Acid and enzymatic hydrolysis of autohydrolyzed lignocellulosic substrates
by David Allen Lamar

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Chemical Engineering
Montana State University

Abstract:
Four biomass residues (barley straw, wheat straw, lodge pole pine, and Douglas fir) were analyzed for effects of an autohydrolysis pretreatment on lignin extractibility and cellulose hydrolysis, both acid and enzymatic catalyzed. A pure cellulose substrate, Chromedia, was also used for hydrolysis tests.

The conditions used for the autohydrolysis were 205 °C and 10 minutes. These conditions were those found to be optimal for lignin extractibility from wheat straw during previous work at this laboratory.

The extractibility of lignin (by an ethanol-water solvent) following pretreatment was very similar for barley straw and wheat straw. A total of about 75% of the lignin was removed during the autohydrolysis and subsequent solvent extraction. The amounts of lignin removed from the two woods were also very similar with about 30% of the lignin being removed.

Experiments were performed to determine the effects of lignin content and substrate morphology on acid hydrolysis of lignocelluloses. These experiments involved hydrolyses on lignin-free substrates and substrates containing lignin. The delignification procedure resulted in a substrate that was no more hydrolyzable than a non-pretreated substrate with the lignin intact. Acid hydrolyses on ball-milled Chromedia revealed that as the amorphous content of the substrate increases the rate of hydrolysis also increases.

Pretreated and non-pretreated substrates were hydrolyzed using both sulfuric and hydrochloric acids and enzymes to determine the pretreatment effect on degree of hydrolysis. Pretreatment of wood substrates resulted in only slightly increased carbohydrate conversion via acid hydrolysis over that observed for non-pretreated woods. No increase in hydrolysis rate was observed for pretreated straw substrates when acids were used as the catalytic agents. When mixed enzymes were substituted for acids in wheat straw hydrolysis, the cellulose conversions increased dramatically for pretreated substrates, with values in excess of 90% observed.

A theory based upon the solubility of reaction products is presented to explain higher cellulose conversions with mixed enzymes versus acid hydrolysis results. This theory leaves open the possibility that autohydrolysis pretreatment renders all substrates investigated more subject to hydrolysis.

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APPROVAL

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David Allen Lamar

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citation, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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Date May 27, 1987

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ABSTRACT

Four biomass residues (barley straw, wheat straw, lodge pole pine, and Douglas fir) were analyzed for effects of an autohydrolysis pretreatment on lignin extractibility and cellulose hydrolysis, both acid and enzymatic catalyzed. A pure cellulose substrate, Chromedia, was also used for hydrolysis tests.

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The extractibility of lignin (by an ethanol-water solvent) following pretreatment was very similar for barley straw and wheat straw. A total of about 75% of the lignin was removed during the autohydrolysis and subsequent solvent extraction. The amounts of lignin removed from the two woods were also very similar with about 30% of the lignin being removed.

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INTRODUCTION

The realization that petroleum supplies are not limitless has awakened the U.S. to the need of an alternative hydrocarbon source for fuel and chemical feed-stocks. A search has been focused on determining a hydrocarbon source that does not compete with food supplies or other valuable raw materials. An ideal source of hydrocarbon might be waste or by-product biomass from agricultural operations or wood product industries. The utilization of this biomass would benefit the above industries by converting low value materials into more valuable commodities.

The energy requirement of the U.S. is roughly 76 quadrillion BTU's (Quads) per year. The generation of this energy would require 40 million barrels of oil per day. A major goal is to replace expensive petroleum used in energy production with low cost biomass [1].

Presently, two to three Quads of the U.S. energy requirements are supplied by biomass utilization. Combustion of forest products is the primary source for this energy. A conservative estimate of the energy that will be supplied by utilization of biomass by the end of this century is 15 Quads [1].

Biomass for energy could be supplied from direct and indirect sources. Direct sources might include farms developed to grow plants solely for energy uses, while indirect sources would include agricultural and forest waste or by-products mentioned above. The indirect sources are of primary interest in this study.

It is estimated that 278 million dry tons of agricultural by-products and 108 million dry tons of unused mill and logging residues are produced annually [1]. Theoretically, if these materials were converted to glucose and the glucose fermented to alcohol, approximately 30 billion gallons of ethanol could be produced per year. This ethanol would meet the entire current industrial demand and provide ethanol for gasoline blending

as well. Most of the present industrial grade ethanol is now produced from petroleum-based feedstocks. This use of biomass would therefore reduce the demand for petroleum [1].

The above estimate of ethanol from lignocellulose requires a 90 percent conversion of the cellulose to glucose. Barriers exist in lignocelluloses that prevent such high conversion of cellulose to glucose. Existing technologies only provide about a 50 percent cellulose conversion. A higher conversion is desirable to economically produce cellulose-derived products.

Research Objectives

The first objective of this investigation is to test three lignocellulosic substrates for degree of lignin removal and enhanced hydrolysis of their cellulose to glucose after a novel pretreatment. The substrates to be investigated are barley straw, lodge pole pine, and Douglas fir. These materials will be pretreated by autohydrolysis at conditions found to be optimum for lignin removal from wheat straw during previous work at this laboratory.

The second objective is to investigate reasons that might explain the low acid hydrolysis yields observed with pretreated wheat straw. Meeting this objective entails applying new hydrolytic catalysts and/or conditions to the hydrolyses in an attempt to increase yields.

STRUCTURE OF LIGNOCELLULOSE

Forest and agricultural residues consist of several primary components. These components include cellulose, hemicellulose, lignin, protein, and miscellaneous extractibles. Table 1 summarizes the percent composition of individual components by weight.

Table 1. Major Component Composition of Lignocellulose [2].

Component	% Composition
Cellulose	45-50
Hemicellulose	20-25
Lignin	20-30
Extractible and Protein	0-10

Together these components make the basic structural unit of biomass, the plant cell. A cell, in simple terms, is composed of two basic parts, the lumen and the cell wall. The lumen contains the living matter of the cell. Once dead the cell's lumen is either void space or filled with extractibles [2]. The cell wall serves as a mechanical divider between individual cells. Rigidity of a plant stalk is the direct result of its cell walls.

The cell wall also consists of two parts, the primary wall and the secondary wall. The primary wall is very thin in comparison with the secondary wall. The secondary wall consists of three distinct layers termed the outer (S_1), middle (S_2) and inner (S_3) layers [3]. Surrounding the primary wall and separating adjacent cells is the middle lamella.

Figure 1 [1] is a representation of the basic structure of a plant cell. Cellulose is mainly in the microfibrils, shown as lines in the diagram. Orientation of the fibrils is different in the respective portions of the cell wall.

The amount of cellulose in the plant is highest in the secondary wall and decreases toward the middle lamella. Hemicellulose has its

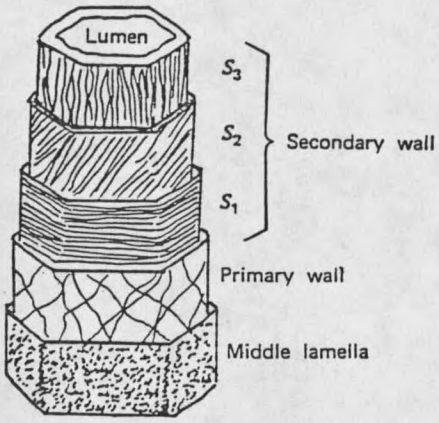

Figure 1. Basic Structure of a Plant Cell [1].

highest percentage in the middle lamella and decreases toward the lumen. Hemicellulose and lignin form a matrix that surrounds the cellulose fibrils. Lignin and hemicellulose are also found in the spaces between the crystalline regions of the microfibrils, the amorphous (non-crystalline) regions [2].

Cellulose

Cellulose is a linear polymer of D-anhydroglucose molecules serving as the monomer. These monomers are linked by β-1-4-glucosidic bonds. Figure 2 is a schematic of a cellulose molecule. Cellulose degree of polymerization ranges from 3,500 to 14,000 glucose units when in a native form. The average length of cellulose molecules range from 2,500 nm to 5,000 nm [2,5].

The linear molecules lay one on another forming bundles of molecules, fibrils, that are held together by lateral hydrogen bonding. The large number of hydrogen bonds result in crystalline regions of about 60 nm in length that comprise 67 to 90% of the cell wall. Since the cellulose molecule is longer than 60 nm, the molecules pass through several crystalline and amorphous regions [2]. Fibrils are surrounded by a sheath of hemicellulose and lignin [5].

The apparent morphology of cellulose depends on the methods of analysis and also the source of the cellulose. At present cellulose is

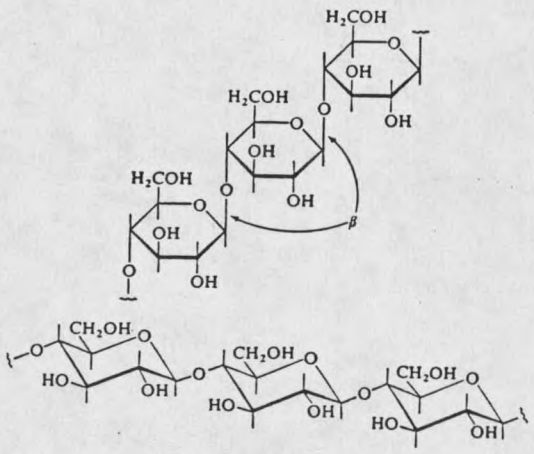

Figure 2. Chemical Structure of Cellulose.

categorized into four distinct types, cellulose I, II, III, and IV. Each group is based on the aggregation of molecules in the crystalline solid. Degree of crystallinity decreases with cellulose type (i.e., Cellulose I is more crystalline than Cellulose II, and so forth). The class to which a particular cellulose belongs depends on the method used to produce the pure cellulose. The class is identified by the x-ray diffraction pattern of the sample [4].

Cellulose in its native form is classed Cellulose I. Cellulose II is a cellulose that has been regenerated from solution at ambient temperatures or one that has been mercerized with caustic solution in excess of 15% sodium hydroxide. Cellulose I and II are the most common morphologies. Treatment of cellulose with anhydrous ammonia or one of several different amines produces cellulose III. Heat treatment of cellulose II or regeneration of cellulose from solutions at elevated temperatures results in cellulose IV [4].

Hemicellulose

Hemicellulose is a polymer of simple sugar molecules like cellulose, though it consists of more than one type of sugar. The backbone of the polymer is a linear chain containing D-xylose sugar units linked together by β-1-4-glucosidic bonds. Unlike cellulose, hemicellulose is not a linear homopolymer. It contains side chains branching from

the main chain of D-xylose sugars. The branches are usually bonded to the xylose molecules via 1-3 glycosidic links, but they can also contain 1-4 and 1-6 glycosidic bonds. The side chains can contain glucose, xylose, galactose, mannose, arabinose, and uronic acids of glucose and galactose. Composition of a particular hemicellulose varies from source to source, not only with plant species, but also with climate and location of the particular plant. Degree of polymerization of hemicellulose ranges from 100 to 200 molecules but rarely exceeds 200 [1,2].

Hemicellulose does not form crystals like cellulose and is found only in an amorphous state. It is usually in intimate contact with plant lignins. It is thought that the hemicellulose and lignin are chemically bonded together.

Lignin

Lignin is a highly complex, three-dimensional polymer of various phenolic acids connected by ether linkages. Unlike the other components discussed so far, lignin has no set pattern of structure. Figure 3 [1] presents a possible structure of lignin.

Lignin acts as the cement that holds the cellulose fibrils together. It is an integral part of a system that gives plants their strength and rigidity. This polymer not only acts as a cement but also as a protective shield against elements that would otherwise destroy the plant by attacking the cellulose. Investigations have shown that lignin can limit the microbial degradation of plant polysaccharides [6].

Structure as It Relates to Hydrolysis

A lot of work has been done to determine how the chemical and physical structure of lignocellulose inhibits hydrolysis of the carbohydrate components. The following is an overview of this work and some of the conclusions that have been drawn.

Many different aspects of plant structure and chemical make-up

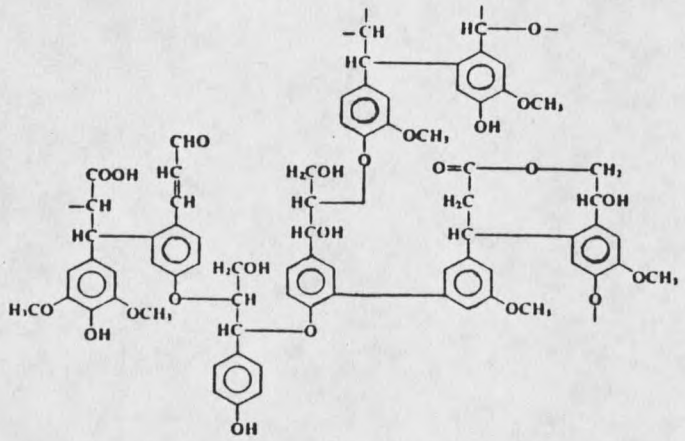

Figure 3. Structure of a Portion of Lignin [1].

have been identified as detrimental to the hydrolysis of biomass. These are the hemicellulose-lignin barrier, crystallinity of the cellulose, surface area, degree of polymerization of cellulose, and pore size distribution. Some of these properties act synergistically to prevent hydrolysis. The ramifications of individual aspects will be discussed here.

Hydrolysis of cellulose can be catalyzed by either acids or enzymes. Although chemically the results are the same, the mechanisms are quite different. A successful acid catalyzed hydrolysis (see Figure 4) takes place when the oxygen atom of the β-1,4-glycosidic bond is attacked by a hydrogen ion. This attack results in a positive charge on the oxygen which then pulls electrons from the oxygen-carbon bond resulting in a partial positive charge on the carbon atom. Non-bonding electrons of a water oxygen atom are attracted to this partial positive charge. Electrons from the original carbon-oxygen bond form a bond with the attacking hydrogen ion, and hydrogen-oxygen bonding electrons from the water molecule form a bond between the carbon atom and the attacking oxygen atom releasing a hydrogen atom. This series of events results in the breaking of the β-1,4-glycosidic bond by the addition of a water molecule in between two glucose monomers of the cellulose molecule. A proposed mechanism for an enzyme catalyzed hydrolysis is presented later in this work.

