peroxide pretreatment utilizes oxidative delignification to detach and solubilize the lignin and loosens the lignocellulosic matrix thus improving enzyme digestibility. The extent by which lignin degradation occurred in this study was not as high as expected. Pretreatment of sugar cane bagasse with 2% hydrogen peroxide showed a 50% decrease in lignin and solubilization of most of the hemicellulose within 8 hours at 30°C (Azzam, 1989). Shorter residence times up to 1 ½ hours but higher temperatures were used for the cotton stalks. The average percentage of lignin in the pretreated solids ranged from 25.6% (2%, 60

min, 121°C/15 psi) to 31.9% (1%, 90 min, 121°C/15 psi) on average as seen in Table 4.4. There were no significant ($p>0.05$) differences in lignin content for any combination of time, temperature, and concentration.

Table 4.4 Composition of hydrogen peroxide pretreated cotton stalks[1]

Time (min), Concentration (%), Temperature (C)	Lignin %	Xylan %	Glucan %	Solids Recovered %
30, 0.5, 90	29.63 (0.38)	8.92 (0.35)	29.83 (2.95)	83.16 (1.57)
30, 1.0, 90	28.28 (1.45)	9.01 (0.59)	28.44 (0.47)	82.27 (1.41)
30, 2.0, 90	27.83 (0.46)	9.22 (0.32)	30.12 (1.21)	83.51 (0.52)
60, 0.5, 90	30.47 (1.71)	10.26 (0.44)	32.64 (0.71)	86.02 (1.37)
60, 1.0, 90	28.58 (1.12)	10.30 (0.08)	31.42 (0.35)	84.28 (1.61)
60, 2.0, 90	27.82 (1.68)	10.09 (0.57)	32.32 (1.65)	81.93 (2.21)
90, 0.5, 90	28.35 (1.24)	10.06 (0.57)	29.25 (4.78)	85.59 (5.83)
90, 1.0, 90	30.16 (0.92)	9.25 (0.55)	30.43 (0.39)	83.21 (1.39)
90, 2.0, 90	27.43 (2.22)	10.87 (0.14)	34.15 (1.92)	80.44 (2.43)
30, 0.5, 121/15psi	27.70 (0.75)	9.55 (0.98)	31.39 (3.08)	84.12 (1.36)
30, 1.0, 121/15psi	28.36 (2.78)	9.45 (0.85)	30.48 (2.22)	81.64 (0.81)
30, 2.0, 121/15psi	25.66 (2.15)	8.69 (0.74)	30.12 (1.99)	76.83 (0.82)
60, 0.5, 121/15psi	28.11 (1.75)	9.12 (0.70)	30.72 (0.50)	83.97 (2.19)
60, 1.0, 121/15psi	28.51 (1.33)	9.26 (0.96)	30.69 (2.73)	81.15 (4.83)
60, 2.0, 121/15psi	25.59 (2.30)	10.00 (0.26)	34.53 (0.86)	74.42 (2.90)
90, 0.5, 121/15psi	30.98 (0.95)	9.53 (0.38)	30.21 (2.11)	85.04 (1.55)
90, 1.0, 121/15psi	31.94 (1.03)	10.03 (0.54)	30.97 (1.11)	79.26 (1.76)
90, 2.0, 121/15psi	29.00 (1.38)	10.52 (0.31)	33.41 (0.65)	72.59 (0.60)

[1] Composition percentages are on a dry-weight basis
[2] Standard deviations of three replicates in parentheses

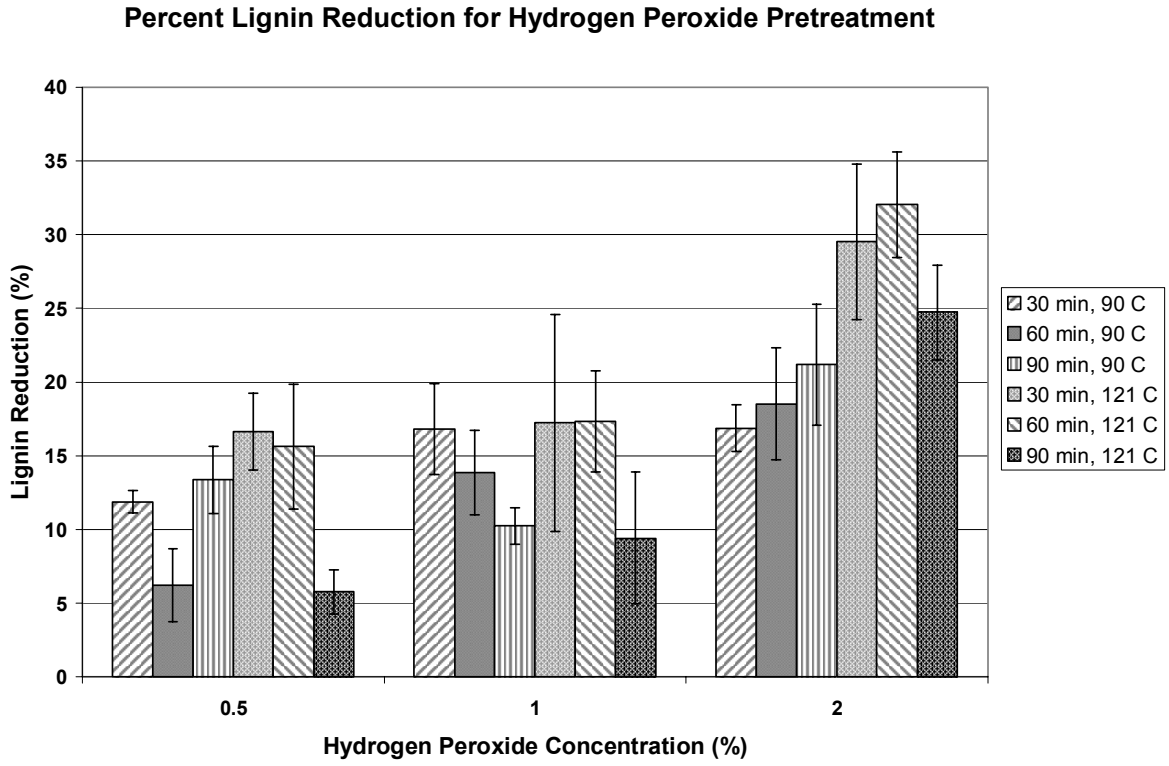

Figure 4.7 Percent lignin reduction for hydrogen peroxide pretreatment

Delignification ranged from 6.22% (0.5%, 90 min, 90°C) to 32.01% (2%, 60 min, 121°C/15 psi) on average (Figure 4.7), which were lower percent lignin reductions than expected. When the simple treatment effects for delignification were determined, there was a significant ($p \leq 0.05$) concentration effect for all combinations of time and temperature except for 30 minutes at 90°C. In other words, increasing the concentration from 0.5 to 2% did not significantly increase delignification for 30 minutes at 90°C probably because the residence time was too short at the lower temperature. The simple time effect was only significant for 121°C/15psi at 0.5 and 1% H_2O_2, which indicates that increasing the time from 30 to 90 minutes only showed significant improvements for the two lower concentrations at the higher temperature. Temperature only played a significant role in improving delignification for 0.5% at 60 min and 2% at 30 and 60 minutes and the increase in

temperature significantly decreased the mean delignification for 0.5% at 90 minutes. The results for hydrogen peroxide pretreatment showed the most variation and had trends that were unexpected. For example, the most severe pretreatment in the autoclave at 121°C for 90 minutes with 2% acid had lower levels of delignification than the treatments at 30 and 60 minutes at 0.5 and 1%. One would expect the opposite result, however, upon further investigation, it was realized that when hydrogen peroxide is exposed to heat, it undergoes the following decomposition reaction $2H_2O_2 ==> 2H_2O + O_2$ where the hydrogen peroxide decomposes to water. From this, one could surmise that 90 minutes at 121°C is a high enough temperature for a long enough period of time to allow the decomposition of all or most of the hydrogen peroxide to water, thus diminishing the oxidative delignification effects.

The amount of xylan remaining in the pretreated samples ranged from 8.69% (2%, 30 min, 121°C/15psi) to 10.87% (2%, 90 min, 90°C) (Table 4.4) and the solubilization of xylan averaged between 8.18% (0.5%, 60 min, 90°C) and 30.56% (2%, 30, 121°C/15psi) (Figure 4.8). Concentration had a significant effect ($p \leq 0.05$) on xylan solubilization for 90 minutes, 90°C and 30 minutes, 121°C. The simple temperature effect was significant for xylan solubilization for 0.5% at 60 min, 1.0% at 60 min, and 2% at 30, 60, and 90 min. The percentage of glucan in the pretreated solids (Table 4.4) ranged from 28.4% for (1%, 30 min, 90°C) to 34.1% for (2%, 90 min, 90°C). Glucan solubilization averaged from 14.91% (0.5%, 60 min, 90°C) to 29.10% (2%, 30 min, 121°C/15psi) as presented in Figure 4.9. Concentration did not have a significant effect on glucan solubilization. Significant differences ($p \leq 0.05$) between percent glucan solubilization means due to changes in temperature were noted for 2% at 90 min, while time played a significant role for 0.5 and 1% H_2O_2 at 90°C.

