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ABSTRACT

THE EFFECTS OF PHYSICAL AND CHEMICAL CONSTRAINTS ON THE ENZYMATIC HYDROLYSIS OF LIGNOCELLULOSIC MATERIALS

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Mixed hardwood (90 % birch, 10 % maple) was pretreated by four methods which produce substrates differing substantially in chemical composition and structural configuration. The pretreatments included alkaline peroxide delignification, solvent delignification with ethylenediamine, steam autohydrolysis in a plug flow reactor, and dilute acid hydrolysis in a plug flow reactor. The pretreated substrates were then hydrolyzed with Rutgers C30 cellulase at 40.8 FPU/g wood and their crystallinities, lignin contents, and surface areas available to solutes within the cellulase size range determined. The initial glucose yield was found to depend linearly on the surface area available to enzymes with diameters ranging from 24 to 77 Å, regardless of the method of increasing the surface area. Empirical correlations were also derived which also showed that crystallinity and lignin content have little effect on the initial rate of enzymatic hydrolysis.

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CHAPTER I: INTRODUCTION

Low value lignocellulosic materials such as wheat straw, sawdust and other agricultural residues are produced at a rate of about one billion tons (dry weight) annually in the United States[1]. It has been estimated that about 20 % of this biomass can be economically collected and fed into the production of chemicals[1]. It is evident that this represents a renewable resource that would be largely useful if properly utilized. Many chemicals can be produced from these lignocellulosic materials, including ones useful in producing energy, such as ethanol[2,3], ethylene, propylene and related compounds[4]. Other uses include production of high energy animal feeds[5], polymer feedstocks[4] and organic solvents[4].

Lignocellulose is a highly heterogeneous 3-dimensional matrix comprised of cellulose, hemicellulose and lignin. Cellulose, the major component of the matrix, comprising about 40-50 %, is a linear glucose polymer consisting of β-D-1,4-anhydroglucopyranose units and is the source of glucose from which many chemicals could be derived. Hemicellulose, comprising about 20-30 % of the matrix, is a mixed polymer consisting mostly of xylose (in hardwoods) or mannose (in softwoods) and some galactose, glucose, arabinose, and glucuronic and galacturonic acids. The remainder of the matrix is mostly lignin, a polymer of various phenolic momomers[6] and is dispersed throughout the matrix. This lignin barrier serves to protect the cellulose and hemicellulose from microbial degradation.

The lignocellulosic matrix makes up the cell walls of plants. Surrounding the cell is the middle lamella (see Figure 1.1), a highly lignified region containing lignin and hemicellulose in a ratio of about 70 %:30 %[8]. In the cell wall, there are four distinct morphological layers. The outermost layer, or primary wall, contains approximately one-third cellulose mixed with lignin and hemicellulose in a loose and random network[8]. The secondary wall is composed of three layers: S1, S2, and S3[9]. In the outer layer, or S1, the cellulose microfibrils are wound in flat helices, while in the middle layer (S2) the microfibrils are tightly packed in a parallel helix[8]. It is not clear how the microfibrils are arranged in the innermost (S3) layer. Typically the secondary wall contains a majority of cellulose[8].

This type of arrangement presents a problem of accessibility for cellulases, which degrade the cellulose polymers, since these enzymes are generally very large in comparison with the spaces between the fibers into which they must diffuse. In addition, it has long been thought that the arrangement of the cellulose in the secondary wall, or its crystallinity, might have some exclusion effects on the enzymatic action since direct contact between the enzyme and fiber are necessary for cellulolytic activity[9,10].

It is a well accepted notion[9] that some sort of pretreatment of the lignocellulosic material is necessary to make the cellulose in the matrix more susceptible to cellulolytic attack. Various types of