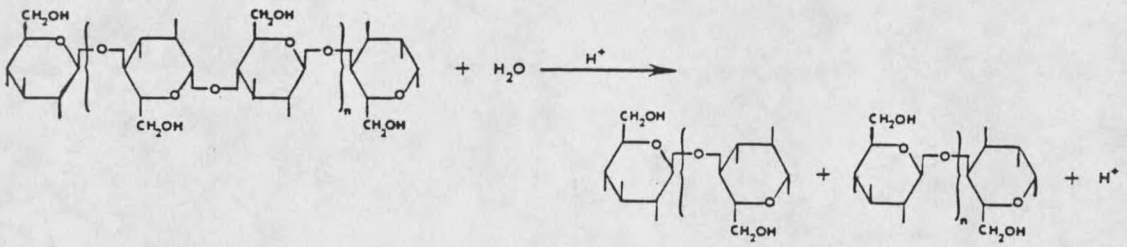

Figure 4. An Acid Catalyzed Hydrolysis Reaction.

Hemicellulose and lignin are believed to be chemically bonded in biomass. These two components form a sheath that surrounds and protects the cellulose [3]. The lignin-hemicellulose barrier prevents contact of hydrolytic agents (such as enzymes and acid) and cellulose molecules, thus preventing hydrolysis. Removing either hemicellulose, lignin, or both, improves access to the cellulose increasing the hydrolizability of the cellulose [7,8,9,10,11].

It has been proposed that cellulytic enzymes irreversibly attach themselves to lignin molecules. This adsorption removes the affected enzymes from the reaction mixture [12]. Increasing enzyme charge to the hydrolysis mixture only increases hydrolysis up to a point. This implies that enzyme adsorption is not the only inhibitory mechanism of lignin.

Several researchers have shown that some aromatic compounds can chemically inhibit enzymatic hydrolysis of hemicellulose [13]. Aromatic compounds having an inhibitory affect are products derived from the breakdown of lignin molecules or hot water extracts of lignocellulose. As an example, wheat extract normally contains p-coumaric acid, ferulic acid, and vanillic acid [14]. These compounds have been shown to be detrimental to digestion, and hence enzymatic hydrolysis, of cellulose in rumenic animals [6].

The hemicellulose-lignin barrier is not the only characteristic preventing conversion of cellulose to glucose. Evidence of this can be

ascertained from the hydrolysis of cotton. Cotton contains virtually no lignin and only small amounts of hemicellulose [3]. The lack of lignin and low amounts of hemicellulose should render cotton cellulose highly susceptible to hydrolytic attack by either enzymes or acids. Several investigators in Italy have shown that this is not the case. After a 24-hour enzymatic hydrolysis they found only a 22% conversion of cellulose to sugar [15]. This indicates that the hemicellulose-lignin barrier is not always the major inhibitor of hydrolysis.

Saddler et al. [8] and Fan et al. [16] have studied the effects of cellulose crystallinity on degree of hydrolysis. They separately reported that crystallinity may represent a major block to cellulose conversion. They found that highly crystalline cellulose resists hydrolysis, whereas amorphous cellulose is readily hydrolyzed. Their experiments show that as a hydrolysis proceeds, the unhydrolyzed substrate crystallinity increases. This suggests that amorphous regions of cellulose are preferentially hydrolyzed leaving crystalline substrate intact. As the crystallinity increases, the rate of conversion slows [10,16]. Although these researchers have placed crystallinity high on the list of barriers to hydrolysis, they agree that it is not the greatest block to hydrolysis [8,10]. Their data correlations seem to indicate that surface area has the largest affect on hydrolysis.

Grethlein [17] has suggested that the enzyme-catalyzed hydrolysis of cellulose is not dependent on the crystallinity in cellulose. He believes surface area is the most important characteristic contributing to the rate of hydrolysis. The enzymatic hydrolysis for a given surface area is directly dependent on the pore size distribution. There may be a large surface area, but pore size may limit the amount of surface accessible to enzymatic attack. Grethlein discusses a pore size of 51 Å based on the size of hydrolytic enzymes.

Acid molecules are generally very small in comparison with enzyme molecules. Therefore, pore size limitations should not have as great an effect on the rate of acid hydrolysis. Several researchers at Dartmouth College have found the rate constants for acid hydrolysis of

cellulose to glucose for a wide variety of substrates. These constants are all within the same order of magnitude at a given temperature and acid concentration. The substrates varied in crystallinity, surface area, and pore size distribution [18].

Degree of polymerization of cellulose is a characteristic that some believe has an effect on hydrolysis. Their belief is that long chain molecules are less accessible to hydrolytic agents than are shorter chain molecules. This lowered access to the glucosidic bonds of cellulose results in slowed hydrolysis rates and less conversion. These researchers have shown that as chain length increases, hydrolysis rate decreases [19].

There is a general consensus that the hemicellulose-lignin barrier slows the hydrolysis of cellulose, especially when enzymes are used as a catalyst. A disagreement exists pertaining to the degree that crystallinity and surface area effects cellulose hydrolysis. The purpose of this research study was to investigate the effect of a pretreatment on degree and ease of conversion of cellulose to glucose. This researcher was aware of crystallinity and surface area as inhibitory factors, but the degree of individual importance was not investigated.

PRETREATMENTS TO ENHANCE HYDROLYSIS

Different treatments prior to a hydrolysis have been studied to effect an increase in the susceptibility of lignocellulose to hydrolytic agents. These pretreatments can be chemical or physical in nature. Physical pretreatments are primarily milling or grinding operations that reduce particle size. Chemical pretreatments involve subjecting the lignocellulose to a chemical agent. Some pretreatments involve a combination of physical and chemical elements.

A novel method of using physical treatments simultaneously with hydrolysis has been studied at the University of Montana. Their process involved a simultaneous wet ball milling and enzymatic hydrolysis of various cellulose substrates. This process continuously produces active sites for hydrolytic agents to attack [7]. Although physical pretreatments may enhance the hydrolysis of lignocelluloses, they are very energy intensive operations. High energy consumption makes the economic feasibility of this type of pretreatment doubtful for commercialization. Therefore, the primary work of developing a pretreatment process has been focused on chemical pretreatments. Some studies are looking at combinations of chemical and physical disruption of lignocellulosic structure.

As stated above, chemical pretreatments involve subjecting lignocellulose to a chemical agent. Some of the more common agents are solutions of acids or alkali, organic solvents, and hot water, both liquid and vapor. A primary advantage of chemical pretreatments, over physical pretreatments, is the ability of some chemicals to remove lignin from the substrate. Chemical treatments can also swell the carbohydrate-lignin matrix, thus decreasing the mass transfer limitations in hydrolysis.

Alkaline solutions (of which NaOH is the most common) are thought to increase hydrolysis by swelling the substrate. Although water can swell lignocellulose, it does so primarily by entering regions between crystalline units. Sodium Hydroxide expands the cellulose matrix by

breaking bonds in the crystalline units of the cellulose resulting in an increase in the amount of amorphous cellulose [20]. Gharpuray et al. have shown that caustic removes both lignin and hemicellulose in addition to its swelling action [10]. This component removal allows greater access to the cellulose molecules by increasing surface area and pore size. One problem with caustic pretreatments is the consumption of alkali during the pretreatment. This loss of reagent detracts from the economics of a process using caustic pretreatments [21].

Dilute acid pretreatments at elevated temperatures have been shown to increase the hydrolysis rate of the pretreatment residue. This type of pretreatment could be called a prehydrolysis since it hydrolyzes the hemicellulose fraction, thus removing it from the matrix. Since the hemicellulose is removed, lignin-hemicellulose bonding no longer exists. This condition allows the lignin to be removed by solvation if so desired. Grethlein et al. noted however that delignification was unnecessary [18]. The disruption of the carbohydrate-lignin matrix by dilute acid was sufficient to render the residue highly susceptible to an enzymatic hydrolysis. Acid pretreatments increase the pore volume allowing greater penetration of cellulase enzyme molecules into pretreated substrates. The increased access of enzymes results in higher rates of cellulose to glucose conversion.

Engineers at the University of Arkansas have studied the use of moderate temperature, dilute acid prehydrolysis in an acid hydrolysis process. This pretreatment hydrolyzes the hemicellulose without substantial product degradation. The prehydrolysis is followed by a concentrated acid hydrolysis of the cellulose residue. The easily hydrolyzed hemicellulose fraction would be largely converted to its degradation product, furfural, if only a concentrated acid step was used. Furfural and 5-hydroxymethyl furfural (HMF), the acid catalyzed degradation products of glucose, are poisonous to yeast cells that would ferment sugars to ethanol or other products [22].

A joint project between the University of Pennsylvania and General Electric Corporation has developed a solvent delignification pretreatment. This pretreatment involves a hot aqueous ethanol treatment of a

substrate. This results in a solid consisting of cellulose, partially degraded hemicellulose, and lignin [23]. This residue is then enzymatically hydrolyzed and results in cellulose conversions of 80 to 90% [1].

The pretreatment schemes that seem most promising are those that involve a steam or high temperature water treatment. These processes are known as autohydrolyses. The term autohydrosis comes from the fact that in the presence of water at an elevated temperature acetyl groups in hemicellulose (are thought to) break down and form acetic acid. Therefore, an automatic acid catalyzed hydrolysis takes place in the pretreatment mixture. During an autohydrolysis hemicelluloses are hydrolyzed and the lignin is rendered into a form that is easily solvated. A benefit of autohydrolysis is that considerable amounts of lignin can be removed while the hemicellulose is simultaneously hydrolyzed [24]. Rupture of the protective lignin-cellulose linkage is sufficient to allow a relatively easy hydrolysis of the cellulose. The residual cellulose from autohydrolysis can then be enzymatically converted to glucose with yields of 90 to 95% [25].

A pretreatment related to autohydrolysis is the steam explosion of lignocellulose. In this process a substrate is subjected to high pressure steam (≈500 psig), cooked at this temperature for a short time (≈5 seconds), and then the mixture is flashed to atmospheric pressure. This explosive flashing greatly disrupts the structure of the cellulose increasing the pore volume and surface area of the substrate. A process that uses this pretreatment is the Iotech process. In this procedure, the steam explosion is followed by an enzymatic hydrolysis with cellulose conversions of about 90% and hemicellulose conversions of approximately 80%. The lignin need not be extracted from the substrate but can be left to be removed at the end of the hydrolysis.

Of all the pretreatments and processes discussed here, the use of a dilute acid pretreatment, autohydrolysis, or the steam explosion technique of the Iotech process seem the most commercially promising.

EXPERIMENTAL

This section describes the substrates that were used and the experimental procedures that were followed during this study.

Substrates

Substrates used for this investigation were wheat straw, barley straw, lodge pole pine chips, Douglas fir chips, and Chromedia. Wheat straw was a spring wheat of the Pondera variety and the barley straw was Clark's barley. Two bales of each of these substrates were obtained from Larry Van Dyke of Manhattan, Montana. The straws were harvested in November of 1982. Lodge pole chips were supplied by the Brand-S Lumber Company of Livingston, Montana. Willow Creek Lumber Company, also of Livingston, provided the Douglas fir chips. The chips of both varieties of woods were sawmill residues from the harvesting of live timber in December of 1983. It was approximately four weeks between the timber harvest and the acquisition of the chips. All four of the above substrates were stored in sealed plastic bags until used. A fifth substrate, Whatman Chromedia, was used in several experiments. This material was essentially a pure cellulose in powdered form designed for use as a column packing in chromatography. The Chromedia was manufactured by W & R Balston, LTD of England.

All of the substrates, except the Chromedia, were prepared for use as follows. First, the materials were air dried for approximately 48 hours. Next, the substrates were milled in a Wiley hammer mill equipped with a 1-mm discharge screen. The mill was stopped periodically and allowed to cool to prevent overheating of material being ground so that substrate would retain its natural integrity. The milled material was then screened to isolate the 35 to 60 mesh fraction. All ASTM and TAPPI standard methods required this size fraction for analysis. This fraction was used in all subsequent characterizations and experiments. After grinding and screening, the substrates were stored in containers open to the atmosphere.

Characterization of Substrates

Substrates used for this research were characterized before pretreatment experiments were performed. The materials were characterized by relative amounts of ash content, moisture, extractibles, lignin, cellulose and hemicellulose.

Ash

The ash content in the substrates was determined using a slight variation in the ASTM D 1102-56 Standard Test Method for Ash in Wood [26]. This procedure involved weighing about 2 grams of the substrate in a tared, previously ignited (at 600°C) porcelain crucible and lid. The material was weighed to the nearest 0.1 milligram. The crucible and detachable lid were placed in an ashing oven with a starting temperature below 100°C. The oven was slowly heated to the 600°C ashing temperature and allowed to remain there for 30 minutes. At the end of this period the lid was placed on the crucible and the crucible was then transferred to a drying oven to cool. (The drying oven temperature was maintained between 105°C and 110°C.) The crucibles were cooled further in a desiccator and weighed to the nearest 0.1 milligram. The percent ash was corrected for moisture in the initial sample and reported to the nearest 0.01%.

Moisture

The moisture analysis used ASTM D 1102-56 Standard Test Method for Ash in Wood [26]. This test specifically analyzes for ash content but it also describes a procedure for determining moisture content. The standard method entailed weighing a sample of substrate to the nearest 0.1 milligram using a tared glass weighing vial. The vial containing the sample was placed in an oven controlled between 105°C to 110°C. Two different procedures were used from this point.

The first procedure followed the standard method by drying the material for 2 hours and then transferring the vial unstoppered to a desiccator. The vial was allowed to cool and was then stoppered and reweighed to the nearest 0.1 milligram. The vial was unstoppered and

returned to the oven for one hour, then cooled in the desiccator and reweighed. This procedure was repeated until a constant weight was obtained.

It was found that after about four hours of oven drying the samples had reached a constant weight. Therefore, for convenience a second method of moisture content was used. For this method, the vial was kept in the oven overnight (eight to twelve hours), then transferred from the oven to a desiccator for cooling. When the vial was cool, it was stoppered and weighed to the nearest 0.1 milligram.