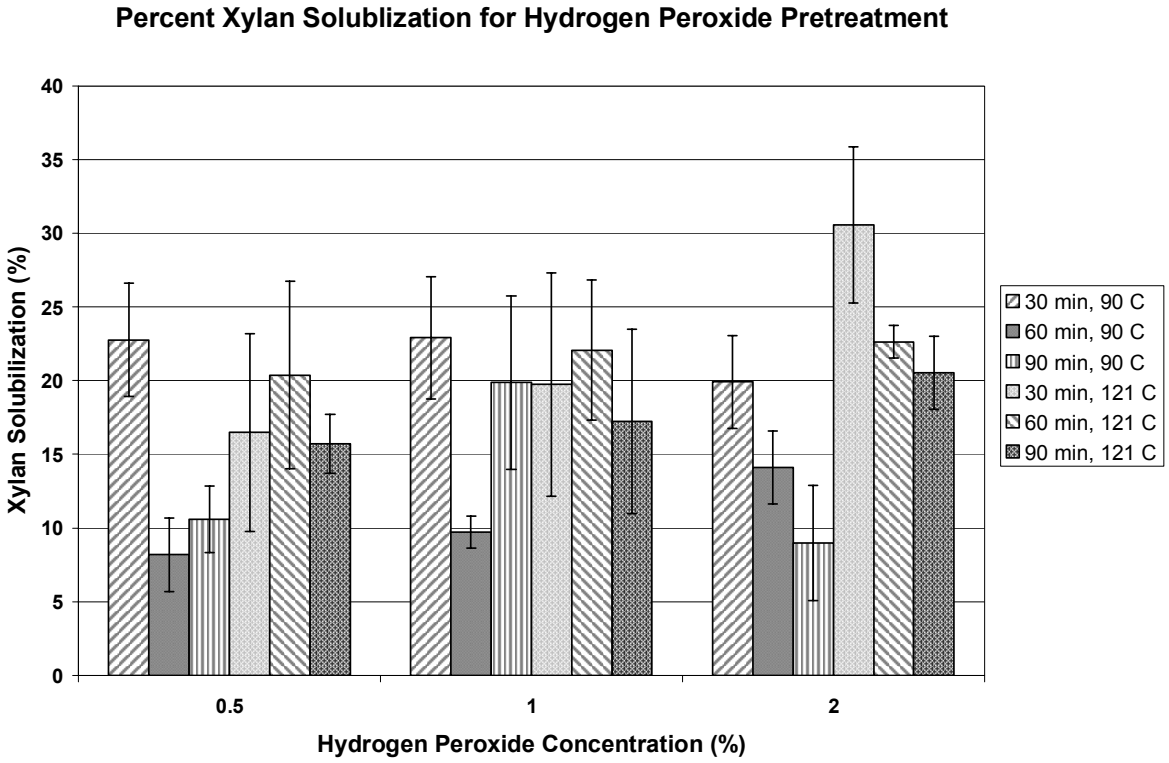

Figure 4.8 Percent xylan solubilization for hydrogen peroxide pretreatment

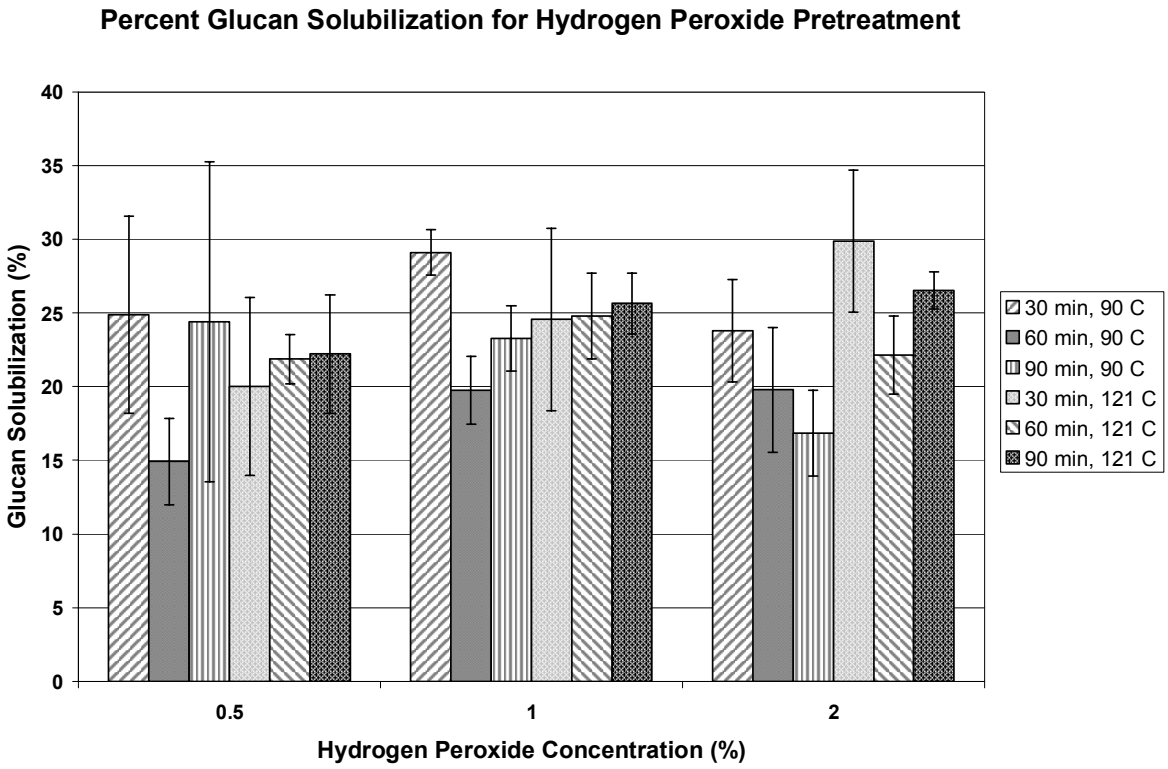

Figure 4.9 Percent glucan solubilization for hydrogen peroxide pretreatment

4.2.4 Ozone

Pretreatment of lignocellulosic biomass with ozone gas has been reported to reduce both the lignin and hemicellulose contents of the treated materials. The most substantial effect of ozone pretreatment is on degradation of the lignin polymer, followed by hemicellulose and cellulose solubilization, respectively (Quesda et al., 1999). The concentrations of ozone in pure water after 30, 60, and 90 minutes were 16.96, 17.74, and 18.52 ppm, respectively. The amount of lignin in the solids after ozone pretreatment for 30, 60 and 90 minutes was 27.21, 26.31, and 25.95%, xylan percentages for the pretreated solids were 8.86, 9.70, and 10.31%, and glucan percentages were 30.45, 31.12, and 33.51% for 30, 60, and 90 minutes, respectively. Ben-Ghedalia et al. (1980) reported a 50% decrease in both

lignin and hemicellulose in ozone treated cotton stalks. However, in this study, reduction in lignin ranged on average from only about 11.97% to 16.6% with no significant difference ($p > 0.05$) noted among treatment times of 30, 60, and 90 minutes. The amounts of glucan removed during ozone treatment were 16.6, 13.7, and 7.2% for 30, 60, and 90 minutes, respectively, while the amount of xylan removed was 16.7, 10.6, and 1.9% for 30, 60, and 90 minutes, respectively as shown in Table 4.6. The percent solubilization of xylan and glucan for 90 minutes was significantly ($p<0.05$) lower than the solubilization for 30 and 60 minutes.

Table 4.5 Composition of ozone pretreated cotton stalks[1]

Time (min)	Lignin %	Xylan %	Glucan %	Solids Recovered %
30	27.21 (0.77)[2]	8.86 (0.92)	30.45 (3.13)	90.44 (2.47)
60	26.31 (1.28)	9.70 (1.06)	31.12 (1.54)	88.66 (2.82)
90[3]	25.95 (0.99)	10.31 (0.27)	33.51 (0.04)	91.66 (0.47)

[1] Composition percentages are on a dry-weight basis
[2] Standard deviations of three replicates in parentheses
[3] Only two samples were used for 90 minutes because the third replicate was an outlier

Table 4.6 Percent lignin reduction and xylan and glucan solubilization during ozone pretreatment

	Reduction %		
Time (min)	**Lignin**	**Xylan**	**Glucan**
30	11.97 (2.91)	16.76 (7.32)	16.62 (7.80)
60	16.63 (2.60)	10.61 (8.12)	13.74 (3.64)
90[1]	15.15 (3.02)	1.92 (2.89)	7.19 (0.36)

[1] Only two samples were used for 90 minutes because the third replicate was an outlier

Possible explanations for the differences between the results from this study and those from past studies include insufficient treatment times, inadequate ozone concentration, and poor distribution of ozone gas throughout the cotton stalks because of inefficient sparging.

Inadequate ozone concentrations could have been due to a low gas flow rate or possible decomposition of O_3 due to reactions with dirt and other organic matter. The sparger was submerged in the mixture of cotton stalks and water; however, the bubbling of ozone gas was more intense near the top layer of cotton stalks. The stalks at the bottom were darker in color with a color gradient going from dark to light from the bottom to the top of the sample. This indicates possible variations in the distribution of ozone throughout the sample thus increasing the probability of non-homogeneous changes in lignin, cellulose, and hemicellulose throughout the samples. If lignin and sugar analyses were performed on samples from the bottom versus the top, there could potentially be considerable differences between replicates. A possible improvement for this situation would be constant mixing of the sample during ozonation.

4.3 Modeling

4.3.1 Empirical Models

Modeling with experimental data was done to predict xylan solubilization for sulfuric acid pretreatment and lignin reduction for sodium hydroxide pretreatment. These two treatment agents were chosen for modeling because they have been widely studied in the past and seem to be the most promising pretreatments for use on cotton stalks. Xylan solubilization predictions were made for sulfuric acid because the main mode of action during this pretreatment is hemicellulose solubilization, which can be directly related to improvements in enzymatic hydrolysis. Predictability of delignification was investigated for sodium hydroxide pretreatment because lignin was significantly reduced during pretreatment and could possibly be used as a measure for predicting improvements in enzyme hydrolysis.

Empirical quadratic models using time, temperature, and concentration as numeric variables were developed in order to predict percent lignin reduction and percent xylan solubilization. The models used to predict both lignin reduction for sodium hydroxide and xylan solubilization for sulfuric acid were of the form:

$$y = \beta_0 + \beta_1 T + \beta_2 t + \beta_3 C + \beta_4 Tt + \beta_5 CT + \beta_6 CTt + \beta_7 Ct + \beta_8 t^2 + \beta_9 C^2 \quad (4.1)$$

Where: T = temperature (°C)
t = time (min)
C = concentration (%)
β_n = estimated regression coefficients, n=0,..,8

The squared temperature term (T^2) was not included in the model because there were only two temperatures used during the experiments. This did not provide a sufficient number of degrees of freedom to estimate a regression coefficient for a squared term, and was thus left out of the model. After eliminating the insignificant terms ($p>0.05$) from the model based on the p-values from the Type III Sum of Squares ANOVA table (Table 7.11), the reduced model used to predict the percentage of xylan removed from the cotton stalks during sulfuric acid pretreatment with a squared correlation coefficient (R^2) of 0.964 was:

$$\% \text{ xylan solubilization} = -117.6194 + 1.0798\,T + 0.2644\,t - 22.6728\,C + 0.6347\,CT - 11.0451\,C^2 \quad (4.2)$$

The p-value for concentration in the ANOVA table was not significant ($p>0.05$), however the interaction between temperature and concentration and concentration^2 were both significant, so the concentration term was left in the model.

The percent lignin reduction model for sodium hydroxide containing significant terms from the Type III Sum of Squares ANOVA table (Table 7.12) had an R^2 of 0.924 and was:

$$\% \text{ lignin reduction} = -1.3705 + 0.0002\,T + 0.5554\,t + 49.6254\,C + 0.0904\,Ct - 15.9216C^2 - 0.0047\,t^2 \quad (4.3)$$

The appropriate values for C, T, and t were plugged into the equations and the predicted vs. actual values for xylan solubilization (Figure 4.10) [rs4]and percent lignin reduction (Figure 4.11) from the quadratic regression models were plotted to verify the validity of the model predictions. If the predicted values were exactly the same as the experimental values, then the equation of the line relating the two would be y=x with an R^2 of 1. If the slope is greater than 1 or less than 1, this indicates over or under prediction of the model and helps with assessing the validity. The intercept of the line was forced through zero, which increased or decreased the slope accordingly. The closer the actual intercept was to zero, the better the predictability of the model and the closer the slope would be to 1. The R^2 of the models was calculated by plotting a linear regression in MS Excel (MS Office, Microsoft, Inc.). The R^2 for the percent xylan solubilization model for sulfuric acid pretreatment was 0.96 with a slope of 0.97 and the R^2 for the percent lignin reduction model for sodium hydroxide pretreatment was 0.90 with a slope of 0.97. Both models had high R^2-values and slopes close to 1 thus indicating good predictive abilities of the models.