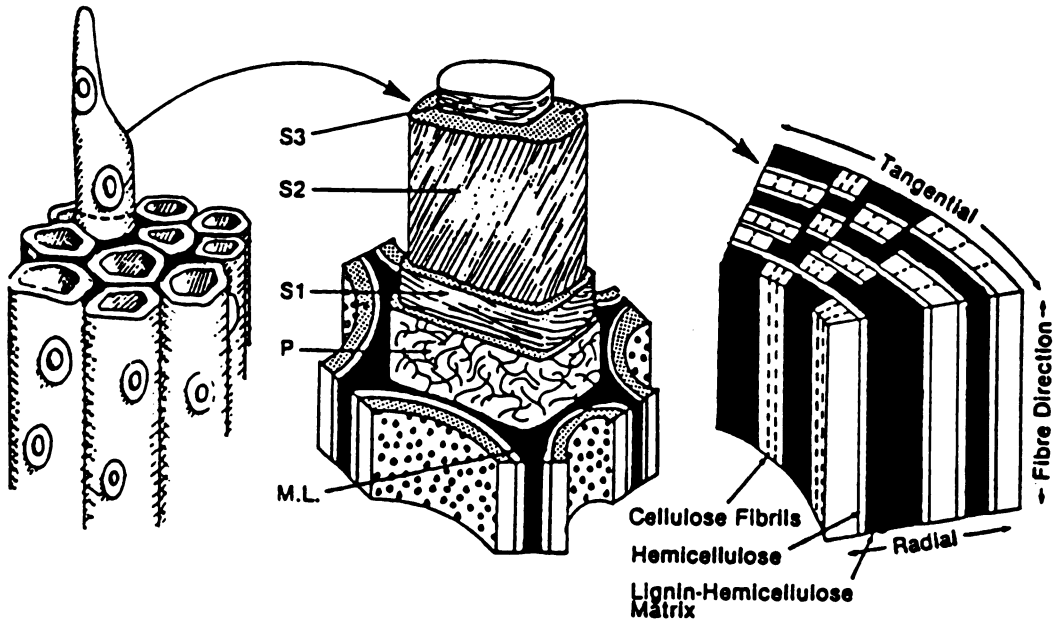


Figure 1.1: The cell wall of wood is composed of three distinct layers, S1, S2, and S3, arranged as shown [7].

pretreatments have been employed, such as treatment with acids[11,12], alkalis[13,14], microwaves[15], and many others. Most have produced an increase in enzymatic glucose yield, although to varying degrees. The unifying question among the pretreatments, however, is not the fact that they all increase glucose yield but how they do it. In all cases, some component of the matrix is removed, completely or in part, to open up the pores of the matrix to the cellulase[9]. Yet some doubt still exists as to the importance of the various factors which are thought to influence the rate of cellulolytic hydrolysis, such as crystallinity[10], lignin content[16], and cellulose surface area available to the cellulase enzyme[17]. In addition, it is often difficult to compare the results of pretreatments, since researchers invariably use different cellulases and/or different substrates to test pretreatment effectiveness.

In this work, a common substrate, mixed hardwood (90 % birch, 10 % maple), was pretreated by four methods which modify the structure and composition of the lignocellulosic matrix, each in a different manner. These included dilute acid hydrolysis in a plug flow reactor, steam autohydrolysis in a plug flow reactor, organosolvent delignification with ethylenediamine, and alkaline peroxide pretreatment. The lignin content, crystallinity and surface area available to a *T. reesei* cellulase for each pretreated substrate were determined and combined with enzymatic hydrolysis yields at various times of hydrolysis. These data were used to show a linear

correlation between initial glucose yield from enzymatic hydrolysis and surface area available to the cellulase enzyme. Empirical fits of the initial rate data with crystallinity index, glucan to lignin ratio, and available surface area were also determined. These fits also showed that available surface area is the limiting parameter in the initial stages of enzymatic hydrolysis, regardless of how it was created.

This thesis is divided into five chapters, including this first chapter (Introduction). Relevant literature are presented in Chapter II, focusing on cellulases, factors limiting cellulolytic hydrolysis of cellulosics and lignocellulosics, and pretreatments to enhance cellulolytic hydrolysis. The third chapter is devoted to pretreatment and analytical protocols for all techniques used to obtain data for this work. Numerical results of this study are presented in Chapter IV, while their interpretation and significance are discussed in Chapter V. Finally, pertinent equations are derived in Appendix A, and data used in figures are listed in tabular form in Appendix B.

CHAPTER II: LITERATURE SURVEY

2.1 Introduction

Lignocellulosic biomass occurs in nature in a variety of forms. Agricultural residues such as corn stover, wheat straw and chaff, rice straw and chaff, and bagasse (from sugarcane or other sources) are produced at a rate of nearly one billion tons annually in the United States alone, although only 20 % of this can be economically collected and utilized[1]. The lumber and pulp and paper industries also produce large amounts of sawdust, bark, wood chips, and waste paper pulp. In addition, urban refuse contains large amounts of waste paper products, such as newspaper, cardboard, and so on. These lignocellulosic wastes constitute a significant renewable resource, and thus considerable research efforts have gone into developing economically feasible processes for the conversion of lignocellulosics into useful products[3,9,18].