With either method the amount of moisture in the sample was calculated as the weight loss between the dried and the undried samples. The moisture is reported to the nearest 0.1%.

Extractibles

The extractibles were defined as the components in the lignocelluloses soluble in benzene-ethanol, ethanol, and water. These components are gums, resins, waxes, and in some substrates, catechol. The test for extractibles used TAPPI T 12 os-75, Preparation of Wood for Chemical Analysis (Including Procedures of Removal of Extractives and Determination of Moisture Content) [27].

Approximately 80 grams of the substrate was weighed to the nearest 0.01 gram. The substrate was then placed into a 250 ml Soxhlet extraction thimble with a coarse fritted glass disc. This thimble was placed in a Soxhlet extraction apparatus and the solvent reservoir charged with 350 ml of benzene and 175 ml of 95% ethanol. The reservoir was placed in a Variac-controlled heating mantle. The Variac was adjusted so the boil-up rate of the extraction solvent resulted in 6 to 7 refluxes per hour. The extraction was continued for 6 hours. At the end of this period, the substrate was suctioned to remove as much of the solvent as possible. The substrate was then washed with fresh ethanol and suctioned dry.

A second extraction was performed by charging the reservoir with 95% ethanol while maintaining 6 to 7 refluxes per hour as before. This

extraction only proceeded for 4 hours. The substrate was then suctioned dry and washed with deionized water. At this time the sample was split into two similar portions. The two portions were placed in 500 ml Erlenmeyer flasks and 500 ml of boiling deionized water was added. They were kept at 80°C for one hour with frequent stirring via a glass rod. The substrate was filtered from the water extract and allowed to air dry. A moisture analysis was performed on the air dried extracted substrate (extract-free substrate). The amount of extractibles was determined by a simple weight loss between the starting material and extracted material. Extractibles were reported on a moisture-free basis to the nearest 0.1%.

Lignin

The next constituent analyzed for was lignin. The method used was a variation of ASTM D 1104-56 Standard Test Method for Holocellulose in Wood as described by Browning [28]. The method involved weighing about 2.5 grams of the extract-free substrate to the nearest 0.1 milligram. The sample was placed in a 30 ml coarse porosity fritted glass filtering funnel. The funnel was placed in a bath of ice water while chlorine gas was slowly passed through the sample (see Figure 5). After 3 minutes, the chlorine gas flow was interrupted so the sample could be stirred with a glass rod. The chlorine gas flow was then continued for 2 more minutes. Enough 1,4-dioxane was added to the funnel to cover the sample. The ice water was then drained and the dioxane suctioned off. Next the sample was washed twice with a 50°C solution of 5% monoethanolamine in 1,4-dioxane. Each wash was allowed to remain for 2 minutes before being suctioned off. The sample was then washed twice with room temperature 1,4-dioxane.

The sample was transferred to a 60 ml fritted glass Buchner filtering funnel of coarse porosity and washed twice with room temperature deionized water. The sample was then transferred back to the original funnel. This procedure prevented the fritted discs from clogging. The funnel was reassembled into the configuration necessary for use of the chlorine. The gas was passed through the sample for 3

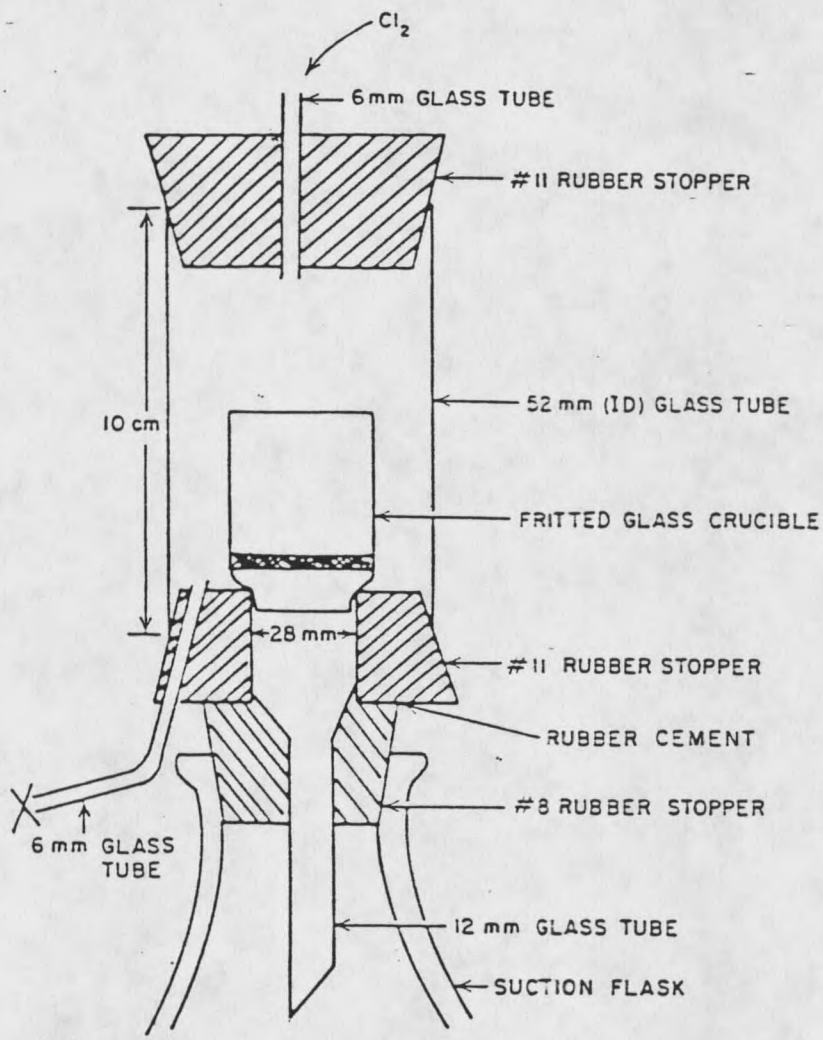

Figure 5. Funnel Setup used for Lignin Determination.

minutes and the washing procedure repeated.

This sequence of chlorinating and washing was continued until the substrate no longer underwent a color change upon monoethanolamine solution addition or until the substrate was white. The color change is that associated with the 5% ethanolamine wash. The sample was washed with dioxane until neutral (≈7 pH) and then washed twice with 30 ml aliquots of diethyl ether. After the ether wash, the sample was washed with deionized water and transferred to a tared Petri dish for air drying. The lignin content was determined as the moisture-free weight loss between the extract-free substrate and the delignified sample.

Cellulose and Hemicellulose

The percentage of total cellulose was determined by assuming that the mass not accounted for by the lignin, moisture, and ash was cellulose.

The procedure used to analyze for the relative amounts of cellulose and hemicellulose was TAPPI standard method T 203 os-74, Alpha-, Beta-, and Gamma-Cellulose in Pulp [29]. It was assumed that the alpha-cellulose fraction was crystalline cellulose, the beta-cellulose was amorphous cellulose, and the gamma-cellulose was hemicellulose.

This method suggests use of 1.5 gm of delignified pulp, weighed to the nearest 0.1 milligram. However, it was not possible to obtain 1.5 gm of pulp during an actual experimental run due to the small amounts of sample that could be generated. The reagents were adjusted for the smaller amount of sample used. The following procedure was based on 1.5 grams of sample.

The sample was placed in a 125 or 250 ml Erlenmeyer flask with 100 ml of 5.21 ± 0.005 N carbonate-free sodium hydroxide (NaOH). The flask was placed on a magnetic stirrer and stirred to insure complete suspension of the pulp in the NaOH. Stirring was done in a manner to insure that no air was drawn into the mixture. The flask was then placed in a water bath with the temperature maintained at 25 ± 0.2°C.

When 30 minutes had passed, 100 ml of deionized water was added to the pulp-NaOH mixture. The diluted mixture remained in the water bath for 30 minutes. The mixture, while in the bath, was stirred occasionally with a glass stirring rod.

After the 60 minute period, the mixture was filtered through a coarse porosity Buchner filtering funnel. The first 10 to 20 ml of the filtrate was discarded and the rest of the filtrate was collected for later analysis.

Alpha-Cellulose Analysis Ten milliliters of the clear filtrate and ten milliliters of 0.5 N potassium dichromate ($K_2Cr_2O_7$) solution were placed in a 250 ml Erlenmeyer flask. Thirty milliliters of concentrated sulfuric acid (H_2SO_4) were slowly added to the mixture while the flask was swirled. The resulting solution was kept hot for 15 minutes. Fifty milliliters of deionized water were then added and the flask was cooled in a room temperature water bath.

A blank was made using 12.5 ml of deionized water and 12.5 ml of the 5.21 N (17.5% by weight) NaOH. Fifty milliliters of concentrated sulfuric acid were slowly added to the blank.

The pulp filtrate and blank were titrated with 0.1 N ferrous ammonium sulfate ($Fe(NH_4)_2(SO_4)_2$) solution with the end point determined potentiometrically. A phenanthroline ($C_{12}H_8N_2$) -ferrous sulfate ($FeSO_4$) ('Ferrion') indicator solution was also used to confirm the potentiometric end point. Ferrous ammonium sulfate solution is unstable and was standardized before each use. Standardization was accomplished by a potentiometric titration with 0.1 N potassium dichromate solution.

The alpha-cellulose percentage was calculated as follows:

$$\text{Alpha-cellulose \%} = 100 - \frac{6.85\ (V_2 - V_1) \times N \times 20}{A \times W}$$

where V_1 = Titration of pulp filtrate, milliliters

V_2 = Blank titration, milliliters

N = Exact normality of the ferrous ammonium sulfate solution

A = Titration of the pulp filtrate used in the oxidation, milliliters

W = Oven-dry weight of the pulp specimen, grams

Beta- and Gamma-Cellulose Analysis Fifty milliliters of the pulp filtrate were pipetted into a 100 ml graduated cylinder equipped with a ground glass stopper. Fifty milliliters of 3 N sulfuric acid were added to the cylinder and with the stopper in place the cylinder was inverted several times to insure complete mixing. The cylinder was placed in an 80°C water bath for several minutes to aid the coagulation of beta-cellulose, which precipitates upon acidification. The cylinder was either allowed to stand overnight or was centrifuged to settle the precipitate.

Fifty milliliters of the clear supernatant liquid was pipitted off, being careful that none of the precipitate was removed. The liquid was placed in a 250 ml Erlenmeyer flask. Ten milliliters of 0.1 N potassium dichromate were added to the flask. Then 90 ml of concentrated sulfuric acid were carefully poured into the flask while swirling. This solution was kept hot for 15 minutes and then cooled in a room temperature water bath. A blank was prepared in the same manner except a solution of 12.5 ml of deionized water, 12.5 ml of 17.5% NaOH, and 25 ml of 3 N sulfuric acid was substituted for the clear supernatant liquid.

These solutions were titrated by the same method used during the alpha-cellulose determination. The relative fractions of beta- and gamma-cellulose were calculated as follows:

$$\text{Gamma-cellulose \%} = 100 - \frac{6.85\ (V_4 - V_3) \times N \times 20}{25 \times W}$$

where V_3 = Titration of the solution after precipitation of beta-cellulose, milliliters

V_4 = Blank titration, milliliters

Beta-cellulose % = 100 -(α-cellulose % + γ-cellulose %).

Autohydrolysis and Lignin Extraction

The autohydrolysis and lignin extraction pretreatments were carried out in an Autoclave Engineers ZipperClave with a one liter capacity. The autoclave has two 500-watt heating bands and was rated to 2000 psi at 450°F. An air driven agitator shaft extended into the pressure vessel to stir the reaction mixture during an experiment. Temperature inside the autoclave was monitored using a Cole Palmer Model 8530 digital thermometer and a Type K (Cromel-Alumel) thermocouple installed in a stainless steel thermowell.

Substrate was held in eight small packets that were made of 200 mesh stainless steel wire cloth fastened with flat pieces of aluminum (Figure 6). These packets were held in a radial arrangement by two metal discs. The aluminum closures were fitted into holes drilled in the discs. The two discs were separated by a 1/2 ID stainless steel pipe (Figure 6). This pipe, with the eight packets of substrate, was mounted on the agitator shaft of the autoclave. The packets could then be rotated during the run to minimize mass transfer limitations that might affect either the autohydrolysis or the lignin extraction.

A 500 ml Parr bomb was plumbed to the autoclave and served as a high pressure steam generator. Plumbing from the bomb to the autoclave was low pressure stainless steel tubing with Swagelock fittings. This line had an Autoclave Engineers low pressure valve with high temperature packing (model number 6V81U4TG) to control the injection of steam. The steam line was heated with heat tape to prevent cooling of the steam during steam injections. Cooling of the autoclave was attained by venting to a cold trap submerged in an ice bath. Venting was controlled by a valve of the same type used in the steam line. Figure 7 is a diagram of this experimental set up.