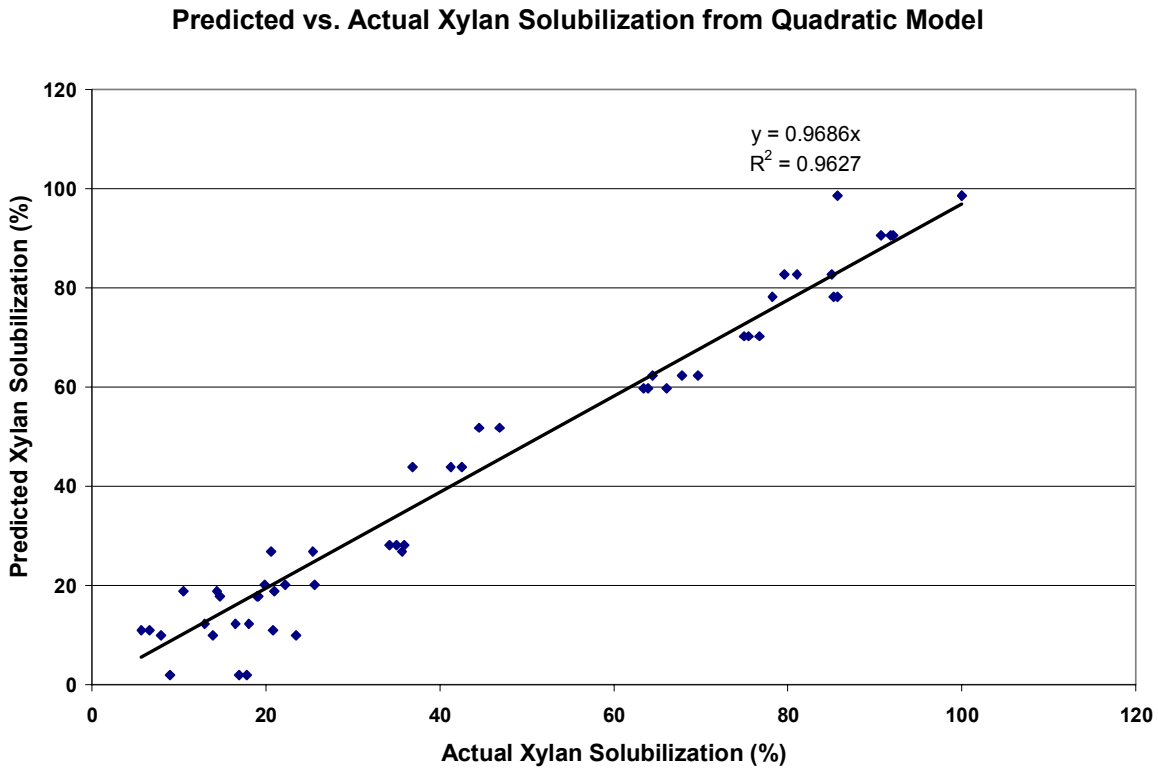

Figure 4.10 Predicted xylan solubilization vs. actual xylan solubilization for sulfuric acid pretreatment using quadratic model

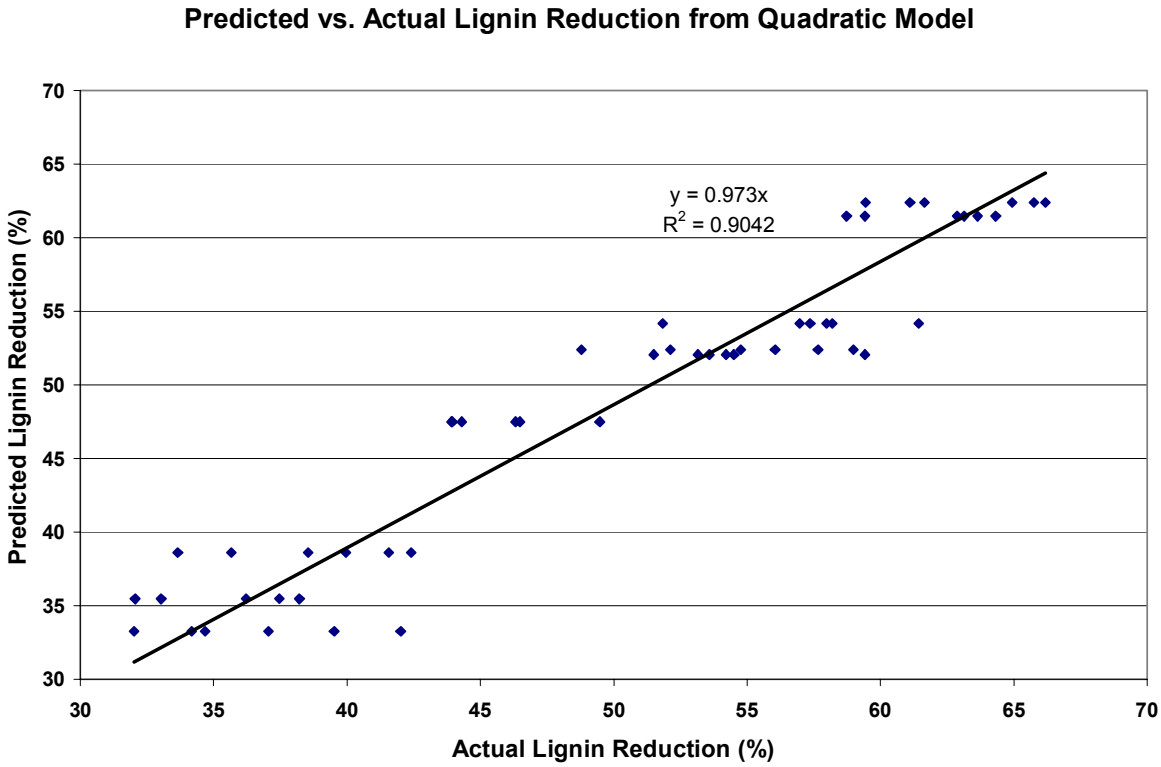

Figure 4.11 Predicted lignin reduction vs. actual lignin reduction for sodium hydroxide pretreatment using quadratic model

4.3.2 Models Based on Modified Severity Parameter

Linear models relating a modified severity parameter (described in Section 3.5) that combines the effects of time, temperature and concentration to the % solubilization of xylan for sulfuric acid pretreatment and to the % reduction in lignin for sodium hydroxide pretreatment resulted in R^2 values of 0.89 and 0.78, respectively (Figure 4.12-4.13). The n-values used in the modified severity parameter equation (Eqn. 3.16) were determined by making an initial guess for 'n', calculating the corresponding values for M_0 and plotting the % xylan solubilization (data from Figure 4.2) vs. log (M_0) (graph not shown). The predicted xylan solubilization percentages were then calculated using the linear regression equation

generated from this plot. The squared difference between the experimental and predicted values was minimized using Solver in MS Excel by allowing the n-value to change. Once the optimum n-value was calculated, the % xylan solubilization was plotted against the new log (M_o) values to generate the linear regression model used to predict percent xylan solubilization (Figure 4.12). A similar approach was employed for the % lignin reduction prediction model. The n-values for sulfuric acid and sodium hydroxide that provided the best model fits while keeping log (M_o) positive were 0.849 and 3.90, respectively.

$$M_o \text{ (sulfuric acid)} = t * C^{0.76} * \exp\left[\frac{Tr - 100}{14.75}\right]$$

$$M_o \text{ (sodium hydroxide)} = t * C^{3.90} * \exp\left[\frac{Tr - 100}{14.75}\right]$$

The model equation determined for the solubilization of xylan during sulfuric acid pretreatment using n=0.849 to calculate M_0 was:

$$\% \text{ xylan solubilization} = 53.508 * \log(Mo) - 55.043 \quad (4.4)$$

While the model equation for the reduction of lignin during sodium hydroxide pretreatment using n=3.90 to calculate M_0 was:

$$\% \text{ lignin reduction} = 8.6438 * \log(Mo) + 33.68 \quad (4.5)$$

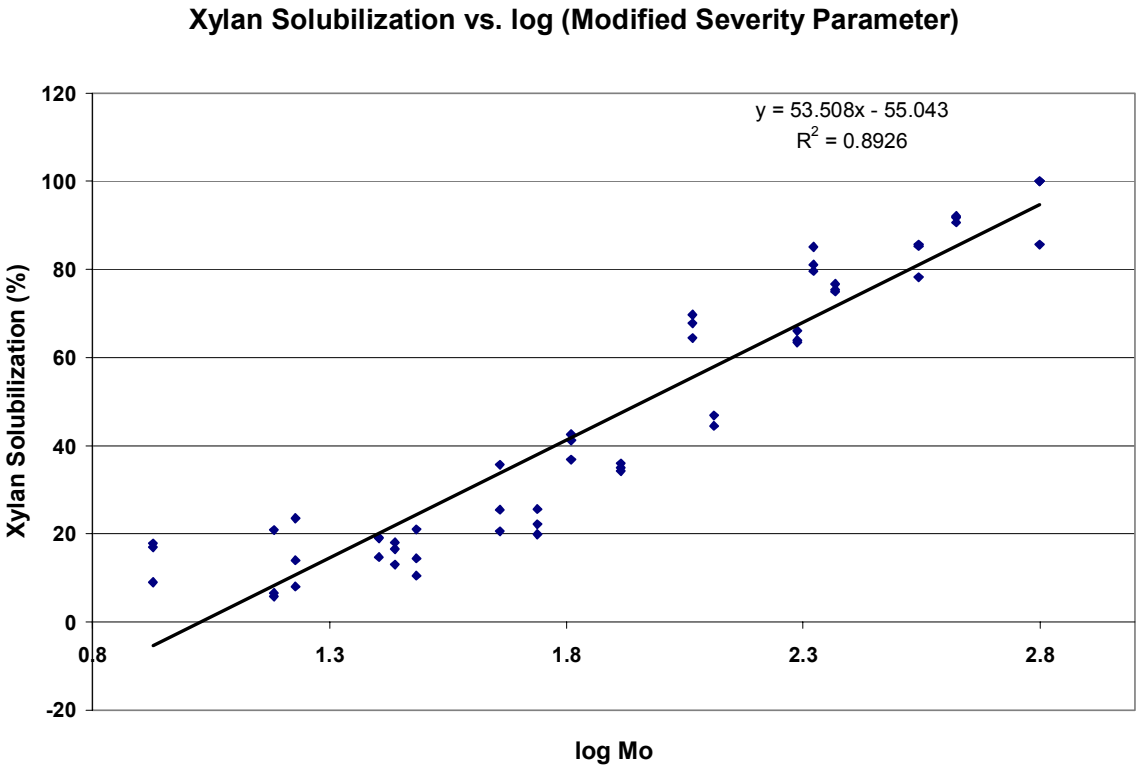

Figure 4.12 Xylan solubilization vs. log (modified severity parameter) for sulfuric acid pretreatment