The major barrier to these efforts, however, is that the complex lignocellulosic structure, engineered by nature to protect plants from microbial attack, also presents a barrier for the degradation of these materials to their substituents by man. When broken down, cellulose yields glucose, which can be fermented to ethanol or used to produce other chemicals[4]. Hemicellulose gives a number of five- and six-carbon sugars, several of which can be fermented to ethanol[2]. Lignin, which is a complex polymer made up of a variety of phenylpropane units[9], gives aromatic molecules which could serve as octane

enhancing additives for conventional fuels. Some of the more relevant topics in the conversion of lignocellulosics will be reviewed in this literature survey, with particular emphasis given to factors affecting cellulolytic conversion of the cellulose in the lignocellulosic matrix, and pretreatments for this conversion.

2.2 Pretreatment Rationale

Most of the research to date on the conversion of lignocellulosics has gone into the conversion of the cellulose to glucose, since glucose can easily be fermented to ethanol using common yeasts[9]. Dilute acid hydrolysis of the cellulose to glucose is a relatively simple process which can give moderately high yields in relatively short times[9]. Protons are small, so accessibility of the catalyst to the glucosidic bonds is not a problem. The sugar monomers produced, however, can undergo acid-catalyzed reactions to form degradation products. The two most common degradation products, furfural (from xylose) and hydroxymethylfurfural (from glucose), can condense to form insoluble polymers which precipitate and cause reactor problems, besides reducing yields. Unfortunately, while yields increase with increasing hydrolysis temperature, so does the formation of these degradation products[19]. Cellulase enzymes, on the other hand, are specific for cellulose and form only glucose. The hydrolysis conditions are mild (50 °C, pH 4.80)[9] and thus there are no degradation products. Cellulases, however, are relatively large enzymes, much too large to fit into the spaces in the lignocellulosic

matrix[18]. Some researchers have tried using methods such as reducing cellulase size[20] to increase accessibility and using surfactants to increase cellulase adsorption on the available surface area[21], but it is a generally accepted notion that some sort of pretreatment must be employed to increase the accessibility to cellulases[3,9,18]. Although opinions differ as to which property of lignocellulosics is most important, a "good" pretreatment will normally remove one or more components of the matrix, in addition to swelling the matrix and the crystalline cellulose microfibrils[9].

2.3 Cellulases

2.3.1 Sources of Cellulases

Extracellular cellulases are produced by a number of wood and plant degrading fungi and bacteria. While many microorganisms are capable of growing on cellulose, only a relatively small fraction of these can produce extracellular cellulases[22]. Fungi that produce extracellular cellulases include several *Trichoderma* species, such as *T. reesei*, *T. viride*, *T. kongii*, and *T. lignorum*. Other fungal species include *Penicillium funiculosum*, *A. wentii*, and several others. Bacterial species which produce extracellular cellulases include *Cellulomonas* and *Clostridium thermocellum*. In the early 1950s, it was found that *T. reesei* (thought to be a *T. viride* at that time) produces a cellulase complex which has excellent activity on crystalline cellulose[23]. *T. reesei* cellulases, as well as cellulases produced by mutant strains of *T. reesei*, are among the most common

cellulases used today[9]. In particular, the *T. reesei* QM9414 and *T. reesei* Rut-C30 strains have high cellulase productivities[9], and *T. reesei* Rut-C30 has the added benefit that it is resistant to catabolite repression[24].