Autohydrolysis

The tared wire mesh packets were filled with extract-free substrate and weighed to 0.1 milligram. Each packet held about one gram; therefore, approximately eight grams of substrate were pretreated per

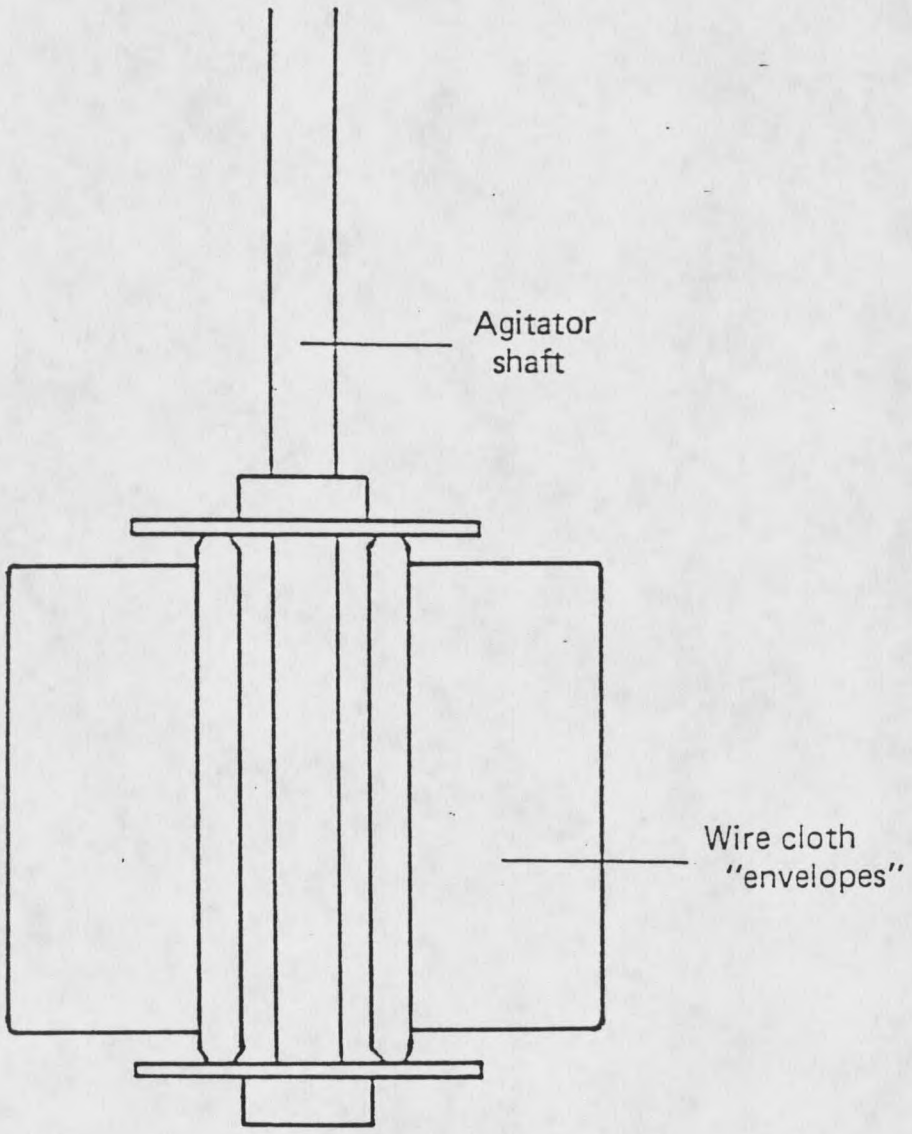

Figure 6. Sample Basket Apparatus.

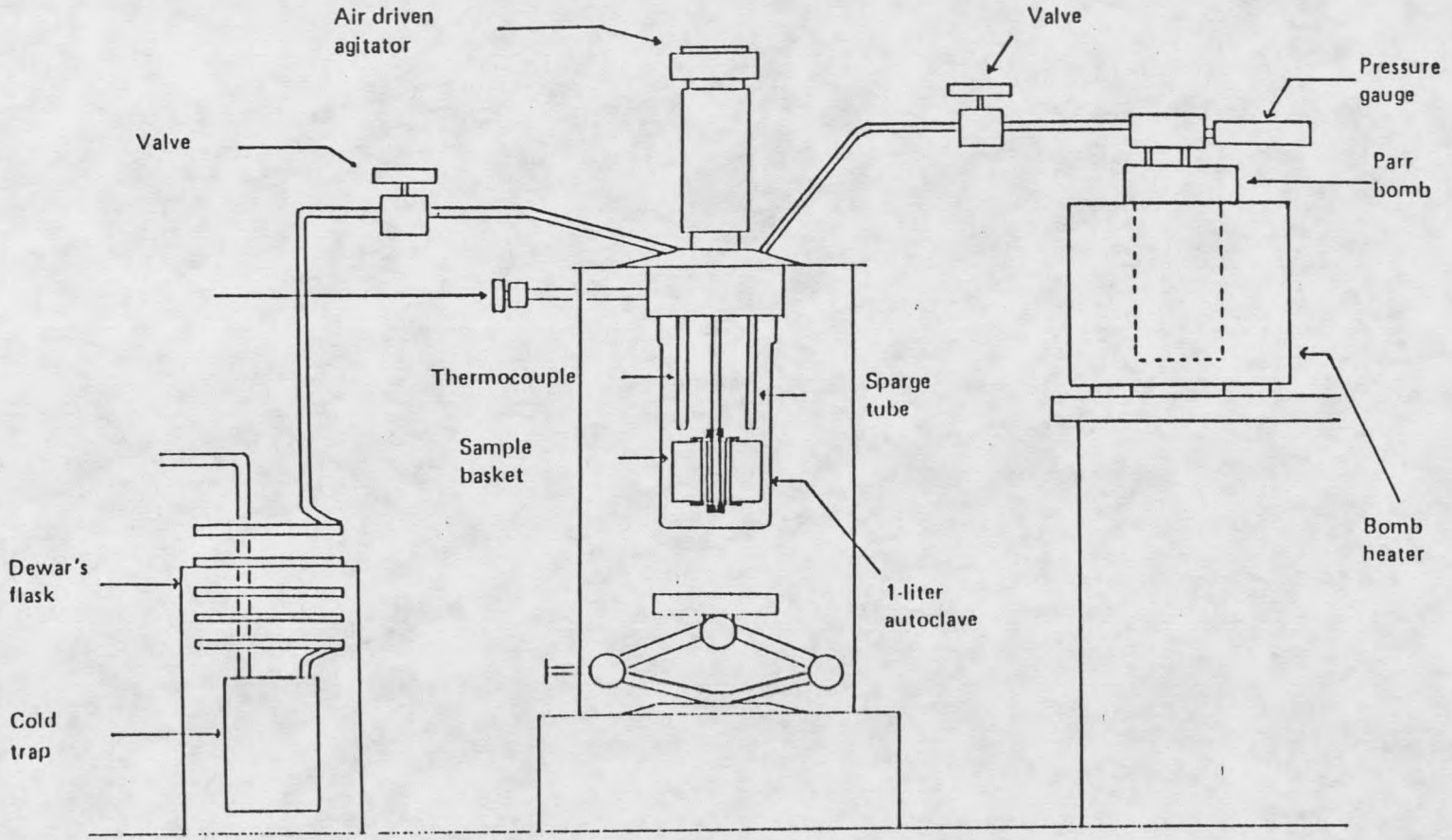

Figure 7. Setup used for Autohydrolysis and Extraction Experiments.

run. The packets were assembled and mounted on the agitator shaft. Either 300 or 350 ml of deionized water were charged to the Parr bomb and heated to a pressure of 1200 psig (1500 psig for some runs). The autoclave was charged with 600 ml of deionized water and heated to 150°C. This volume was required to insure that the sample and the thermowell were submerged. A temperature of 150°C was chosen because similar work by Waymen and Lora indicated that at and below 150°C the substrate does not undergo any significant change [30].

Once the bomb was at the desired pressure and the autoclave at 150°C, the agitator was started and adjusted to 300 rpm. Steam was then released into the autoclave. The injection of high pressure steam allowed for very rapid heat-up of the substrate. (This rapid heating was desired so that accurate time-at-temperature effects could be ascertained.) When the autoclave reached operating temperature, the steam injection was discontinued. Temperature of the autoclave was maintained within ± 2°C of the chosen operating value by venting vapor to cool or adding steam to heat.

When the predetermined time had elapsed the autoclave was vented to the cold trap, being careful not to allow any uncondensed vapors to escape the trap. This procedure allowed for rapid cooling of the autoclave and its contents. Average heat-up and cool-down times observed were 141 ± 65 seconds and 75 ± 15 seconds, respectively.

Once the autoclave had been properly vented it could be opened and the assembly of packets removed. Four packets were saved for delignification tests, the other four packets were opened and the contents quantitatively removed. The autohydrolyzed substrate was washed with copious amounts of deionized water, suctioned to remove the excess water, and then allowed to air dry. When the substrate was air dry it was characterized as described earlier. However, the ethanol-benzene extractibles test was not run.

The amount of liquid in the autoclave, cold trap, and Parr bomb was measured to trace the liquid's movement during the autohydrolysis. Air dried material was sealed in a sample bottle for subsequent testing.

Delignification

The next step in the pretreatment process was an alcoholic extraction to remove lignin that was rendered soluble by the autohydrolysis. A 50:50 volumetric solution of 95% ethanol and deionized water was used.

The four remaining packets from the autohydrolysis were reassembled into their holder and placed in the autoclave. The cold trap was reconnected to the system and as before placed in an ice bath. Six hundred milliters of the 50:50 ethanol-water solution were added to the autoclave, the autoclave was heated to 150°C, and the agitator adjusted to 300 rpm. Temperature was controlled by the methods used during the autohydrolysis. The autohydrolyzed substrate was left in the alcohol mixture for one hour. At the end of the hour the autoclave was vented to the cold trap, the autoclave was opened, and the contents removed. The substrate was quantitatively washed from the pouches with deionized water, vacuum filtered to remove the excess water, and then air dried. Once the substrate was air dry it was characterized using the procedures previously described. Again, the extractibles were not determined.

The air dried sample was then sealed in a sample bottle for future experiments.

Ball Milling

Chromedia, one of the substrates used, was ball milled in an attempt to produce an amorphous cellulosic material. The ball milling was carried out with both wet and dry substrate.

The initial Chromedia substrate, the dry ball milled Chromedia, and the wet milled Chromedia were analyzed to determine the relative amounts of the various cellulose types present. Acid hydrolysis tests were performed on all three materials. Since Chromedia is essentially pure cellulose, lignin determinations were unnecessary.

Dry Ball Milling

Steel ball bearings of various sizes (1/4 inch diameter to 1/2 inch diameter) were used to mill the dry Chromedia. The ball bearings were loaded into a porcelain grinding jar and Chromedia was added to fill the void spaces between the balls. The total space occupied in the jar was approximately one third of its volume. The jar was sealed and placed on a tumbler rotating between 30 and 40 revolutions per minute. The temperature was approximately 20°C. Total milling time was 96 hours with periodic down times to scrape the substrate from the corners of the jar.

The dry ball-milled substrate was stored in Nalgene sample jars.

Wet Ball Milling

Wet ball milling was carried out using porcelain cylinders (20 mm by 20 mm) in the same jar used in the dry milling operation. Chromedia was mixed with deionized water to form a slurry. The slurry was vacuum filtered to remove excess water, then placed in the jar with the cylinders. Chromedia slurry was added to just cover the porcelain cylinders. The jar was filled as before to about one-third capacity. The jar was rotated with the same velocity and the same length of time as the dry milling operation. The milling operation was again stopped periodically to scrape the material from the corners of the jar. Once the 96-hour period had passed the substrate slurry was washed into a flask and centrifuged. The water was decanted off and the Chromedia transferred to glass sample jars so it could be stored wet for future analysis.

Acid and Enzymatic Hydrolysis

Pretreated substrates were tested to ascertain the relative ease of hydrolysis among extract-free, autohydrolyzed, and autohydrolyzed-alcoholic extracted materials. The substances were hydrolyzed by several different means during the hydrolyses. One of the methods was a two step acid hydrolysis using either sulfuric or hydrochloric acid. The other methods were various enzymatic hydrolyses using either single

enzymes or mixed enzymes.

Acid Hydrolysis

A variation of ASTM D 1106-56 Standard Test Method for Lignin in Wood [31] was used for the acid hydrolyses. This test uses a two stage acid hydrolysis in which the conditions are strong enough to hydrolyze all of the polysaccharrides leaving the lignin and ash fractions behind. Severity of the conditions were lessened so that only a fraction of the cellulose would hydrolyze. These conditions were arrived at using a trial and error experimental procedure.

Conditions were sought that would readily hydrolyze lignin-free substrate, but would not hydrolyze extract-free substrate to any great extent.

These conditions were not fully achieved (at least with the conditions that were attempted), so conditions were chosen where the experimental work-up of the sample was easiest. As the acid hydrolysis conditions increased in severity, colloidal suspensions would form. These suspensions were very difficult to filter, thus making sample work-up difficult.

The conditions chosen for sulfuric acid were:

1. 100:1 weight ratio of actual acid to substrate using 16 N acid solution
2. 30°C for one hour
3. Dilute to 0.7 N acid strength
4. 80°C for two hours

Acid hydrolyses were run in a 250 ml Erlenmeyer flask with approximately 300 milligram of substrate. When 2 hours had lapsed for hydrolysis, substrate that had not hydrolyzed was transferred to a tared Gooch crucible with a coarse fritted disc. The non-hydrolyzed material was washed with copious amounts of deionized water and the crucible placed in a drying oven with the temperature between 105°C and 110°C. The crucible was kept in the oven overnight before weighing. The extent of hydrolysis was reported as the moisture-free weight loss of the sample.

Enzymatic Hydrolysis

Three methods of enzymatic hydrolysis were used. One method hydrolysis used a cellulase enzyme, another used a mixture of a cellulase and a cellobiase, and the third used a mixture of three enzymes, a cellulase, a cellobiase, and a hemicellulase.

The enzyme solutions were made by mixing the desired amounts and types of enzymes with a 0.1 M citrate buffer solution. Buffer solutions of the desired pH, 4.8, were mixed from 0.1 M stock solutions of citric acid and sodium citrate. To obtain a solution that buffered to a pH of 4.8, 46 volume parts of citric acid solution were mixed with 54 volume parts of sodium citrate solution.

About 100 milligrams of the substrate were placed in a 200 x 25 mm test tube and wetted with one milliter of the citrate buffer. Once the wetting was complete, four milliters of the desired enzyme solution were added to the substrate. The test tube was submerged in a 50°C water bath and stirred for the time of hydrolysis via a magnetic stirrer and stir bar. At the end of the desired time period, the contents of the tube was transferred to a 100 x 13 mm test tube and centrifuged. The clear supernatant liquid was frozen for future reducing sugar analysis.