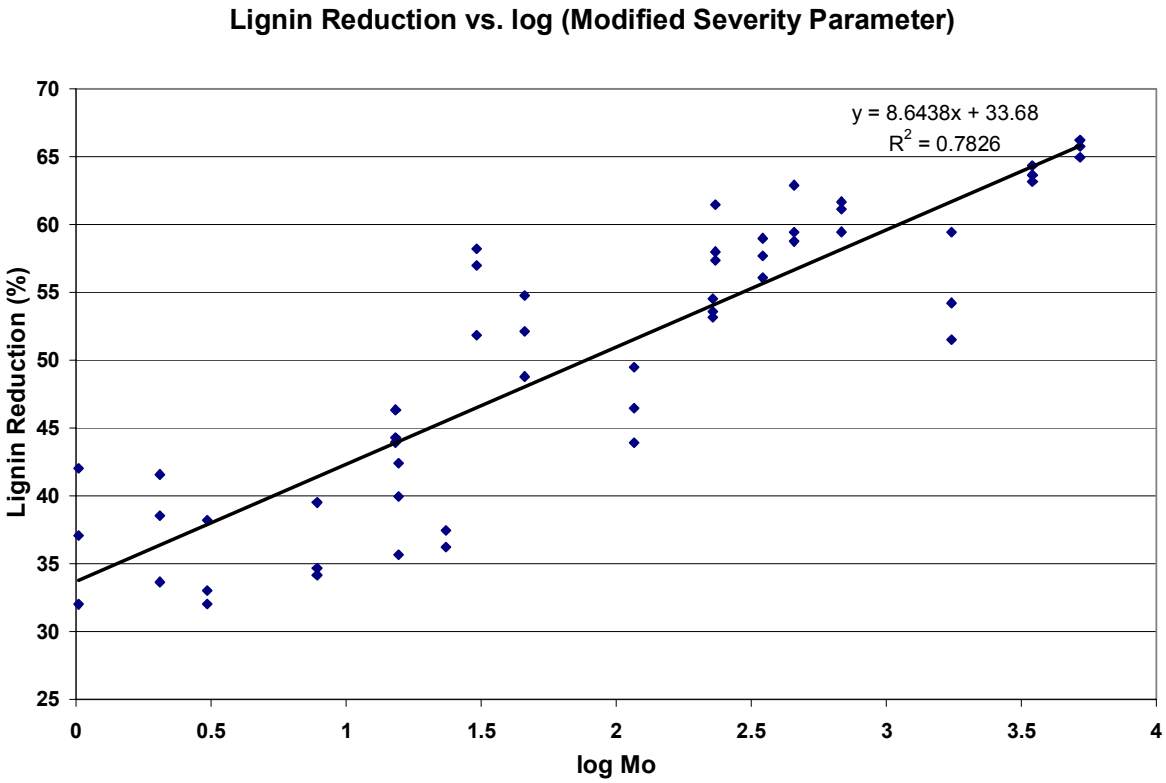

Figure 4.13 Lignin reduction vs. log (modified severity parameter) for sodium hydroxide pretreatment

The predicted vs. actual values for percent lignin reduction and xylan solubilization using the modified severity parameter were plotted to verify the validity of the model predictions based on the same procedure used for validation of the quadratic models. The graph for experimental vs. predicted % xylan solubilization is shown in Figure 4.14. The R^2 for the model was 0.88 and the slope was 0.952 indicating good predictive ability of the model. Variation between replicates due to possible experimental error and the heterogeneity of the cotton stalks samples probably decreased the level of predictability of the model.

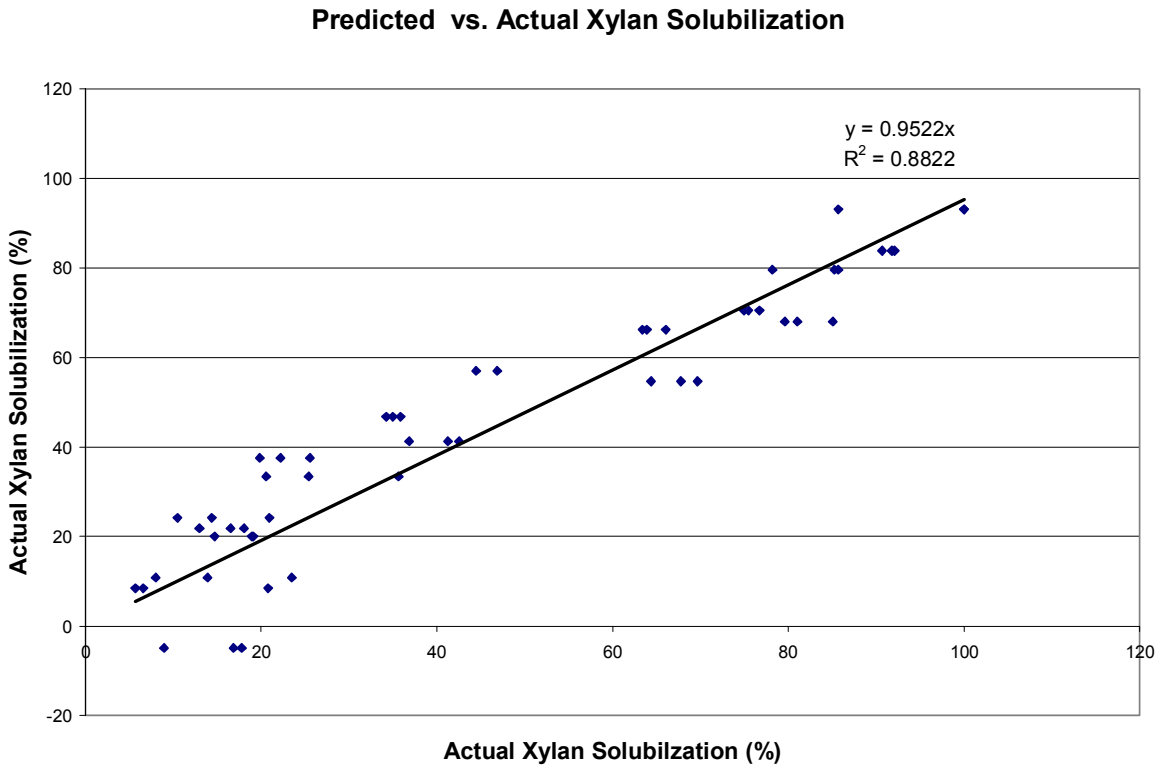

Figure 4.14 Predicted xylan solubilization vs. actual xylan solubilization for sulfuric acid pretreatment using modified severity parameter

The graph for comparing the predicted and experimental values for % lignin reduction during sodium hydroxide pretreatment is shown in Figure 4.15. When forcing the intercept through zero, the R^2 was 0.72 and the slope was 0.99. The predictions from this model were not as good as those made by the xylan prediction model since only 75% of the variability between the experimental data and the predicted data can be explained by this model.

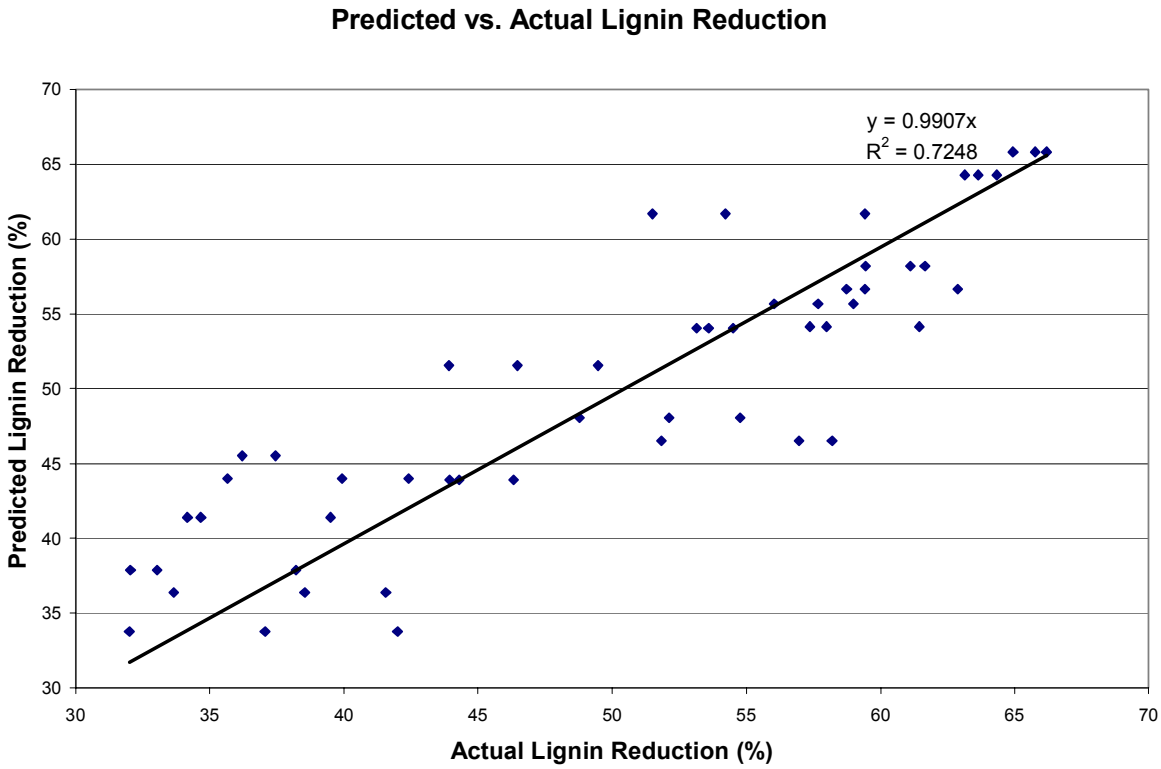

Figure 4.15 Predicted lignin reduction vs. actual lignin reduction for sodium hydroxide pretreatment using modified severity parameter

4.4 Enzymatic Hydrolysis

Sulfuric acid, sodium hydroxide, and hydrogen peroxide pretreatments were chosen for enzymatic hydrolysis. For acid pretreatment, the selection was based on examining differences among glucose content in pretreated samples and the samples resulting in maximum glucose availability were chosen for enzyme hydrolysis (2% H_2SO_4, 60 min, 121°C/15psi). This selection criterion was based on the fact that acid pretreatment has little effect on lignin degradation and the main treatment effect is on hemicellulose and cellulose solubilization.

The selection for sodium hydroxide pretreatment was based on finding the pretreatment with a compromise between having the lowest percentage of lignin in the

pretreated solids, while maintaining a high percentage of glucan in the solids as well. The pretreatment chosen for enzyme hydrolysis (2% NaOH, 60 min, 121°C/15psi) met this criteria. The 60 minute treatment was chosen instead of 90 minutes because there was no significant ($p>0.05$) difference in lignin or glucan content between 60 and 90 minutes, and the operating costs would be lower for the shorter residence time.

For hydrogen peroxide, there were no significant differences between percentage glucan, xylan, or lignin in the pretreated solids for any of the treatments. Hence, the treatment with the highest percentage of glucan and the lowest percentage of lignin was chosen. The pretreatment combination chosen for was 2% H_2O_2 for 60 min at 121°C/15psi.

After the 72-hour enzyme hydrolysis, a sugar analysis was performed on the supernatant liquid and the percentages of glucose and xylose detected are reported in Table 4.7. The results showing the average percent cellulose conversion of the three replicates for each hydrolysis treatment are depicted graphically in Figure 4.16. Sodium hydroxide pretreatment had the highest cellulose conversion of 60.8%, followed by hydrogen peroxide (49.8%) and then sulfuric acid (23.8%). Differences in mean cellulose conversions for all of the treatments were statistically significant ($p\leq0.05$), with cellulose conversions for both NaOH and H_2O_2 being more than two times the conversion obtained for H_2SO_4.

Table 4.7 Percent glucose and xylose in enzyme hydrolysis supernatant liquid[1] and percent glucose and xylose in pretreated solids[2]

Pretreatment Agent	**Glucose (%)[1,2]**	**Xylose (%)[1,2]**
Sulfuric acid	11.03 (0.66) 46.30 (2.89)	1.43 (0.16) 0.00 (0.00)[rs5]
Sodium hydroxide	30.57 (0.56) 50.33 (1.84)	8.34 (0.15) 12.13 (0.40)
Hydrogen peroxide	17.21 (0.84) 34.53 (0.86)	0.90 (0.14) 10.00 (0.26)

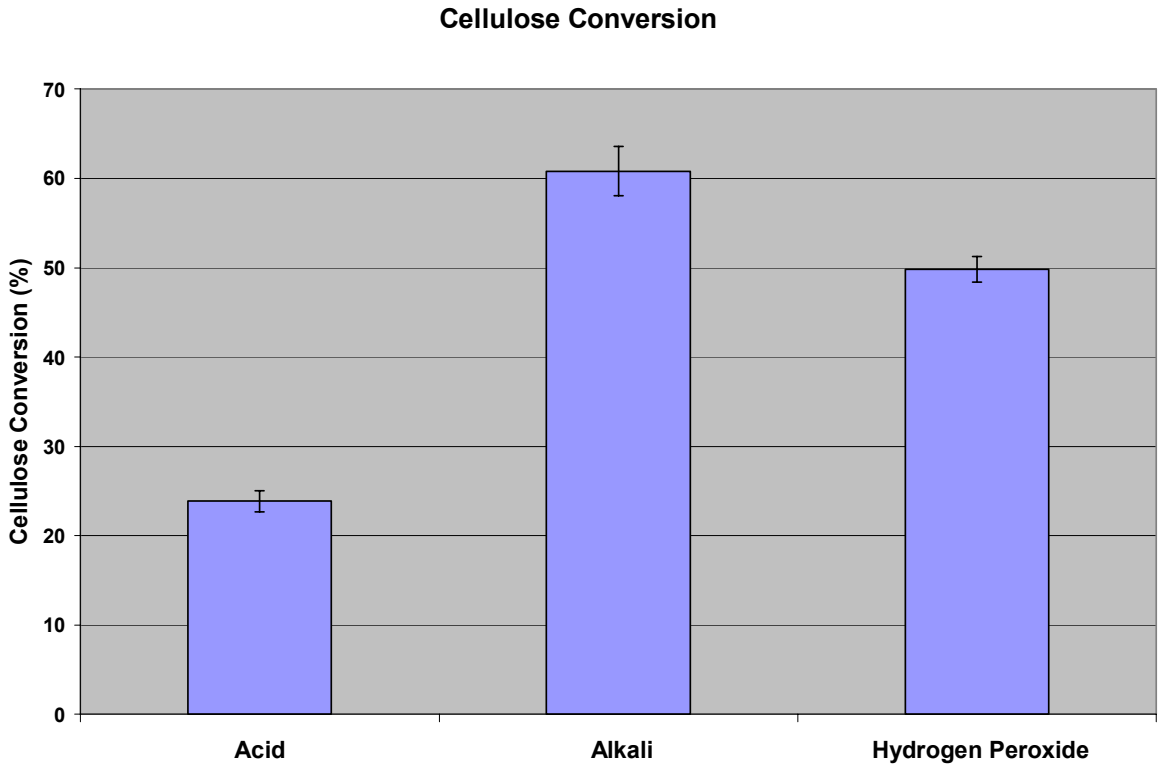

Figure 4.16 Percent cellulose conversion with enzymatic hydrolysis based on glucan available in pretreated sample

In addition, the sodium hydroxide pretreatment resulted in the highest xylan to xylose conversion (Table 4.8), with an average of 62.57% conversion for the three replicates, whereas hydrogen peroxide averaged 7.78% conversion. For the acid pretreated samples (2%, 60 min, 121°C/15psi), no xylan was detected in the solids during the initial carbohydrate analysis (Table 4.2), but an average of 14.3 mg xylose/g dry biomass or 1.43% was detected in the supernatant after enzymatic hydrolysis (Table 4.7). This confirms the hypothesis mentioned earlier that there was xylan in the stalks after pretreatment, but the amount was so low that it was not detected during the sugar analysis. Xylose was detected after enzyme hydrolysis because the dry sample weight was 75 times greater than that used for the initial carbohydrate analysis. Assuming complete xylan conversion during saccharification would

result in a maximum xylan solubilization during pretreatment of 91.5%. This value was determined by using the average of 1.43% xylan detected during enzyme hydrolysis to calculate the percent xylan solubilization during pretreatment by following the same method used for the all other pretreatment calculations as outlined in Section 3.2.

Table 4.8 Percent xylan conversion with enzymatic hydrolysis based on xylan available in pretreated solids

Pretreatment Agent	**% Xylan Conversion (based on pretreated solids)**[1]
Sulfuric acid	0.00 (0.00)[rs6]
Sodium hydroxide	62.57 (2.57)
Hydrogen peroxide	7.78 (1.13)

[1]Standard deviations of three replicates in parentheses

There are a few possible causes for the large differences in the amount of cellulose converted to glucose during enzymatic hydrolysis. Firstly, the amount of lignin in the samples was different depending on the pretreatment agent. The acid pretreated samples had about 40% of the solids as lignin (acid insoluble material), the samples treated with NaOH consisted of only 18% acid insoluble material and hydrogen peroxide pretreated samples had approximately 25% lignin. The sulfuric acid pretreated samples had 2.2 times the amount of acid insoluble material and converted 2.5 times less cellulose to glucose than the sodium hydroxide pretreated samples. The hydrogen peroxide treated samples had 1.4 times the amount of lignin and converted 1.2 times less cellulose to glucose than sodium hydroxide pretreated samples during enzyme hydrolysis. In addition, the amount of lignin in the untreated sample is about 3 times greater than the amount of xylan, so reducing the amount of lignin in the sample would most likely have a larger impact than the solubilization of xylan.

Chapter 5: Conclusions

5.1 Summary

Cotton stalks with known composition were pretreated with sulfuric acid, sodium hydroxide, hydrogen peroxide at three different concentrations and times and at two temperatures. Ozone pretreatment was also performed by sparging ozone gas using three different reaction times. Research focused on studying the effects of the pretreatments on the removal of lignin, glucan and xylan from the pretreated solids. Enzyme hydrolysis was performed on one set of samples treated with sulfuric acid, sodium hydroxide, and hydrogen peroxide. The selection of the pretreatment parameters for enzyme hydrolysis was based on a statistical analysis of the percentage of glucan, xylan, and lignin in the pretreated solids.

5.2 Conclusions

The conclusions drawn from this study are as follows:

1. Cotton stalks are a heterogeneous biological material. Compositional analysis data of pretreated cotton stalks can vary highly depending on the ratio of cotton to woody fibers in the samples.
2. The most substantial effect of sulfuric acid pretreatment on cotton stalks is the solubilization of xylan. There is a linearly increasing relationship between the solubilization of xylan and pretreatment severity.
3. The most substantial effect of sodium hydroxide pretreatment is delignification. There is a linearly increasing relationship between lignin reduction and pretreatment severity.
4. Hydrogen peroxide pretreatment resulted in lower lignin and xylan solubilization than expected. This was probably due to decomposition of hydrogen peroxide to water at high

temperatures. Sodium hydroxide pretreatment was more effective at delignification than hydrogen peroxide.

5. Ozone pretreatment did not perform as expected. Possible explanations include insufficient time, low ozone concentration, or uneven distribution of ozone throughout the sample.
6. There was substantial solubilization of glucan during pretreatment. The direct exposure of free cotton fibers to the pretreatment agents probably contributed to the higher percentages of glucan solubilization.
7. Concentration of the pretreatment agent had the most significant effect on lignin reduction for sodium hydroxide pretreatment.
8. Temperature had the most significant effect on xylan solubilization for sulfuric acid pretreatment.
9. Pretreatment with 0.5% sulfuric acid at 90°C was not effective since there was no significant change ($p>0.05$) in xylan solubilization with increased time.
10. Pretreatment with 0.5% sodium hydroxide at 90°C and 121°C/15psi was not effective since there was no significant change ($p>0.05$) in lignin reduction with increased time.
11. Sodium hydroxide pretreatment resulted in significantly ($p•0.05$) higher cellulose conversion during enzyme hydrolysis than hydrogen peroxide and sulfuric acid pretreatment.
12. Delignification appears to have more effect on enzyme digestibility than xylan solubilization.
13. The empirical quadratic models successfully predicted percent xylan solublization and percent lignin reduction with R^2 values of 0.964 and 0.924, respectively.

14. There was a linearly increasing relationship between the modified severity parameters and percent xylan solubilization for sulfuric acid pretreatment and percent lignin reduction for sodium hydroxide pretreatment.

5.3 Future Work

There are many possibilities for future work on cotton stalks pretreatment, enzymatic hydrolysis, and ethanol production. Different combinations of pretreatment parameters, perhaps using higher temperatures or concentrations and application of pressure could be investigated for optimization of pretreatment. The use of ozone could be further explored by increasing the sample size and using higher ozone concentrations with improved sparging techniques to provide a more thorough examination of the effect of ozone on cotton stalks. Enzymatic hydrolysis using optimized pretreatment parameters could be performed to ensure maximum cellulose conversion. In addition, varying time, enzyme loading, and solids loading to obtain maximum glucose yields while minimizing costs could optimize enzyme hydrolysis. Furthermore, ethanol yields during fermentation could be examined. Fermentation with yeast vs. bacteria could be investigated and the fermentation process could be optimized for cotton stalks. Lastly, the economic feasibility of ethanol production from cotton stalks should be investigated since it could not be addressed in this study.

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Appendices

Appendix 7.1

HPLC Sugar Analysis

The concentration of each sugar (mg/L) was determined by the computer software from Dionex that accompanies the HPLC system. First, a sample of each sugar was run through the system to determine the retention times (Table 8.1). Then, calibration exercises were performed by running carbohydrate samples with known concentrations of fucose, arabinose, galactose, glucose, and xylose. Concentrations of 0, 10, 30, and 50 mg/L respectively, were used and the calibration curves shown in Figures 7.2-7.6 were generated. The linear equations established between the response (based on the peak heights and areas) and the known sugar concentrations were then used to calculate the concentrations of unknown sugars during the carbohydrate analysis.

Table 7.1 Carbohydrate retention times for HPLC analysis

Carbohydrate	Retention Time (min)
Fucose	6.64
Arabinose	12.69
Galactose	14.67
Glucose	16.96
Xylose	19.86

The retention times in Table 7.1 and those shown on the sample chromatograph in Figure 7.1 differ slightly. This is due to changes occurring in the HPLC column over time. The

retention times noted above were recorded for the most recent calibration exercises, while the chromatograph below shows the times for an earlier calibration exercise.