Reducing sugar was analyzed using a method described by Miller [32]. This test used a reagent referred to as Miller's reagent. The reagent was made from the following recipe:

77.75%	Deionized water
20%	Rochelle salt (Potassium sodium tartrate)
1%	3,5-dinitrosalicylic acid
1%	Sodium hydroxide
0.2%	Phenol
0.05%	Sodium Sulfite

For the Miller method, one milliter of test sample and three milliters of Miller's reagent were placed in a 100 x 13 mm test tube. The test tube was then placed in a boiling water bath for fifteen minutes to develop the color for an optical density test. A blank was

generated using two milliters of the buffer solution and six milliters of the Miller's reagent. The optical density of the resulting solutions was found using a Varian DMS 90 UV-Visible Double Beam Spectrophotometer that was interfaced with an Apple II Plus computer. The wavelength was set at 575 nm and the slit width at 4 nm. The spectrophotometer was calibrated using standard solutions of D-glucose; therefore, the concentration units obtained were milligram of apparent glucose per milliters solution and not absolute amounts of reducing sugars. The final units reported were milligram apparent glucose per gram of dry substrate or milligram of apparent glucose per gram of total cellulose in the sample.

The Miller's reagent would not accurately indicate a sugar concentration higher than one mg/ml of solution. Due to this fact, the spectrophotometer was usually calibrated with glucose solutions of about 0.35 mg/ml. Although at times a less concentrated calibration solution had to be employed, because a less concentrated hydrolysis solution needed to be evaluated. Linearity of the relationship between light absorbance and sugar concentration existed only in a narrow range around the calibration concentration. Therefore, samples to be tested were diluted (with buffer solutions) and evaluated until their concentrations were close to the calibration concentration.

The enzyme preparations themselves contained sugar before contact with any substrate. This sugar level was accounted for by preparing a standard enzyme solution subjected to 50°C for the standard hydrolysis time of 6 hours. The amount of apparent glucose was then determined and subtracted from subsequent values of hydrolyzed samples. The final value was the apparent glucose achieved from hydrolysis alone.

RESULTS AND DISCUSSION

Autohydrolysis of Substrates

The first goal of this study was to evaluate hydrolysis characteristics of three substrates. The three substrates were barley straw, lodge pole pine, and Douglas fir. Degree of lignin removal after an autohydrolysis and ethanol-water extraction was determined. Cellulose conversion amounts via an acid hydrolysis and an enzymatic hydrolysis were also determined. Autohydrolysis conditions used for this work were 205 °C and 10 minutes. These conditions were those found to be optimum for the delignification of wheat straw during previous work at this laboratory [24].

Table 2 contains results of the substrate characterizations. Wheat straw composition (generated by Nakaoka [24]) was included for comparison purposes. The ash percentages reported were those from tests performed before the ethanol-benzene extraction of the substrates. Subsequent ashings of ethanol-benzene extracted substrates revealed that some material that tested as ash was removed during the extraction procedure. Table 3 shows the ash percentages after the ethanol-benzene extraction. All subsequent mass balance calculations were made using the Table 3 ash results.

Table 2. Weight Percent Composition of Substrates (Moisture-free basis).

Substrate	Cellulose	Hemicellulose	Lignin	Ash	Extractible
Barley straw	43.6	15.6	23.9	7.7	9.2
Wheat straw	39.3	14.1	28.9	8.3	9.4
Douglas fir	50.8	21.2	26.1	0.2	1.7
Lodge pole pine	48.3	21.3	24.6	0.4	5.4

Table 3. Weight Percent Ash of Ethanol-Benzene Extracted Substrates.

Substrate	Ash
Barley straw	4.2
Wheat straw	5.8
Douglas fir	0.1
Lodge pole pine	0.2

The desired result of the autohydrolysis pretreatment was to hydrolyze most of the hemicellulose and render the lignin highly soluble in an ethanol-water solvent. The cellulose residue from the autohydrolysis (if the above goals were met) should be in a form that allows easy hydrolysis to glucose.

The theory of an autohydrolysis, as discussed before, is that an acid environment results from decomposition of hemicellulose acetyl groups. The acid conditions catalyze the hydrolysis of hemicellulose and some cellulose in the substrate. This hydrolysis produces water soluble products that are easily removed from the lignocellulose matrix during the autohydrolysis.

Autohydrolysis conditions can also break lignin-lignin and hemicellulose-lignin bonds. These reactions are thought to be very fast. During slower reactions, that follow these fast reactions, carbon-carbon bonds are formed between lignin monomers. The first reactions produce products that are soluble in solvents that usually will not dissolve lignin, such as water or ethanol-water solutions. Once the carbon-carbon bonds are formed, the repolymerized lignin becomes highly insoluble in the above solvents [33]. Due to this series reaction mechanism, time-at-temperature is very important for lignin solubility. Wayman and Lora observed this autohydrolysis time-at-temperature effect on residual lignin in aspen woodmeal using a dioxane-water solvent (see Figure 8) [30].

Barley Straw

The autohydrolysis and autohydrolysis/extraction of barley straw produced results similar to those observed with wheat straw [24].

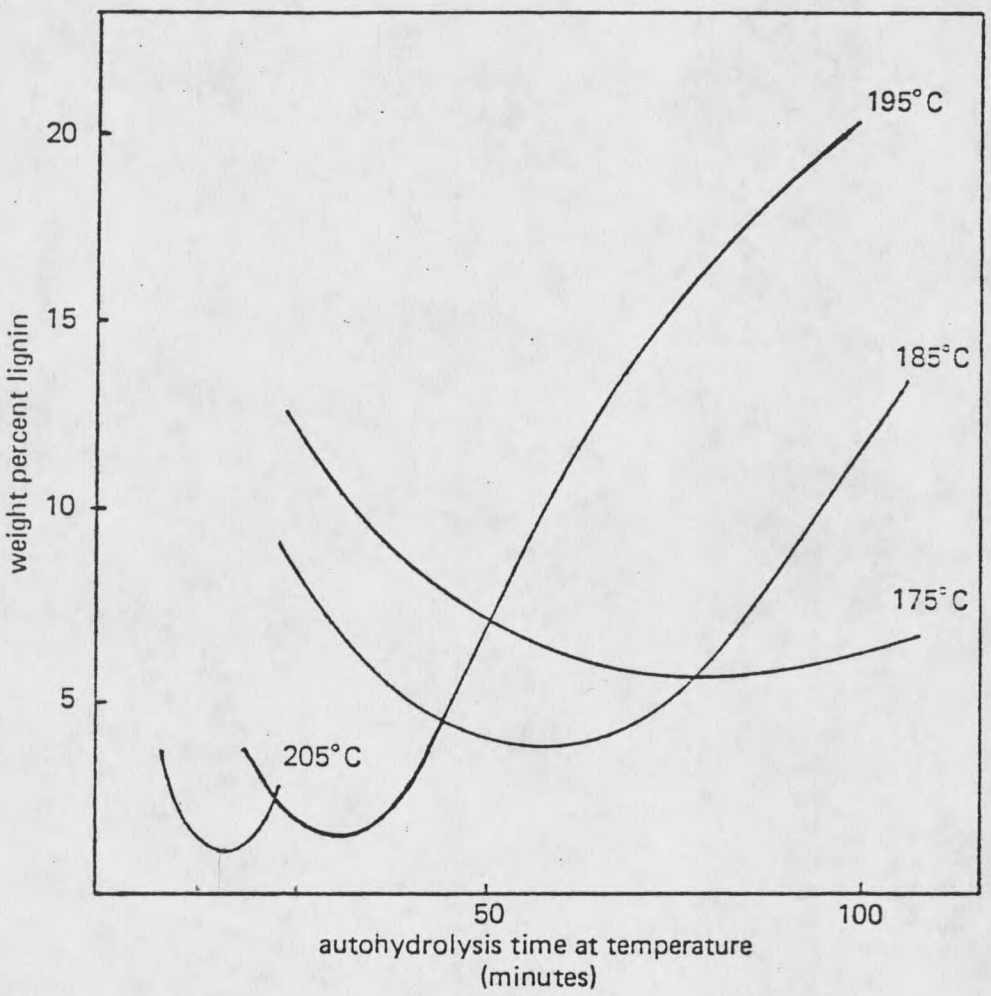

Figure 8. Effects of Autohydrolysis and Dioxane Extraction on Aspen Woodmeal Lignin [30].

Since these two substrates are closely related species, this consistency of results would be expected. Table 4 presents a comparison of autohydrolysis results for wheat and barley straw.

Douglas Fir and Lodge Pole Pine

Table 5 is a summary of autohydrolysis and autohydrolysis/extraction runs on the Douglas fir and lodge pole pine. The overall weight losses from the autohydrolysis and the autohydrolysis/extraction runs for the two woods were within 2% of each other. Removal of components such as cellulose, hemicellulose, and lignin were also reasonably close for these two substrates.

Table 5 shows that removal of lodge pole hemicellulose from the lignocellulose matrix continued during the ethanol-water extraction. This continued dissolution of hemicellulose was not observed with Douglas fir. Lignin analyses seem to indicate that more lignin was removed from Douglas fir during the autohydrolysis/extraction than from lodge pole during the pretreatment. But an inconsistency in the lodge pole lignin data makes this conclusion suspect. Calculations indicated that more lignin was removed from the lodge pole during the autohydrolysis than was removed during the autohydrolysis/extraction, which was not possible. The errant data was probably due to subjective judgments that have to be made during the lignin determination procedure. Due to this apparent inconsistency and the time involved in repeating the run, future work with wood was performed using Douglas fir as the substrate.

Approximately twice as much lignin was removed from the straws than the woods (on weight percent basis) during the pretreatments. The percent of hemicellulose removed from barley straw and woods were similar. Given the intimate relationship of these two constituents in the lignocellulose matrix, together these two facts suggest that the lignin may have repolymerized before its dissolution could occur. Lignin repolymerization would indicate that either the time-at-temperature was too long or the temperature too high.

Table 4. Comparison of Autohydrolysis Experiments for Barley Straw and Wheat Straw.

Substrate	% Weight Loss	% Lignin Removed	% Carbohydrate Removed	% Cellulose Removed	% Hemicellulose Removed
Autohydrolyzed Barley Straw[a]	43.9	66.2	37.6	20.6	85.1
Autohydrolyzed/ Extracted Barley Straw[a]	46.7	75.7	38.0	19.8	88.5
Autohydrolyzed Wheat Straw[b] [24]	42.8	59.3	37.4	no analysis	no analysis
Autohydrolzed/ Extracted Wheat Straw[b] [24]	48.6	78.8	39.0	no analysis	no analysis

a - moisture-free basis
b - air dry basis

Table 5. Summary of Autohydrolysis Experiments on Wood Substrates (Moisture-Free Basis).

Substrate	% Weight Loss	% Lignin Removed	% Carbohydrate Removed	% Cellulose Removed	% Hemicellulose Removed
Autohydrolyzed Douglas Fir	35.2	28.2	37.8	16.1	89.8
Autohydrolyzed/ Extracted Douglas Fir	37.1	34.6	38.0	16.8	88.7
Autohydrolyzed Lodge Pole Pine	34.8	29.4	36.8	18.5	78.3
Autohydrolyzed/ Extracted Lodge Pole Pine	36.1	27.2	39.3	17.2	89.3

Acid Hydrolysis Experiments

Barley Straw

The low percentages of lignin and hemicellulose in barley straw after pretreatment should have resulted in a substrate whose cellulose was highly susceptible to hydrolysis. When pretreated barley straw was subjected to the sulfuric acid hydrolysis conditions less than 10% of the remaining carbohydrate was hydrolyzed to water soluble product. Table 6 shows that weight losses due to acid hydrolyses were very similar for both wheat and barley straw. Carbohydrate weight losses from the overall process (acid hydrolysis plus various pretreatments) were similar for both pretreated and non-pretreated straws although the latter are (surprisingly) somewhat higher. These results seem to indicate the existence of a carbohydrate fraction that is easily hydrolyzed by an acidic environment. Pretreatments as performed here seem to have little effect on the carbohydrate fraction that is not easily hydrolyzed. (Another possible reason for the low weight losses in pretreated materials will be discussed later in this work.)

Douglas Fir and Lodge Pole Pine

Table 7 contains the weight loss results from the acid hydrolysis of the woods. The overall-process weight loss calculations for the woods reveal an interesting result. Carbohydrate weight losses were about 70% higher for pretreated substrates in comparison with those of non-pretreated extract-free woods. This suggests that pretreatments have a greater effect on wood cellulose than on straw cellulose. Even though the data indicated that an autohydrolysis enhances the acid hydrolysis of wood cellulose, the hydrolysis yields were still less than 50%. Therefore, another series of experiments was performed to try to explain the low cellulose conversions.

A possible reason for low hydrolyzability of the wood cellulose was the high amounts of lignin still present in the lignocellulose matrix after pretreatments. On a relative basis, the weight percent

Table 6. Carbohydrate Conversion Results of Acid Hydrolysis on Straw Substrates (Weight %).

Substrate	Total Carbohydrate Converted During Acid Hydrolysis	Total Carbohydrate Converted During Overall Process (Pretreatment + Hydrolysis)
Extract-Free Barley Straw[a]	48.0	--
Autohydrolyzed Barley Straw[a]	8.4	42.9
Autohydrolyzed/ Extracted Barley Straw[a]	5.8	41.6
Extract-Free Wheat Straw[b] [24]	54.9	--
Autohydrolyzed Wheat Straw[b] [24]	11.2	43.5
Autohydrolyzed/ Extracted Wheat Straw[b] [24]	5.4	39.6

a - Moisture-Free Basis
b - Air Dry Basis

Table 7. Carbohydrate Conversion Results of H_2SO_4 Hydrolysis of Wood Substrates (Moisture-Free Basis; % Weight Loss).

Substrate	Total Carbohydrate Converted During Acid Hydrolysis	Total Carbohydrate Converted During Overall Process (Pretreatment + Hydrolysis)
Extract-Free Douglas Fir	24.3	--
Autohydrolyzed Douglas Fir	4.5	40.6
Autohydrolyzed/ Extracted Douglas Fir	3.6	40.2
Extract-Free Lodge Pole Pine	24.3	--
Autohydrolyzed Lodge Pole Pine	5.3	40.2
Autohydrolyzed/ Extracted Lodge Pole Pine	4.6	42.1

lignin in the pretreated woods was higher than that found in the non-pretreated woods.