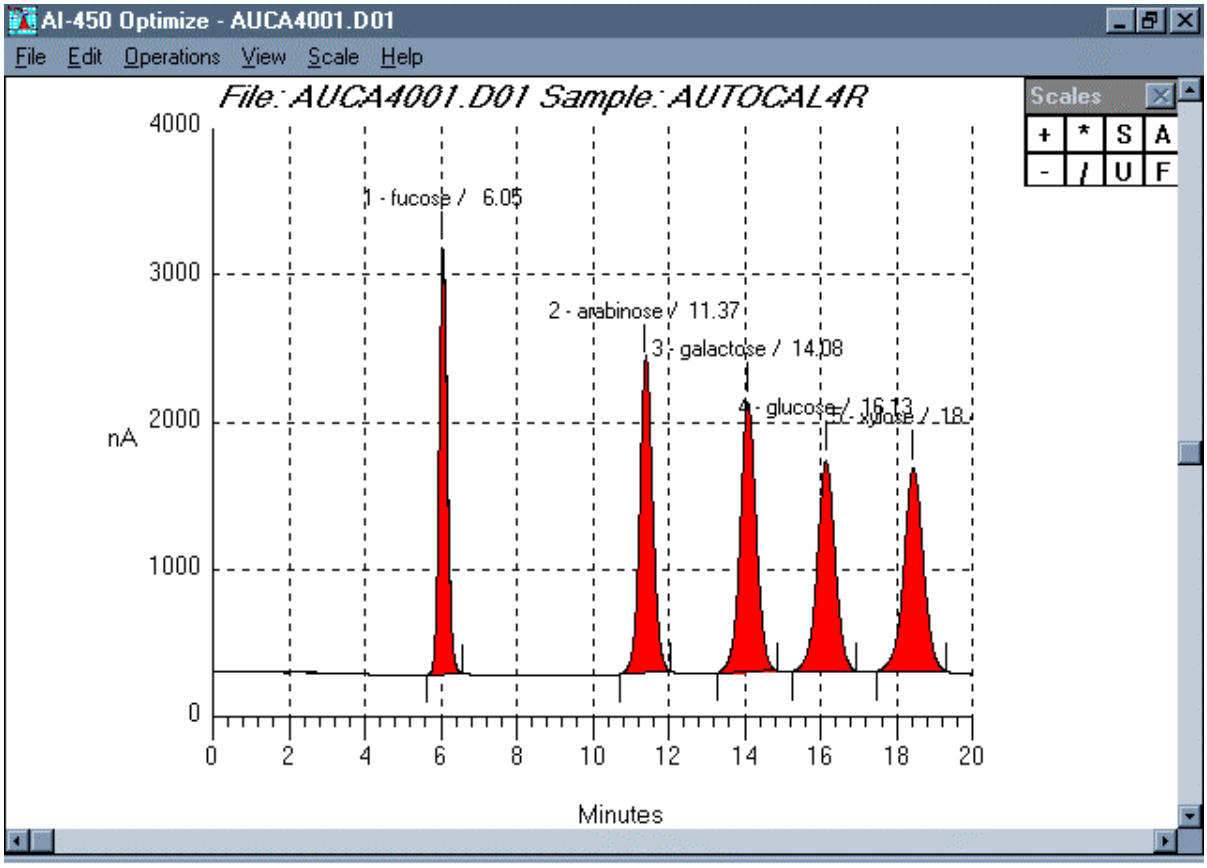

Figure 7.1 Sample chromatograph showing peaks and retention times for fucose, arabinose, galactose, glucose, and xylose during calibration

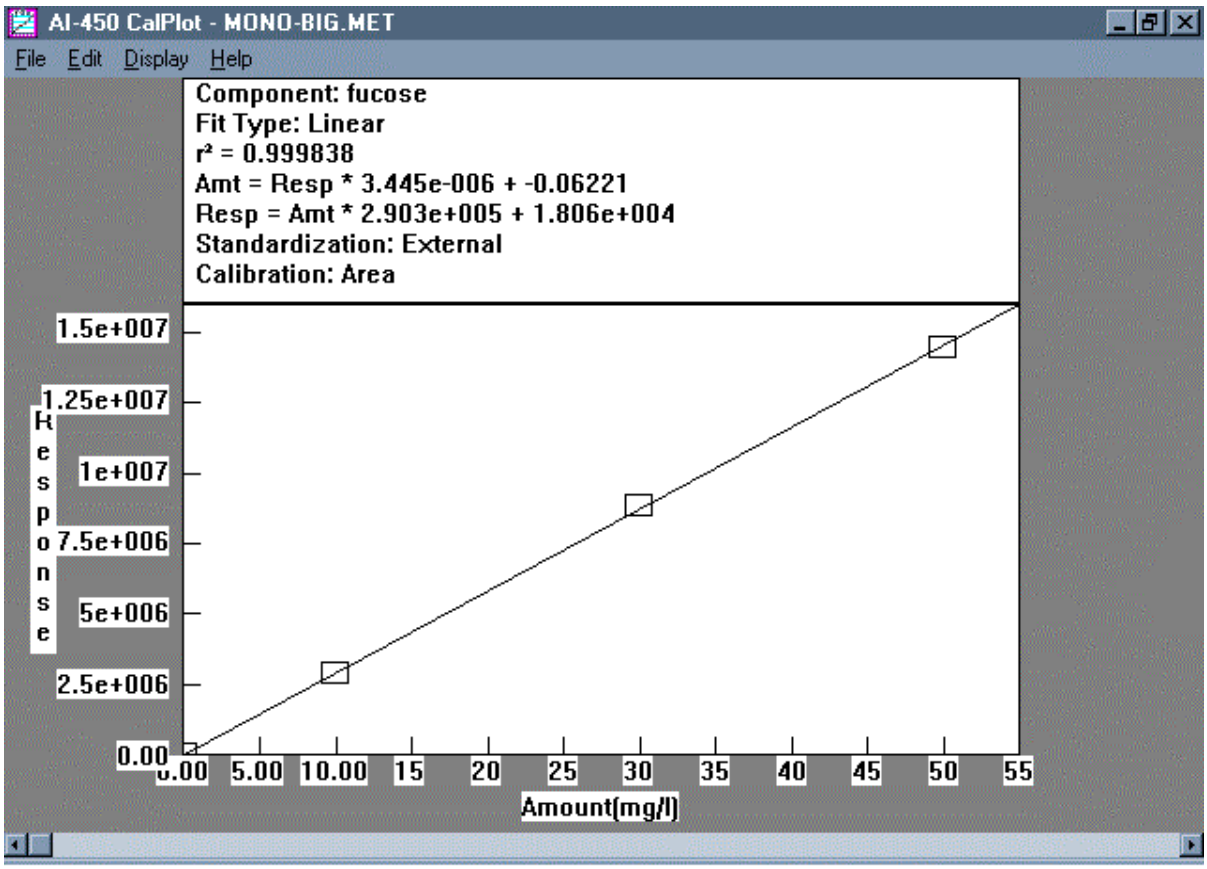

Figure 7.2 Calibration curve for fucose

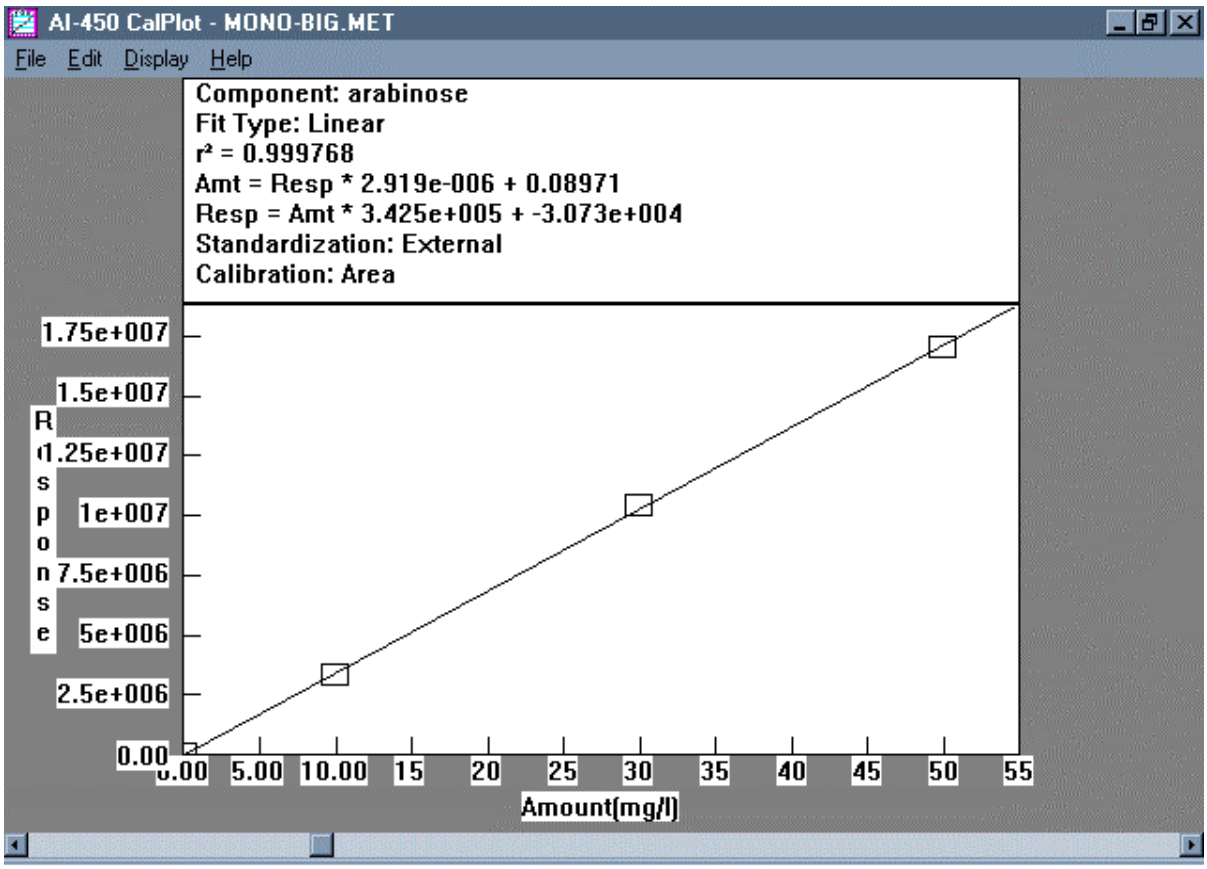

Figure7.3 Calibration curve for arabinose

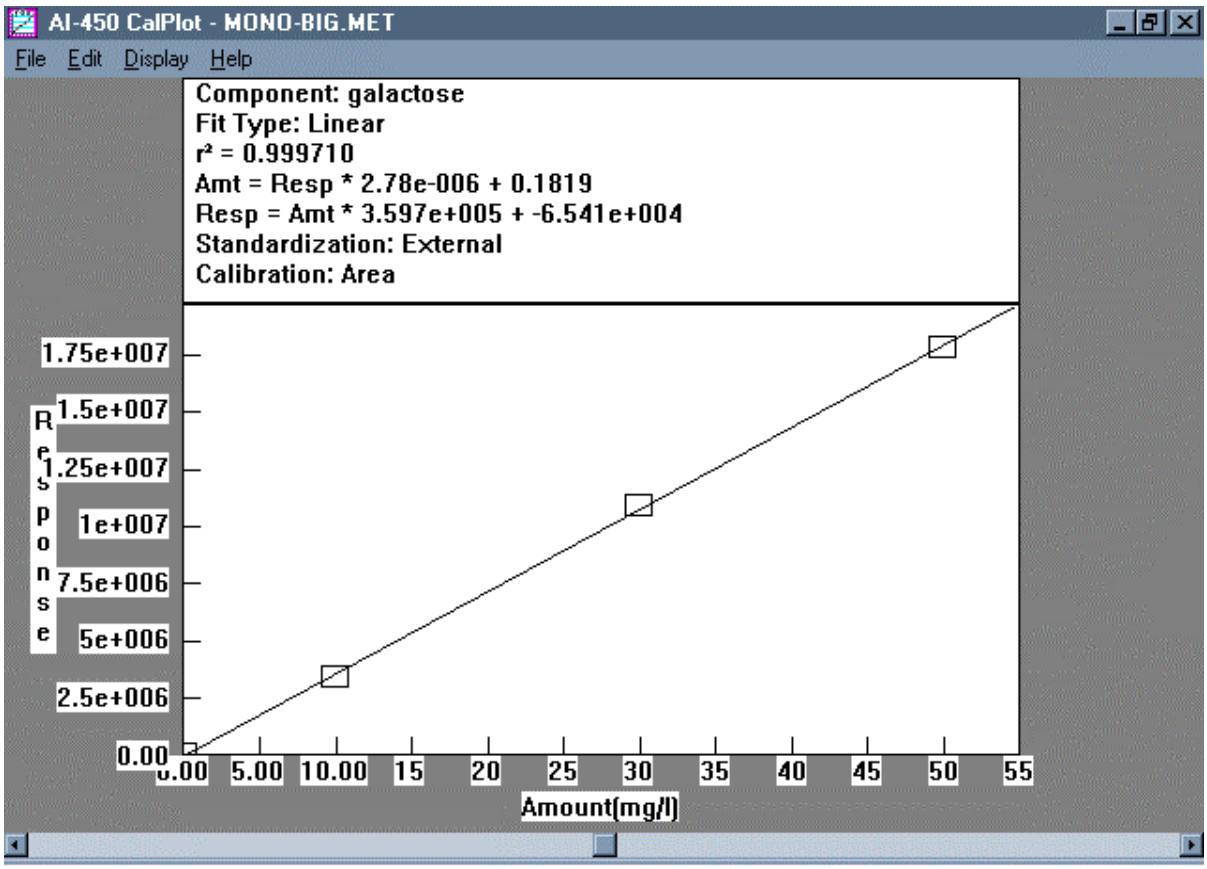

Figure7.4 Calibration curve for galactose

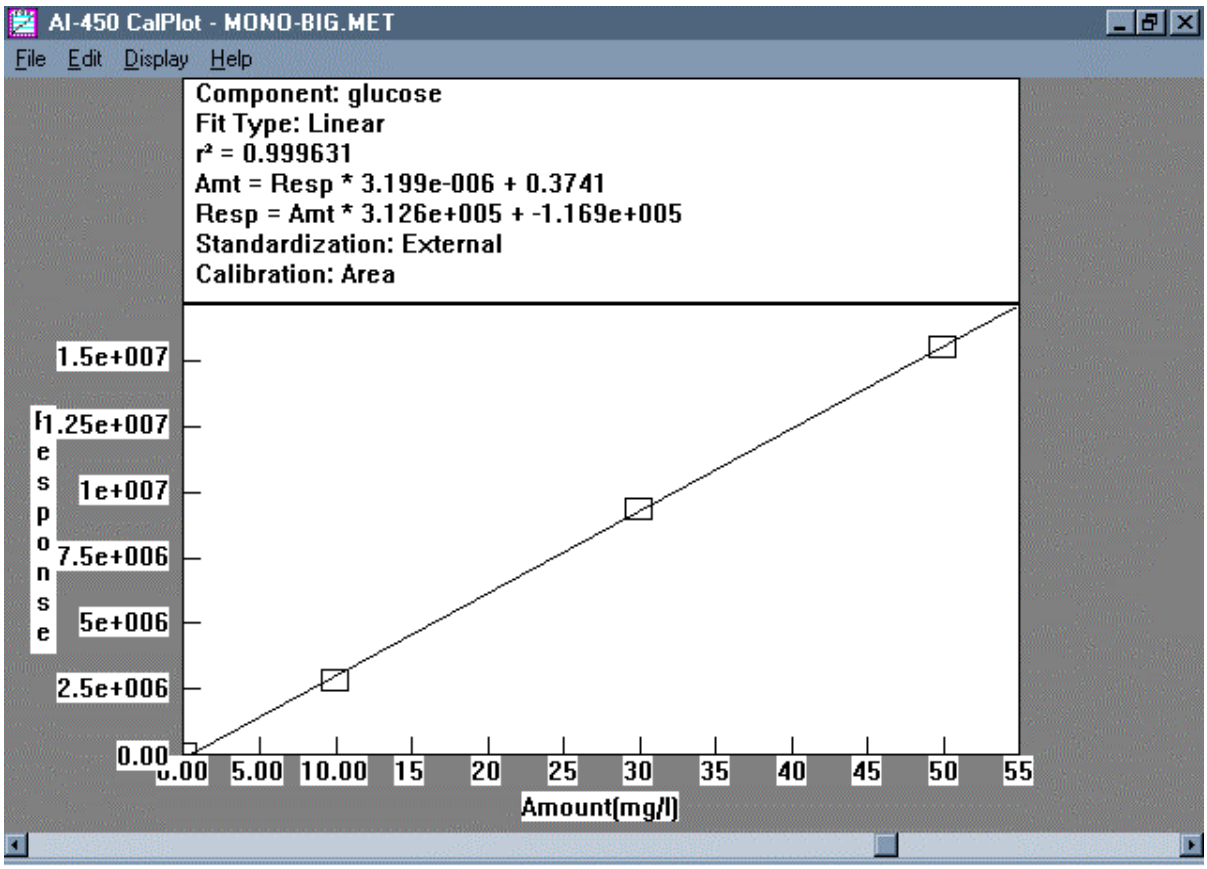

Figure 7.5 Calibration curve for glucose

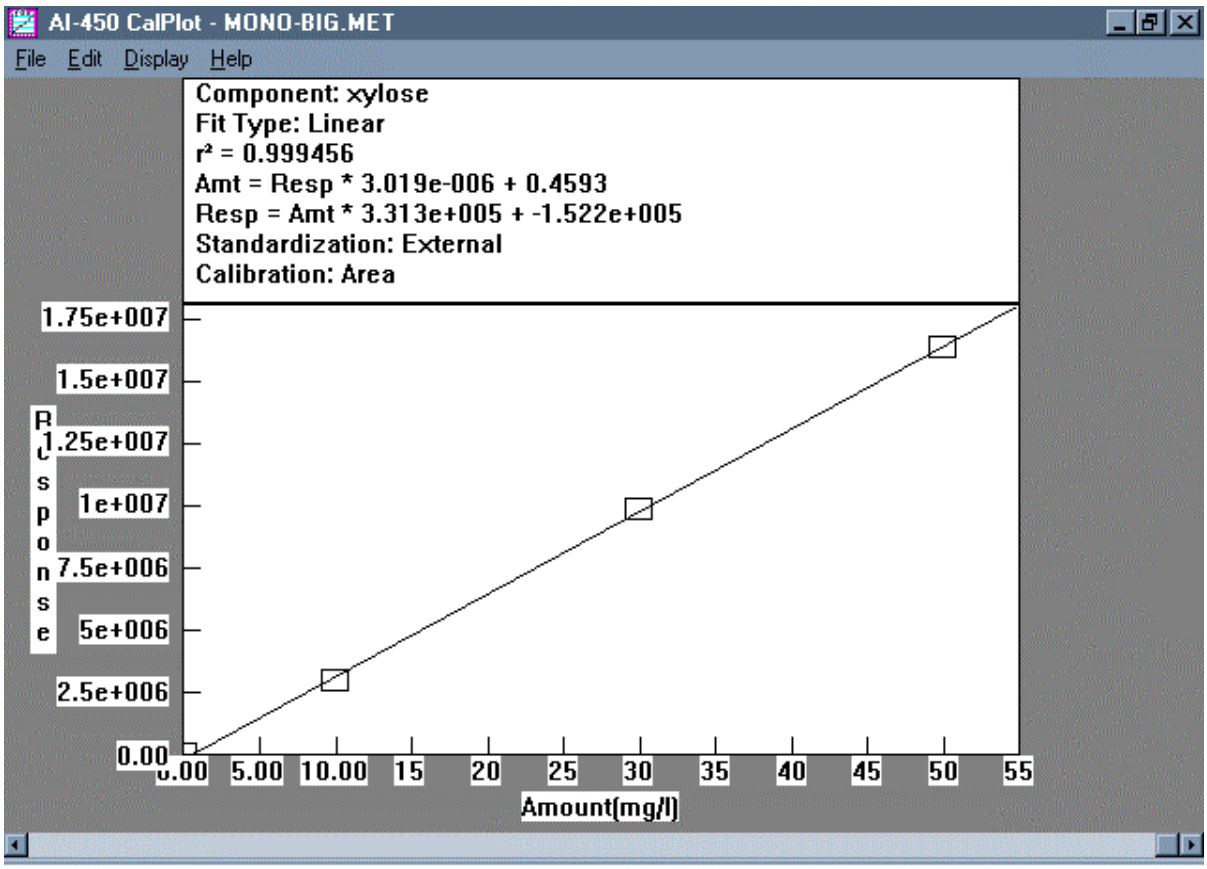

Figure 7.6 Calibration curve for xylose

Appendix 7.2

ANOVA Tables

Table 7.2 ANOVA table for sulfuric acid pretreatment percent xylan solubilization model using class statement

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	47697.77418	2805.75142	78.99	<.0001
Error	35	1243.28453	35.52242		
Corrected Total	52	48941.05871			

R-Square	Coeff Var	Root MSE	xylose_red Mean
0.974596	13.91969	5.960068	42.81754

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Temp	1	38698.47986	38698.47986	1089.41	<.0001
Conc	2	4401.55748	2200.77874	61.95	<.0001
Temp*Conc	2	1937.61252	968.80626	27.27	<.0001
Time	2	1766.75092	883.37546	24.87	<.0001
Temp*Time	2	56.52726	28.26363	0.80	0.4593
Conc*Time	4	119.34448	29.83612	0.84	0.5093
Temp*Conc*Time	4	717.50166	179.37541	5.05	0.0026

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Temp	1	36980.43910	36980.43910	1041.05	<.0001
Conc	2	4680.19800	2340.09900	65.88	<.0001
Temp*Conc	2	1997.97647	998.98823	28.12	<.0001
Time	2	1771.30955	885.65478	24.93	<.0001
Temp*Time	2	40.41729	20.20864	0.57	0.5713
Conc*Time	4	133.49758	33.37439	0.94	0.4526
Temp*Conc*Time	4	717.50166	179.37541	5.05	0.0026

Table 7.3 ANOVA table for sulfuric acid pretreatment percent glucan solubilization model using class statement

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	903.752693	53.161923	1.66	0.0998
Error	35	1119.022431	31.972069		
Corrected Total	52	2022.775125			

R-Square	Coeff Var	Root MSE	glucose_red Mean
0.446789	72.08885	5.654385	7.843633

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Temp	1	310.6676444	310.6676444	9.72	0.0036
Conc	2	302.2108836	151.1054418	4.73	0.0152
Temp*Conc	2	20.1412057	10.0706029	0.31	0.7318
Time	2	70.9554831	35.4777416	1.11	0.3410
Temp*Time	2	27.6324277	13.8162138	0.43	0.6525
Conc*Time	4	72.1227558	18.0306889	0.56	0.6904
Temp*Conc*Time	4	100.0222930	25.0055733	0.78	0.5445

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Temp	1	264.6777941	264.6777941	8.28	0.0068
Conc	2	336.3765692	168.1882846	5.26	0.0101
Temp*Conc	2	30.3385146	15.1692573	0.47	0.6262
Time	2	80.3435973	40.1717987	1.26	0.2972
Temp*Time	2	33.9744385	16.9872192	0.53	0.5925
Conc*Time	4	74.1378749	18.5344687	0.58	0.6793
Temp*Conc*Time	4	100.0222930	25.0055733	0.78	0.5445

Table 7.4 ANOVA table for sulfuric acid pretreatment percent lignin reduction model using class statement

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	1576.989428	92.764084	8.01	<.0001
Error	35	405.540130	11.586861		
Corrected Total	52	1982.529558			

R-Square	Coeff Var	Root MSE	lignin_red Mean
0.795443	40.83406	3.403948	8.336050

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Temp	1	501.9131957	501.9131957	43.32	<.0001
Conc	2	190.0699684	95.0349842	8.20	0.0012
Temp*Conc	2	69.2683774	34.6341887	2.99	0.0633
Time	2	91.9653561	45.9826781	3.97	0.0280
Temp*Time	2	587.9215600	293.9607800	25.37	<.0001
Conc*Time	4	101.1555925	25.2888981	2.18	0.0912
Temp*Conc*Time	4	34.6953777	8.6738444	0.75	0.5656