Wayman and Lora's results with aspen suggested that dioxane-water solvent may be more effective for delignification than the ethanol-water solvent. Therefore, the ethanol-water extraction was replaced with a 9:1 dioxane-water extraction for post-autohydrolysis extractions. See Table 8 for a comparison of the before and after extraction lignin percentages and the percent lignin removed for the two solvents. Although more lignin was removed by the dioxane-water extraction, the final lignin percent in the residue was higher than that found in the ethanol-water extracted sample. This result was attributable to the fact that the autohydrolysis was not as effective removing lignin as that observed during a previous Douglas fir run. Therefore, the autohydrolyzed fir samples contained more lignin than the autohydrolyzed samples used during the ethanol-water extraction experiments. Total amounts of carbohydrates removed during the pretreatments and acid hydrolyses were about the same for both runs (See Tables 7 and 9). This indicates that the small increase in lignin content between this run and the previous run had little effect on the total carbohydrate conversion.

Table 8. Comparison of Douglas Fir Lignin Extractibility by Two Solvents (Moisture-Free Basis; Weight %).

Extraction Solvent	% Lignin Before Extraction	% Lignin After Extraction	% Lignin Removed
Ethanol-Water	29.5	27.6	9.0
Dioxane-Water	34.7	31.6	12.1

To ascertain the extent of the effect of lignin on an acid hydrolysis, two hydrolyses were performed using an extract-free and a delignified extract-free sample of Douglas fir. The delignified sample was prepared using the chlorine gas bleaching procedure of the lignin determination. The carbohydrate conversions for the extract-free sample and the lignin-free sample were 33.4% and 27.7%, respectively.

Table 9. Carbohydrate Conversion Results of Autohydrolyzed Dioxane-Water Extracted Douglas Fir (Moisture-Free Basis; % Weight Loss).

Pretreatment	Total Carbohydrate Converted During Acid Hydrolysis	Total Carbohydrate Converted During Overall Process (Pretreatment + Hydrolysis)
Autohydrolyzed	4.1	44.6
Autohydrolyzed/ Extracted	4.9	44.4

The comparison of these two results seems to indicate that lignin does not have a detrimental effect on cellulose hydrolysis. However, Saddler et al. have shown that as lignin is removed, enzymatic conversion of cellulose increases [34]. If an acid hydrolysis can be compared to an enzymatic hydrolysis, the lower conversion of a delignified sample suggests a problem with the acid hydrolysis test.

Cellulose analyses were performed on the residues from the acid hydrolyses of the two Douglas fir samples just discussed. These analyses were done to ascertain the effects of acid catalyzed hydrolysis on the various cellulose types. Table 10 contains a comparison of the various carbohydrate amounts before and after the hydrolysis. Acid hydrolysis of the delignified sample resulted in the conversion of 19% of the cellulose fraction and only 48% of the hemicellulose fraction. In the extract-free sample all of the hemicellulose was hydrolyzed and only 6% of the cellulose fraction was hydrolyzed. The results for these experiments on cellulose were as expected. The removal of lignin increased access to cellulose resulting in higher cellulose conversion. The fact that all of the hemicellulose was hydrolyzed in the extract-free fir and only 48% was hydrolyzed in the delignified fir was not expected. The expected result would be the reverse, higher hemicellulose conversion for delignified samples. Lignin and hemicellulose are bonded together in untreated lignocellulose. The removal of all the lignin may cause changes in the lignocellulose matrix and the chemical and physical nature of the hemicellulose. These changes in hemicellulose may render it less susceptible to hydrolysis.

Table 10. Cellulose Analysis After Acid Hydrolysis (Basis: 1g Extract-Free Wood, Moisture-Free).

	Extract-Free Douglas Fir		Delignified-Extract-Free Douglas Fir	
Cellulose Type	Before	After	Before	After
α - Cellulose	0.525	0.321	0.525	0.302
β - Cellulose	0	0.173	0	0.121
γ - Cellulose	0.216	0	0.216	0.113

Further acid hydrolyses were performed substituting hydrochloric acid for the sulfuric acid of previous tests. Hydrolysis conditions for these hydrolyses were: 5N HCl, 3 hours, and 80°C. The purpose of these experiments was to examine the effects of HCl, rather than H_2SO_4, and a more severe hydrolysis environment. The substrates hydrolyzed were extract-free Douglas fir, delignified Douglas fir, and autohydrolyzed ethanol-water extracted Douglas fir. Table 11 contains the results of these hydrolyses. The HCl hydrolysis of extract-free fir resulted in a carbohydrate conversion about 25% higher than the 33.4% conversion observed for the previous H_2SO_4 hydrolysis. The hydrolysis conversion of the autohydrolyzed/extracted substrate with hydrochloric acid was essentially unchanged from that observed when sulfuric acid was used. When HCl was used for the hydrolysis, the overall process carbohydrate conversions of extract-free fir and pretreated fir were essentially equal, unlike that observed for the H_2SO_4. This suggests, like that observed with the straws, a certain cellulose fraction exists that is easily hydrolyzed and this fraction is not increased by pretreatment. Like that observed with H_2SO_4, a lower cellulose conversion was observed for delignified wood in comparison to extract-free wood for HCl hydrolyses.

Wheat Straw

Further hydrolyses were performed with hydrochloric acid, but wheat straw was used instead of Douglas fir. The substrate change was made to ascertain the effects of an HCl hydrolysis on a straw substrate. Conditions for these hydrolyses were as follows: 12.4N HCl, 30 °C for one hour, 3 N, 80 °C for two hours. These conditions were similar to those used for the sulfuric acid hydrolyses, except the number of hydrogen ions in solution was ≈50% higher for the first hour and ≈200% higher for the last two hours. The results of these hydrolysis conditions for extract-free wheat, autohydrolyzed wheat, and autohydrolyzed/extracted wheat are presented in Table 12. The extract-free wheat and the autohydrolyzed wheat showed a 30% increase of conversion for the whole processes (pretreatment and acid hydrolysis) over that obtained with H_2SO_4.

Table 11. Carbohydrate Conversions of Douglas Fir Substrates by HCl (Moisture-Free Basis; % Weight Loss).

Substrate	Total Carbohydrate Converted During Acid Hydrolysis	Total Carbohydrate Converted During Overall Process (Pretreatment + Hydrolysis)
Extract-Free Douglas Fir	41.1	--
Lignin-Free Douglas Fir	34.4	--
Autohydrolyzed/ Extracted Douglas Fir	8.4	43.2

Table 12. Carbohydrate Conversions of Wheat Straw Substrates by HCl (Moisture-Free Basis; % Weight Loss).

Substrate	Total Carbohydrate Converted During Acid Hydrolysis	Total Carbohydrate Converted During Overall Process (Pretreatment + Hydrolysis)
Extract-Free Wheat Straw	72.4	--
Lignin-Free Wheat Straw	31.0	56.5
Autohydrolyzed/ Extracted Wheat Straw	32.2	58.8

Cellulose conversion for the autohydrolyzed/extracted sample was 23% higher for the HCl hydrolysis.

Chromedia

The observation that after three hours of acid hydrolysis less than 50% of the amorphous carbohydrate in autohydrolyzed Douglas fir was hydrolyzed by H_2SO_4 and only about 60% by HCl seemed unlikely. Similar results were also observed with other substrates tested (see Table 13). Therefore, a series of experiments was run to test the acid hydrolysis of a pure cellulose substrate with a high fraction of amorphous cellulose.

To produce the highly amorphous cellulose a pure cellulose substrate, Chromedia, was ball milled. Both dry and wet millings were performed. Ball milling the dry Chromedia for 96 hours doubled its amorphous cellulose content, while wet milling reduced the amorphous cellulose fraction to less than 1% of the total cellulose. This was an unexpected result. However, researchers at American Viscose Corporation observed that by wetting an amorphous ball milled cellulose, the crystallinity could be increased to the degree of crystallinity found in the non-ball milled substrate [35]. The recrystallization of wet cellulose may explain the decrease in the amorphous nature of the wet ball milled samples. Because of the apparent recrystallization of wet milled Chromedia, the dry milled sample was chosen for acid hydrolysis comparison with non-milled Chromedia. Table 14 contains a summary of the relative amounts of the carbohydrate fractions found in the three samples (non-milled, wet milled, and dry milled Chromedia).

The acid hydrolysis (with 5 N HCl, 80 °C for 3 hours) of non-milled and dry-milled Chromedia produced the results presented in Table 15. It is evident that doubling the amorphous nature of the substrate had an equivalent effect on the cellulose conversion. The conversion was still very low for samples that contained approximately 40% amorphous cellulose. These low conversions of amorphous cellulose support conclusions concerning the inadequacies of the acid hydrolysis tests.

Table 13. Comparison of Amorphous Cellulose Content versus Amount of Cellulose Converted.

Substrate	Acid	Grams of Amorphous Cellulose Present	Total Grams of Carbohydrate Hydrolyzed
Autohydrolyzed Barley Straw	H_2SO_4	0.125	0.064
Autohydrolyzed/ Extracted Barley Straw	H_2SO_4	0.106	0.046
Autohydrolyzed Douglas Fir	H_2SO_4	0.080	0.031
Autohydrolyzed/ Extracted Douglas Fir	H_2SO_4	0.104	0.026
Autohydrolyzed/ Extracted Douglas Fir	HCl	0.104	0.060
Autohydrolyzed Lodge Pole Pine	H_2SO_4	0.131	0.038
Autohydrolyzed/ Extracted Lodge Pole Pine	H_2SO_4	0.107	0.032

Table 14. Cellulose Analyses on Milled and Non-Milled Chromedia (Weight %).

Milling	Alpha Cellulose	Beta Cellulose	Gamma Cellulose
None	81.2	18.4	0.4
Dry	62.6	36.7	0.7
Wet	90.0	0.4	9.6

Table 15. Results of Acid Hydrolysis on Chromedia (Moisture-Free Basis).

Milling	Cellulose Converted (Weight %)
None	4.1
Dry	7.3

Cellulose conversions observed during acid catalyzed hydrolyses were higher for all non-pretreated substrates versus conversions observed for pretreated substrates. These experimental results suggest that the ease of cellulose hyrolysis is not increased by the pretreatments performed during this investigation. While several investigations presented in the literature have suggested that cellulose conversion could be increased by pretreatments similar to those performed during this study [25,36], these citations and the experimental results from this laboratory indicate that acid hydrolysis may not be indicative of the effect of autohydrolysis as a pretreatment. Therefore, a series of experiments was performed using enzyme catalyzed hydrolyses to ascertain the effects of autohydrolysis on cellulose conversion. Further discussions on the low acid hydrolysis results are presented in the next section.

Enzymatic Hydrolysis Experiments

As a final series of experiments to test the effects of autohydrolysis on rendering a substrate more conducive to hydrolysis, an enzymatic hydrolysis was performed.

The substrate used for the enzymatic hydrolyses was an optimally autohydrolyzed and ethanol-water extracted wheat straw. The sample was left wet after the extraction to avoid adverse effects of drying on enzymatic hydrolyses observed at Colorado State University with loblolly pine (although subsequent experiments showed that drying had no detrimental effect on wheat straw conversion) [36]. The conditions for this hydrolysis were as follows: four milliters of enzyme solution, one milliter of citrate buffer solution, at 50°C for six hours. The enzyme solution consisted of 3.6 gm of a cellulase enzyme dissolved in citrate buffer to make 100 ml of solution. Reducing sugar analysis of an initial hydrolysis liquor revealed 561 milligrams of apparent glucose per gram of dry substrate, which was a conversion of about 74% of the cellulose in the sample. Sulfuric acid hydrolysis of a similar sample (air dried) resulted in only a 5.4 % conversion of cellulose, and a 12.4 N hydrolysis with hydrochloric acid yielded a 32.2% cellulose conversion. Comparing the enzyme and acid hydrolyses indicates that an acid hydrolysis test was a poor measurement of the effectiveness of autohydrolysis as a pretreatment.

A possible reason for the low conversions obtained with acid is that enzymes are very specific in the way that they attack cellulose. A complete cellulase enzyme actually consists of three separate enzymes: an endoglucanase, an exoglucanase, and a cellobiase. β-1,4-glucanohydrolase, an endoglucanase, randomly hydrolyzes cellulose inside the molecular chain, which results in a rapid reduction in the degree of polymerization. The exoglucanase, β-1,4-cellulobiohydrolase, attacks cellulose polymers from the reducing end producing cellobiose, a dimer of glucose which is water soluble. Exoglucanase can attack the ends found in the native cellulose or an end produced by a endoglucanase. The last type of enzyme, β-glucosidase (cellobiase), hydrolyzes cellobiose and other water soluble glucose oligomers to glucose.

Figure 9 is a schematic of this proposed mechanism of enzymatic hydrolysis. Due to this method of cellulose attack, a high percentage of all individual hydrolytic reactions produce a soluble product. Acids attack cellulose in a totally random fashion. Therefore, few hydrolytic reactions yield soluble products. Since the degree of hydrolysis by acids was measured by weight loss, products must be soluble to be measured as conversion.

Clausen and Gaddy obtained data showing a 90% conversion of cellulose could be obtained with 14 N HCl at room temperature in a CSTR (no time frame was given in their literature) [37]. They also found that 8 N HCl converted 90% of the cellulose in 30 minutes at 100°C using a series of CSTR's. Therefore, another possible explanation for low conversions of substrate via acid hydrolyses could be mass transfer limitations, resulting from low shear stirring of the hydrolysis mixtures. Acid hydrolysis mixtures were stirred only periodically with a glass stirring rod, while enzymatic hydrolysis mixtures were stirred throughout the hydrolysis.

Since initial enzymatic hydrolyses resulted in high cellulose conversions, the use of enzymes to evaluate the effects of autohydrolyses was adopted. Enzyme strengths that gave the highest conversion in 6 hours were evaluated.

Celluclast 1.5 L, a cellulase supplied by Novo Corporation, was the first enzyme whose strength was optimized. Celluclast 1.5 L is an enzyme preparation made from the submerged fermentation of a selected strain of the fungus Trichoderma reesei. Figure 10 is a plot of results obtained from the various 6 hour hydrolyses, in which the cellulase charge varied. It can be seen from this graph that a charge higher than 2.8 gm of cellulase preparation per gram of dry substrate would not produce much improvement in cellulose conversion. Therefore, this concentration of cellulase was chosen for future experiments. Wet optimally autohydrolyzed wheat straw was used for this series of experiments.

Experiments evaluating the effects of hydrolysis time on conversion using Celluclast 1.5 L were also performed (see Figure 11).

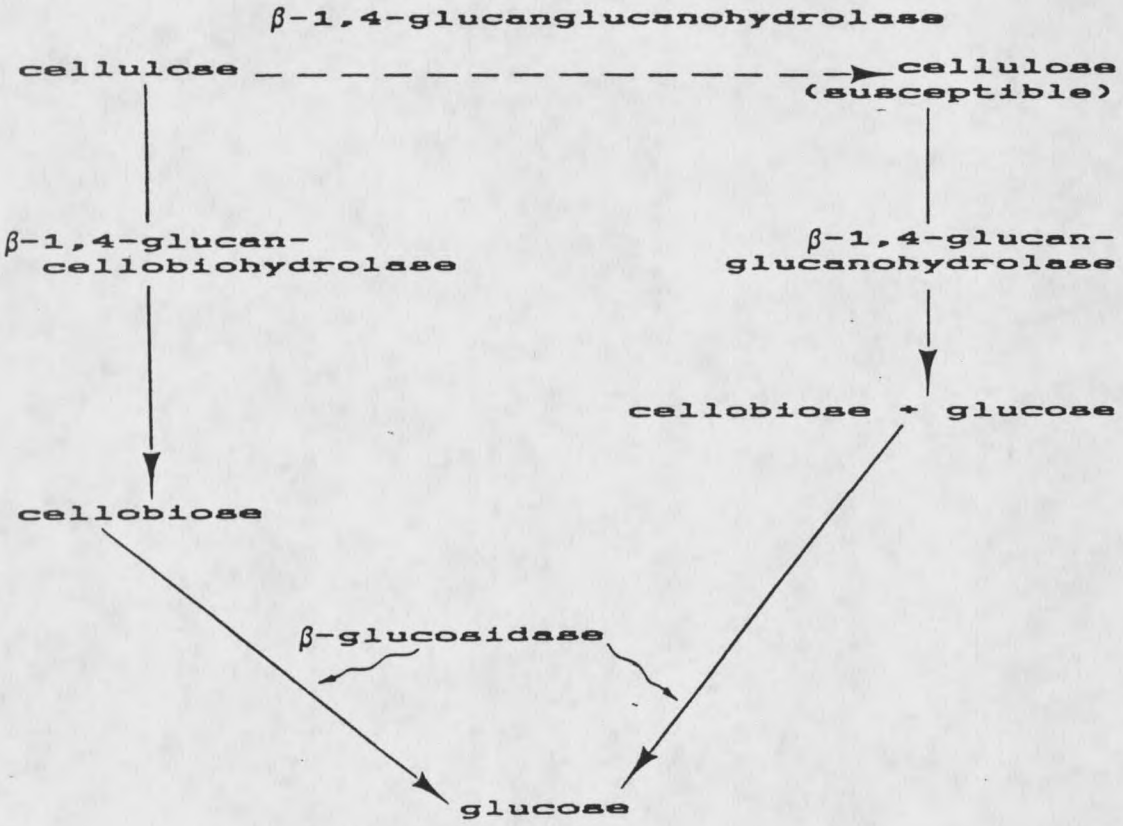

Figure 9. A Proposed Mechanism for an Enzymatic Hydrolysis [5].

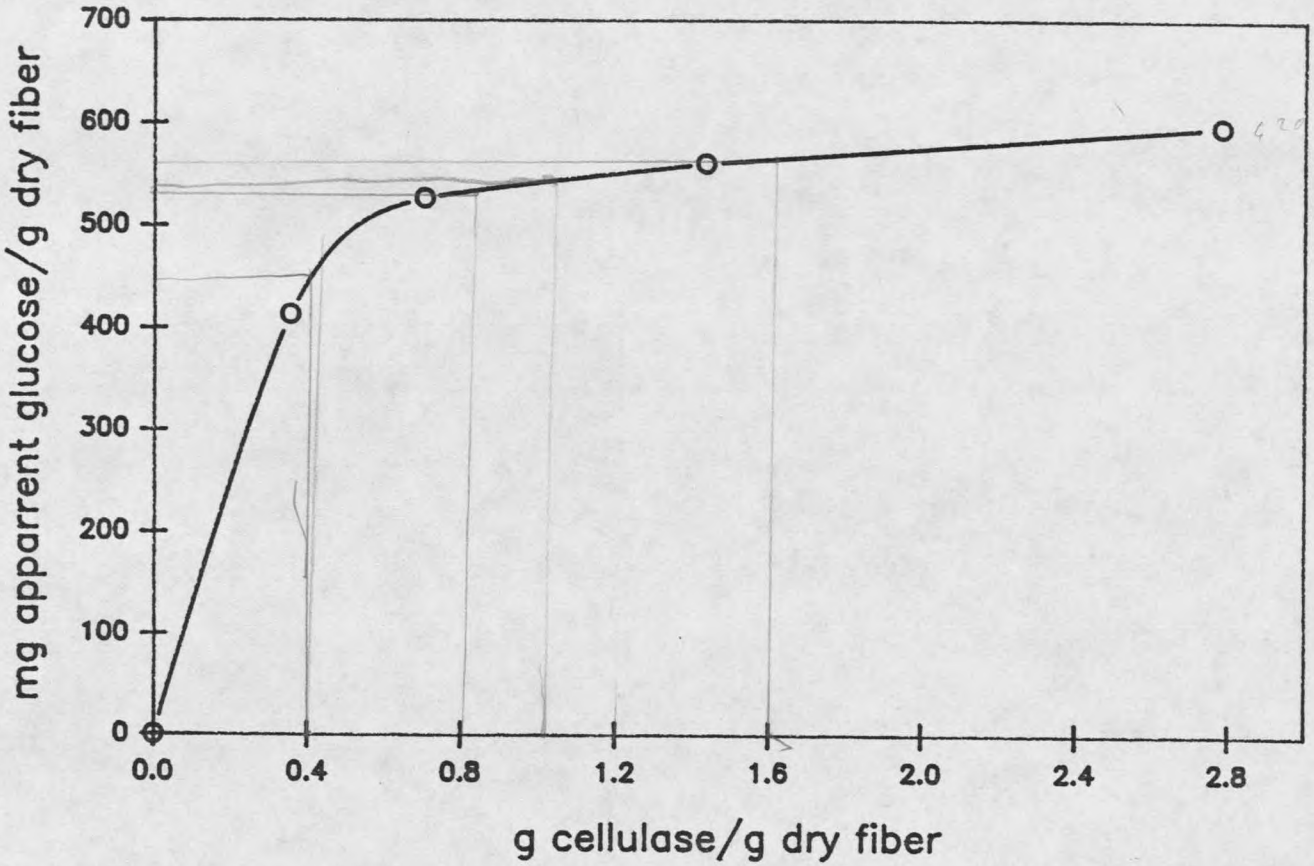

Figure 10. Effects of Cellulase Activity for a 6 hour Hydrolysis.

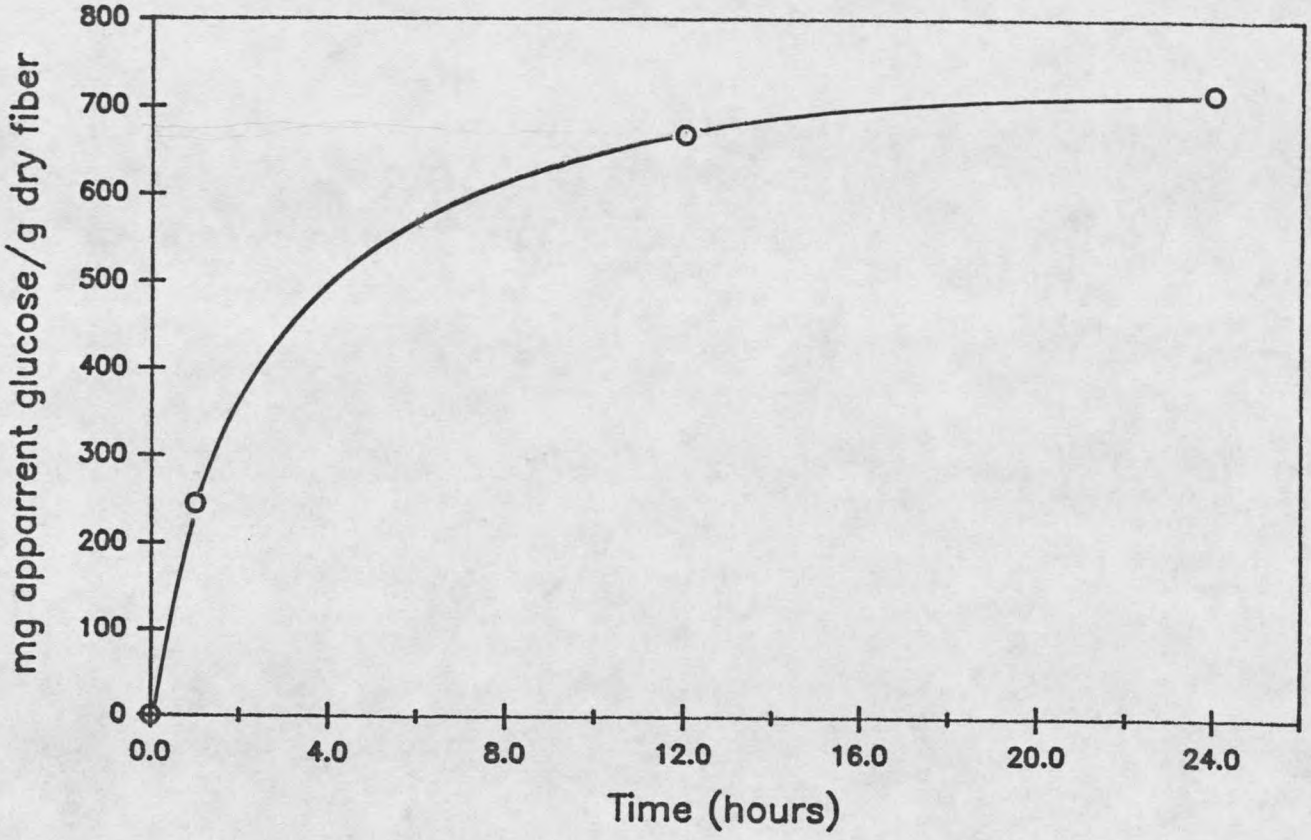

Figure 11. Reaction Time Effects on a Cellulase Hydrolysis.

Increasing hydrolysis time from 6 to 12 hours increased cellulose conversion by 19%. Lengthening the hydrolysis time to 24 hours resulted in 28% more conversion than was observed at 6 hours of hydrolysis. Even though higher conversions were obtained with longer hydrolysis times, practical experimental considerations dictated a hydrolysis time of 6 hours for substrate evaluation. The substrate used for these experiments was again wet, optimally autohydrolyzed wheat straw.

Celluclast 1.5 L contains little cellobiase activity. Therefore, the majority of the hydrolysis products would be cellobiose and not glucose. Since in a reducing sugar test, a cellobiose molecule would appear as only one molecule, a low hydrolytic conversion would be indicated. To alleviate this problem a second enzyme, β-glucosidase, was added to the hydrolysis mixtures. This enzyme preparation was also supplied by Novo Corporation, its trade name being Novozym 188 (or Cellobiase 250 L). It was produced by submerged fermentation of a selected strain of the fungus Aspergillus niger.

The cellobiase preparation was added to the optimized cellulase solutions in varying amounts to obtain the optimum amount of cellobiase. Figure 12 is a graph of the results from varying cellobiase amounts. Charges of cellobiase higher than 0.28 gm of cellobiase preparation per gram of dry substrate had little effect on cellulose conversion. Therefore, the 0.28 gm amount was chosen for charging subsequent hydrolyses. Wet optimally autohydrolyzed/extracted wheat straw was the substrate used for these experiments.

Time studies were also performed with the mixed enzyme solution. As the time increased from 6 hours to 12 hours, cellulose conversion increased 14%. A hydrolysis time of 24 hours did not result in increased conversion over that observed during the 12 hour hydrolysis (see Figure 13). The substrate used for these experiments was an optimally autohydrolyzed and ethanol-water extracted wheat straw.