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Temp	1	450.3200045	450.3200045	38.86	<.0001
Conc	2	210.7894633	105.3947317	9.10	0.0007
Temp*Conc	2	83.2995409	41.6497705	3.59	0.0380
Time	2	99.9832220	49.9916110	4.31	0.0211
Temp*Time	2	589.0572233	294.5286116	25.42	<.0001
Conc*Time	4	100.8221508	25.2055377	2.18	0.0921
Temp*Conc*Time	4	34.6953777	8.6738444	0.75	0.5656

Table 7.5 ANOVA table for sodium hydroxide pretreatment percent xylan solubilization model using class statement

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	3181.254856	187.132639	1.06	0.4296
Error	35	6203.885046	177.253858		
Corrected Total	52	9385.139902			

R-Square	Coeff Var	Root MSE	xylose_red Mean
0.338967	47.71834	13.31367	27.90053

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Temp	1	720.9279080	720.9279080	4.07	0.0514
Conc	2	965.3535345	482.6767673	2.72	0.0796
Temp*Conc	2	79.7202414	39.8601207	0.22	0.7998
Time	2	140.9175763	70.4587881	0.40	0.6750
Temp*Time	2	251.7583103	125.8791551	0.71	0.4985
Conc*Time	4	575.7147503	143.9286876	0.81	0.5261
Temp*Conc*Time	4	446.8625351	111.7156338	0.63	0.6442

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Temp	1	735.3362196	735.3362196	4.15	0.0493
Conc	2	960.7171468	480.3585734	2.71	0.0805
Temp*Conc	2	73.3084312	36.6542156	0.21	0.8142
Time	2	130.2153165	65.1076582	0.37	0.6952
Temp*Time	2	253.5561252	126.7780626	0.72	0.4961
Conc*Time	4	490.3516039	122.5879010	0.69	0.6028
Temp*Conc*Time	4	446.8625351	111.7156338	0.63	0.6442

Table 7.6 ANOVA table for sodium hydroxide pretreatment percent glucan solubilization model using class statement

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	1590.522914	93.560171	2.78	0.0052
Error	35	1178.265150	33.664719		
Corrected Total	52	2768.788064			

R-Square	Coeff Var	Root MSE	glucose_red Mean
0.574447	29.10443	5.802131	19.93556

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Temp	1	721.7326144	721.7326144	21.44	<.0001
Conc	2	350.7042876	175.3521438	5.21	0.0105
Temp*Conc	2	159.9784223	79.9892111	2.38	0.1077
Time	2	76.7649651	38.3824826	1.14	0.3314
Temp*Time	2	52.9772440	26.4886220	0.79	0.4632
Conc*Time	4	110.7750284	27.6937571	0.82	0.5196
Temp*Conc*Time	4	117.5903521	29.3975880	0.87	0.4898

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Temp	1	710.7612888	710.7612888	21.11	<.0001
Conc	2	332.5359674	166.2679837	4.94	0.0129
Temp*Conc	2	163.0438489	81.5219244	2.42	0.1035
Time	2	69.7599148	34.8799574	1.04	0.3655
Temp*Time	2	55.3956887	27.6978444	0.82	0.4475
Conc*Time	4	109.4026956	27.3506739	0.81	0.5258
Temp*Conc*Time	4	117.5903521	29.3975880	0.87	0.4898

Table 7.7 ANOVA table for sodium hydroxide pretreatment percent lignin reduction model using class statement

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	5650.627249	332.389838	43.70	<.0001
Error	35	266.217158	7.606205		
Corrected Total	52	5916.844407			

R-Square	Coeff Var	Root MSE	lignin_red Mean
0.955007	5.510744	2.757935	50.04651

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Temp	1	129.192955	129.192955	16.99	0.0002
Conc	2	4731.863276	2365.931638	311.05	<.0001
Temp*Conc	2	15.936946	7.968473	1.05	0.3615
Time	2	507.269051	253.634525	33.35	<.0001
Temp*Time	2	34.716376	17.358188	2.28	0.1171
Conc*Time	4	230.900563	57.725141	7.59	0.0002
Temp*Conc*Time	4	0.748081	0.187020	0.02	0.9988

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Temp	1	87.093232	87.093232	11.45	0.0018
Conc	2	4705.775854	2352.887927	309.34	<.0001
Temp*Conc	2	16.729509	8.364755	1.10	0.3442
Time	2	500.383523	250.191762	32.89	<.0001
Temp*Time	2	28.240470	14.120235	1.86	0.1713
Conc*Time	4	231.045463	57.761366	7.59	0.0002
Temp*Conc*Time	4	0.748081	0.187020	0.02	0.9988

Table 7.8 ANOVA table for hydrogen peroxide pretreatment percent xylan solubilization model using class statement

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	1855.755258	109.162074	5.76	<.0001
Error	35	663.046083	18.944174		
Corrected Total	52	2518.801341			

R-Square	Coeff Var	Root MSE	xylose_red Mean
0.736761	28.64033	4.352491	15.19707

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Temp	1	437.9632673	437.9632673	23.12	<.0001
Conc	2	131.3870920	65.6935460	3.47	0.0423
Temp*Conc	2	157.3050751	78.6525376	4.15	0.0241
Time	2	556.3742669	278.1871335	14.68	<.0001
Temp*Time	2	238.0706468	119.0353234	6.28	0.0047
Conc*Time	4	94.1566917	23.5391729	1.24	0.3110
Temp*Conc*Time	4	240.4982186	60.1245547	3.17	0.0252

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Temp	1	410.1538850	410.1538850	21.65	<.0001
Conc	2	138.5580722	69.2790361	3.66	0.0361
Temp*Conc	2	159.1027298	79.5513649	4.20	0.0232
Time	2	496.1050830	248.0525415	13.09	<.0001
Temp*Time	2	253.1613169	126.5806585	6.68	0.0035
Conc*Time	4	95.4451271	23.8612818	1.26	0.3043
Temp*Conc*Time	4	240.4982186	60.1245547	3.17	0.0252

Table 7.9 ANOVA table for hydrogen peroxide pretreatment percent glucan solubilization model using class statement

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	888.274777	52.251457	2.26	0.0203
Error	35	809.019423	23.114841		
Corrected Total	52	1697.294200			

R-Square	Coeff Var	Root MSE	glucose_red Mean
0.523348	30.83867	4.807790	15.59013

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Temp	1	102.6146481	102.6146481	4.44	0.0424
Conc	2	79.4694621	39.7347310	1.72	0.1940
Temp*Conc	2	101.1200636	50.5600318	2.19	0.1273
Time	2	270.8785786	135.4392893	5.86	0.0064
Temp*Time	2	83.7517054	41.8758527	1.81	0.1784
Conc*Time	4	70.9513807	17.7378452	0.77	0.5537
Temp*Conc*Time	4	179.4889384	44.8722346	1.94	0.1253

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Temp	1	89.4078602	89.4078602	3.87	0.0572
Conc	2	88.0595826	44.0297913	1.90	0.1640
Temp*Conc	2	104.6635609	52.3317805	2.26	0.1189
Time	2	243.2761068	121.6380534	5.26	0.0100
Temp*Time	2	92.4409988	46.2204994	2.00	0.1506
Conc*Time	4	73.2972784	18.3243196	0.79	0.5379
Temp*Conc*Time	4	179.4889384	44.8722346	1.94	0.1253

Table 7.10 ANOVA table for hydrogen peroxide pretreatment percent lignin reduction model using class statement

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	2809.406867	165.259227	10.44	<.0001
Error	36	569.954031	15.832056		
Corrected Total	53	3379.360898			

R-Square	Coeff Var	Root MSE	lignin_red Mean
0.831343	29.02037	3.978952	13.71089

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Temp	1	284.125656	284.125656	17.95	0.0002
Conc	2	1636.581305	818.290652	51.69	<.0001
Temp*Conc	2	203.797178	101.898589	6.44	0.0041
Time	2	198.649837	99.324918	6.27	0.0046
Temp*Time	2	259.685450	129.842725	8.20	0.0012
Conc*Time	4	124.617778	31.154445	1.97	0.1204
Temp*Conc*Time	4	101.949663	25.487416	1.61	0.1929

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Temp	1	284.125656	284.125656	17.95	0.0002
Conc	2	1636.581305	818.290652	51.69	<.0001
Temp*Conc	2	203.797178	101.898589	6.44	0.0041
Time	2	198.649837	99.324918	6.27	0.0046
Temp*Time	2	259.685450	129.842725	8.20	0.0012
Conc*Time	4	124.617778	31.154445	1.97	0.1204
Temp*Conc*Time	4	101.949663	25.487416	1.61	0.1929

Table 7.11 ANOVA table and parameter estimates for sulfuric acid pretreatment percent xylan solubilization using quadratic model

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	46233.93405	9246.78681	160.54	<.0001
Error	47	2707.12466	57.59840		
Corrected Total	52	48941.05871			

R-Square	Coeff Var	Root MSE	xylose_red Mean
0.944686	17.72489	7.589361	42.81754

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Conc	1	4530.32032	4530.32032	78.65	<.0001
Time	1	1762.02163	1762.02163	30.59	<.0001
Temp	1	38171.40832	38171.40832	662.72	<.0001
Conc*Conc	1	398.30870	398.30870	6.92	0.0115
Conc*Temp	1	1371.87508	1371.87508	23.82	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Conc	1	28.093642	28.093642	0.49	0.4884
Time	1	1762.021631	1762.021631	30.59	<.0001
Temp	1	3390.328791	3390.328791	58.86	<.0001
Conc*Conc	1	428.035646	428.035646	7.43	0.0090
Conc*Temp	1	1371.875084	1371.875084	23.82	<.0001

Parameter	Estimate	Standard Error	t Value	Pr > |t|
Intercept	-122.9901138	16.76880896	-7.33	<.0001
Conc	-11.4500507	16.39489309	-0.70	0.4884
Time	0.2332025	0.04216312	5.53	<.0001
Temp	1.1432757	0.14901681	7.67	<.0001
Conc*Conc	-12.2702396	4.50109598	-2.73	0.0090
Conc*Temp	0.5451015	0.11169282	4.88	<.0001

Table 7.12 ANOVA table and parameter estimates for sodium hydroxide pretreatment percent lignin reduction using quadratic model

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	49814.32757	9962.86551	256.96	<.0001
Error	47	1822.25618	38.77141		
Corrected Total	52	51636.58375			

R-Square	Coeff Var	Root MSE	xylose_red Mean
0.964710	14.32973	6.226669	43.45279

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Temp	1	40574.87914	40574.87914	1046.52	<.0001
Conc	1	4798.56198	4798.56198	123.77	<.0001
Time	1	2264.59933	2264.59933	58.41	<.0001
Conc*Conc	1	315.79447	315.79447	8.15	0.0064
Temp*Conc	1	1860.49266	1860.49266	47.99	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Temp	1	3024.498386	3024.498386	78.01	<.0001
Conc	1	110.154642	110.154642	2.84	0.0985
Time	1	2264.599328	2264.599328	58.41	<.0001
Conc*Conc	1	346.830109	346.830109	8.95	0.0044
Temp*Conc	1	1860.492658	1860.492658	47.99	<.0001

Parameter	Estimate	Standard Error	t Value	Pr > |t|
Intercept	-117.6193920	13.75792082	-8.55	<.0001
Temp	1.0798334	0.12226041	8.83	<.0001
Conc	-22.6727809	13.45114263	-1.69	0.0985
Time	0.2643767	0.03459261	7.64	<.0001
Conc*Conc	-11.0451463	3.69291118	-2.99	0.0044
Temp*Conc	0.6347958	0.09163805	6.93	<.0001