Using the optimum concentrations determined by the above experiments, pretreated and non-pretreated extract-free samples of wheat were evaluated for cellulose conversion. The pretreated samples were wet

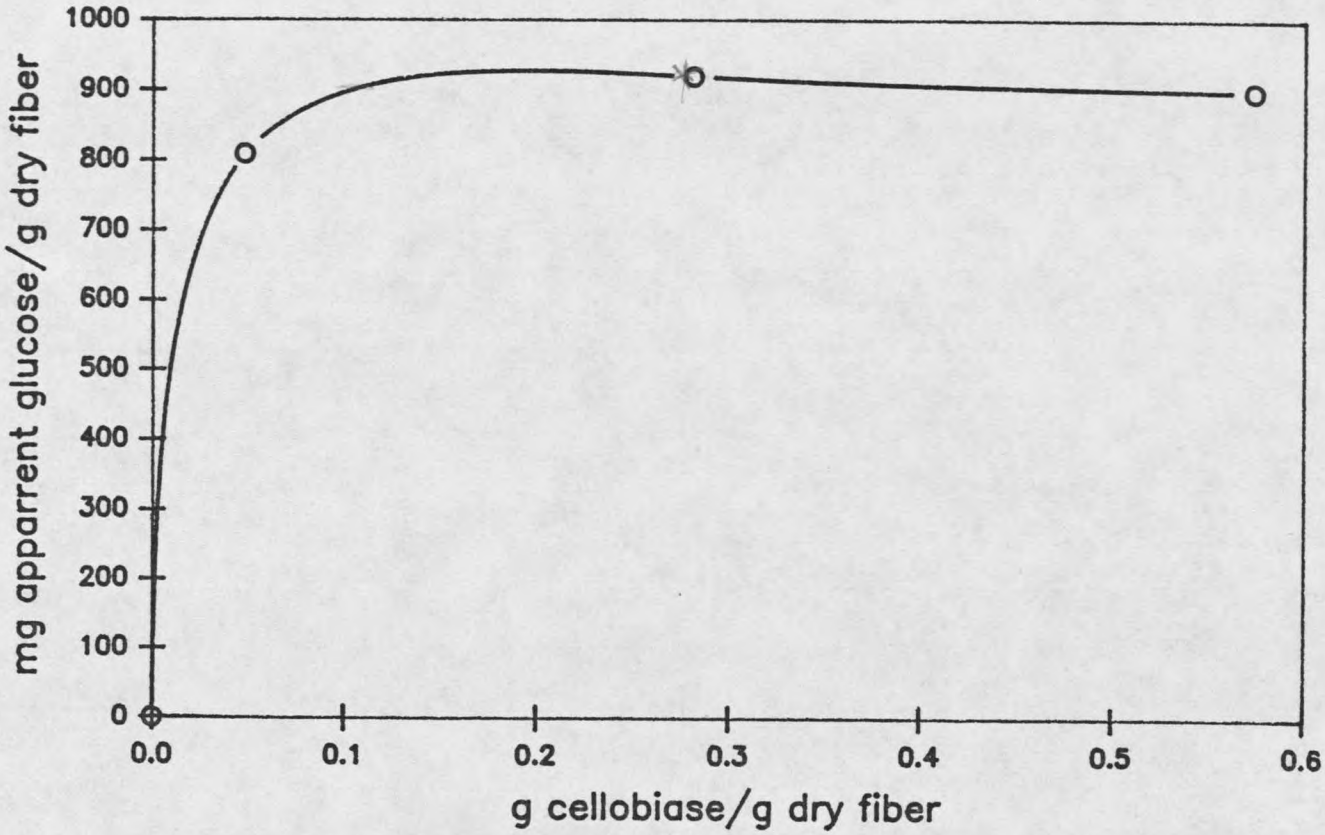

Figure 12. Effects of Cellobiase Activity on a 6 hour Hydrolysis.

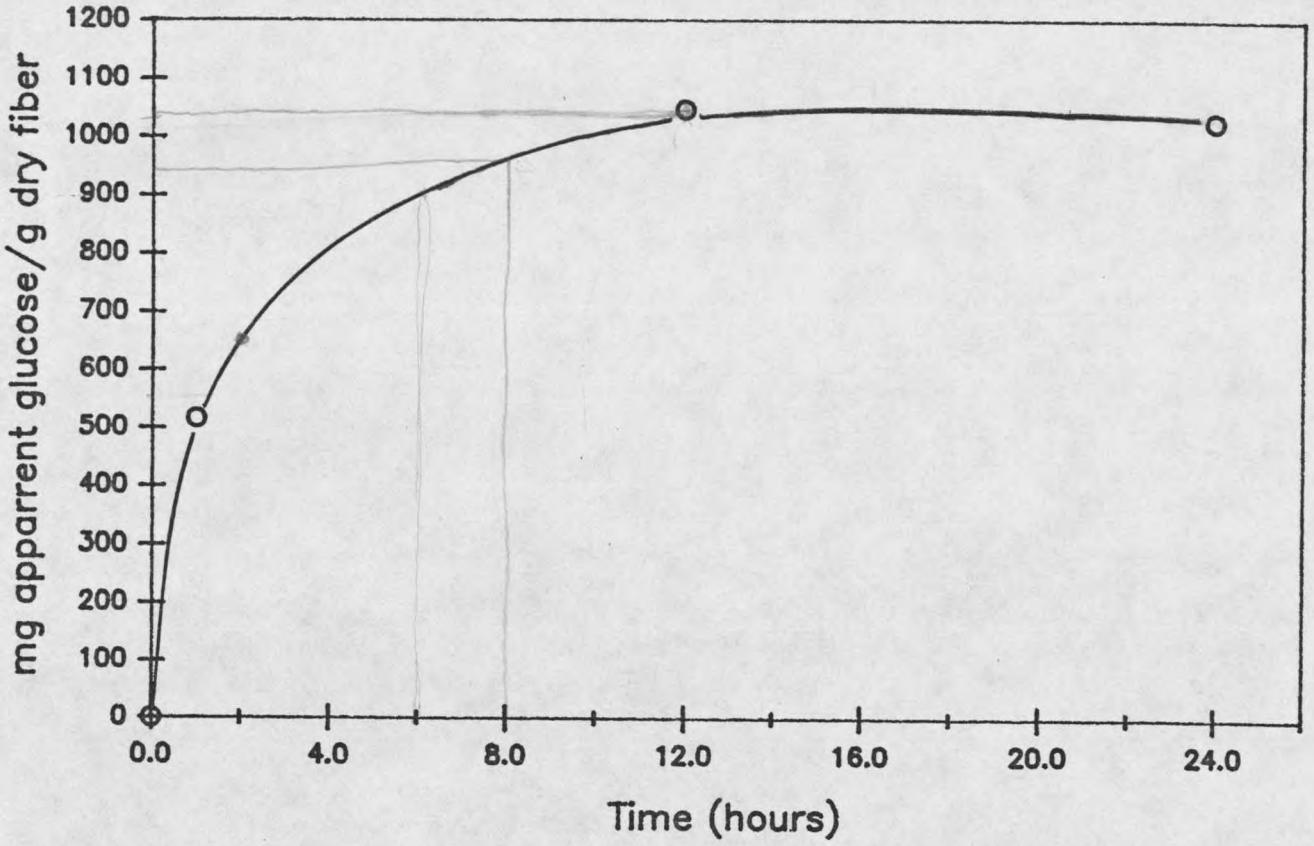

Figure 13. Reaction Time Effects on a Cellobiase-Cellulase Hydrolysis.

autohydrolyzed wheat, wet autohydrolyzed and ethanol-water extracted wheat, dry autohydrolyzed wheat, and dry autohydrolyzed and ethanol-water extracted wheat. Table 16 contains the results of these evaluations. Comparison of the extract-free wheat cellulose conversion with those of the pretreated samples demonstrates that the pretreatments were very effective in increasing the cellulose conversion. Also, the high conversions obtained with substrates that had not been extracted to remove lignin suggests that extraction was an unnecessary pretreatment step. It is also evident from the data that drying had little effect on the enzymatic hydrolysis of wheat straw. Murphy et al., at Colorado State University, also had good conversions of cellulose in autohydrolyzed wheat with dry samples [38]. They did no evaluations on the effects that drying of samples had on enzymatic hydrolysis.

Table 16. Results of Enzymatic Hydrolyses on Wheat Straw.

Substrate	% Cellulose Hydrolyzed
Dried Extract-Free	12.8
Dried Extract-Free Hemicellulase Added to Enzyme Solution	17.4
Dried Autohydrolyzed	94.5
Dried Autohydrolyzed/ Extracted	89.4
Wet Autohydrolyzed	96.5
Wet Autohydrolyzed/ Extracted	98.0

An experiment was performed in which a third enzyme type, a hemicellulase, was added to the hydrolysis mixture. Although the researchers at Colorado State found that the Novo cellulase enzymes had hemicellulase activity, more hemicellulase was added to assure a significant hemicellulase activity [36]. The trade name of this enzyme preparation was Gamanase 1.5 L. Although the Novo Corporation suggests

a dosage of 0.0005 gm of Gamanase 1.5 L per gram of dry substrate, 0.25 gm of enzyme per gram of dry substrate was used for this experiment to assure an excess of enzyme for the hydrolysis. The purpose of this experiment was to determine if an enzyme could remove the hemicellulose from a non-pretreated substrate and thus increase access to the cellulose. Hydrolysis time was increased to 12 hours for this experiment. A carbohydrate conversion of 17.4% indicates that the use of the third enzyme did not attain the effects rendered by an autohydrolysis pretreatment.

Murphy et al. suggested that for a process to be economically feasible, the enzyme charge rate could be no more than 10 IU/gm of substrate [36]. (An IU is an international enzyme unit and is the accepted unit of measure for enzyme strengths. The unit is defined as the release of 1 μmol of glucose per unit.) The final two-enzyme solution used in this work was evaluated for enzyme strength using the Mandels et al. procedure and was found to contain 0.94 IU/ml of enzyme solution [38]. This translates into about 38 IU/gm of substrate, which is high for a commercial process.

An enzyme solution (using the same Novo enzyme preparations) prepared by Bertran and Dale had a strength of 0.55 IU/ml [39]. This solution contained 3 gm of Celluclast 1.5 L and 1.5 gm Novozym 188 per 100 ml of solution. The enzyme used for the final evaluations by this laboratory contained 7.2 gm of Celluclast 1.5 L and 0.72 gm of Novozym 188 per 100 ml of solution with a strength of 0.94 IU/ml. Comparing the strengths of these two enzyme solutions suggests that most of the enzyme strength was the result of the cellulase and not the cellobiase. The steep initial slope of a plot of activity versus cellobiase charge supports the conclusion that the dominant variable determining enzyme strength is cellulase charge (see figure 12). Reducing the amount of cellulase by 75% (to $\approx$0.7 gm cellulase/gm dry fiber) would result in an enzyme strength of about 10 IU/gm of substrate. Data presented in Figure 10 indicates that the decreased enzyme strength would only reduce the 6 hour conversion by about 30%. The cellulase time studies indicate that increasing hydrolysis time to 24 hours (a reasonable time

period for a commercial process) could increase the conversion 20% over that observed in 6 hours (see figure 11). Based on these conclusions, adequate conversions of cellulose could be obtained with reduced enzyme charges by increasing the hydrolysis time.

Therefore, an overall process using autohydrolysis to pretreat agricultural residues followed by enzymatic hydrolysis and yeast fermentation could be a feasible route to obtain useful liquid hydrocarbons. A great deal of further work is needed to confirm the economic potential of such a process, but the basic technology looks quite promising.

CONCLUSIONS

1. When enzymes were used to catalyze the hydrolysis of pretreated wheat straw substrate, higher conversions were observed versus those for non-pretreated straw. However, similar results were observed with both acid and enzyme catalyzed hydrolyses for delignified and non-delignified substrates whether or not the substrates were pretreated before delignification. This indicates that among the effects of pretreatment on the lignocellulose matrix, changes in cellulose morphology have a greater effect in increasing cellulose conversion than does a decreased lignin content.

2. Low conversions of cellulose observed during acid catalyzed hydrolyses versus conversions observed for mixed enzyme hydrolysis resulted from differences in their respective reaction mechanisms. Enzyme systems attack specific locations on a cellulose molecule with a high percentage of these attacks producing soluble reaction products. While, acid catalysts randomly attack cellulose bonds producing few soluble products per individual reaction. Thus, using the weight loss from a cellulose residue to measure extent of reaction, yields low conversions when acids are used to catalyze the reaction.

3. Similar total cellulose conversions observed for acid hydrolysis only versus acid hydrolysis plus autohydrolysis suggests the existence of a carbohydrate fraction that is easily hydrolyzed by acid catalysts. Conversion of this fraction of cellulose does not seem to be increased by pretreatment.

4. Straw lignin was rendered more extractible by pretreatment than wood lignin for the same autohydrolysis conditions. Time-at-temperature effects may have caused wood lignin to repolymerize, thus reducing its solubility in ethanol-water solvent.

SUGGESTIONS FOR FUTURE RESEARCH

1. If the use of acid hydrolysis to determine the effects of cellulose pretreatments is to be continued as an adjunct to enzyme hydrolyses, the following suggestions should be adopted. The hydrolyses should be stirred continuously and vigorously during the experiments, and the reducing sugar analysis method should be adopted to determine the degree of cellulose conversion in all cases.

2. The time-at-temperature conditions used for three substrates (barley straw, lodge pole pine, and Douglas fir) were those found to be optimal for the removal of lignin from wheat straw. These conditions may not be optimal for the other substrates or the optimal conditions for cellulose conversion. Therefore, experiments should be performed to determine the optimal conditions for cellulose conversion for each substrate.

3. The liquors from autohydrolysis experiments should be analyzed to determine if sugars produced by auto-catalyzed hydrolysis reactions are intact in the liquor or if they are destructed to furfurals.

4. Wood substrates of interest should be tested to determine if their enzymatic hydrolysis rate is increased by pretreatment as observed for wheat straw.

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Table 17. Results of the Acid Hydrolysis Development Experiments.

Substrate	Acid Concentration	Temperature (°C)	Time (hr)	% Hydrolyzed
Extract-Free Wheat Straw	10N	40	1.0	4.2
Autohydrolyzed Wheat Straw	10N	40	1.0	3.7
Autohydrolyzed Wheat Straw	12N	40	1.0	3.4
Deligninfied Wheat Straw	10N	40	1.0	1.9
Deligninfied Wheat Straw	14N	70	3.2	19.0
Autohydrolyzed Wheat Straw	14N	70	3.5	12.5
Deligninfied Autohydrolyzed Wheat Straw	18N	70	3.0	80.2
Deligninfied Autohydrolyzed Wheat Straw	18N	80	3.0	89.0
Extract-Free Wheat Straw	18N	80	3.0	56.2

TABLE 17. Results of the Acid Hydrolysis Development Experiments (continued).

Substrate	Acid Concentration	Temperature (°C)	Time (hr)	% Hydrolyzed
Deligninfied Wheat Straw	18N	80	3.0	98.7
Extract-Free Wheat Straw	18N	≈20	3.0	
	0.7N	80	4.0	62.8
Extract-Free Wheat Straw	18N	≈20	1.5	
	0.7N	80	4.0	60.6
Extract-Free Wheat Straw	18N	≈20	1.0	
	0.7N	80	2.0	59.2
Deligninfied Autohydrolyzed Wheat Straw	18N	≈20	1.0	
	0.7N	80	2.0	23.8
Extract-Free Wheat Straw	16N	25	1.0	
	0.7N	80	2.0	48.9
Extract-Free Wheat Straw	18N	25	0.5	
	0.7N	80	2.0	56.0
Autohydrolyzed/ Extracted Wheat Straw	18N	25	0.5	
	0.7N	80	2.0	15.1