2.3.2 The Cellulase Complex

The term "cellulase" refers not to a single enzyme, but to a family of enzymes which act synergistically to degrade the cellulose polymers. The separate components can be grouped into three classes: endoglucanases, exoglucanases and β-glucosidases (or cellobiases). Endoglucanases, or endo-β-1,4-glucanases, attack the glucosidic bonds between molecules in the interior of the chains, forming short oligomers. These enzymes constitute 15-20 % of the total extracellular protein produced when *T. reesei* is grown on cellulose[9]. As many as six endoglucanases have been reported[9]; each showed significant differences in its specificity and in the number of glucose units in its final oligomeric product. Exoglucanases, or exo-β-1,4-glucanases, catalyze the hydrolysis of the glucosidic bonds in an end-wise manner, releasing cellobiose; because of this, exoglucanases are often called cellobiohydrolases. Exoglucanases, of which there are two in *T. reesei* cellulase, comprise 35-85 % of the extracellular protein when *T. reesei* is grown on cellulose[9]. β-glucosidases (cellobiases), as their name implies, cleave cellobiose molecules to give two moles of glucose per mole of cellobiose. These enzymes comprise less than 1 % of the extracellular protein

produced when *T. reesei* is grown on cellulose. For this reason, it is often necessary to supplement *T. reesei* cellulase with β-glucosidase from another source[9], for example *Aspergillus niger*[25]. This helps to keep cellobiose levels low.

2.3.3 Cellulase Dimensions

Cellulases from various sources are quite different in size and molecular weight. Since there are so many different enzymes in the cellulase complex, it is difficult to make a "universal" estimate of cellulase dimensions. Depending on the substrate and the fermentation conditions, different amounts of each component will be produced, resulting in different average dimensions. Cowling[6] compiled data on molecular weights and gel filtration for cellulases from various sources and estimated the dimensions of their cellulase complexes. The results showed that if the cellulases are hydrodynamic spheres in solution, their diameters range from 24 to 77 Å, while if they are ellipsoids with an axial ratio of about six, their sizes range from about 13 × 79 Å to 42 × 252 Å in width and length, respectively[6]. Both spherical and ellipsoidal dimensions have been used by researchers in the past. Van Dyke[26] used ellipsoidal dimensions (35 × 210 Å) in a study of cellulase accessibility, while Grethlein[17] preferred to use the mean of the spherical diameter range (51 Å) in a similar study. Stone *et al.*[27] concluded that the cellulase produced by rumen microorganisms was excluded from pores smaller than 40 Å in swollen cotten linters, using the solute exclusion

technique[28]. All values previously used appear to be within the ranges reported by Cowling[6], although it is still uncertain what steric conformation is maintained in solution by the cellulase complex. A recent study using FPLC and small-angle X-Ray scattering to characterize *T. reesei* cellulases suggests that the shape of one of the cellobiohydrolases from the complex is that of a tadpole, with a maximum width at the head of 44 Å and a total length of 180 Å[29]. The size of this enzyme is therefore well within the reported ranges for cellulase size, although it is only one of the enzymes involved. One possibility is that the separate enzymes are excluded from pores based on their own dimensions and not on the basis of the cumulative cellulase complex dimensions. Then the size ranges reported by Cowling[6] might be viewed as an average over the separate cellulase components.

2.3.4 Cellulase Adsorption

One of the first steps in the enzymatic hydrolysis of cellulose is the physical adsorption of the cellulase onto the substrate. Reese[30] showed that cellulase adsorption is a function of enzyme concentration, available surface area and its nature, physical properties of the enzymes present, and adsorption conditions (for example, pH, salt concentration and temperature). Kim *et al.*[31] reported strong pH effects on the adsorption of *T. viride* cellulase using microcrystalline cellulose as substrate. Adsorption was maximum at pH 4.8, falling sharply with pH in either direction. This

result is contrary to results reported by other researchers[9], in which it was shown that pH had little effect on adsorption in the range 3.5-5.5 although they showed a maximum, also at pH 4.8. The discrepancy may be due to the use of different enzymes, or to differences in the amounts of each component enzyme in the complex, since endoglucanases preferentially adsorb on crystalline substrates[9]. Temperature also has a large effect[9,31] on the adsorption of cellulases. Mandels *et al.*[32] showed that that adsorption rate of cellulase on Solka Floc increased with temperature, but the maximum amount adsorbed showed the opposite trend[33]. Kim *et al.*[31] also also reported a decrease in protein adsorbed with increasing temperature using microcrystalline cellulose, while Bisaria and Ghose[34] found that the specific adsorption increased with temperature up to a maximum at 50 °C, using alkali-treated bagasse as substrate. Perhaps the differences in the trends occur because of choice of substrate; both groups that reported decreasing adsorption with increasing temperature used essentially pure cellulose, while the opposite trend was observed using a lignocellulosic substrate. It was observed[31] that even though adsorbed protein decreased with increasing temperature, enzyme activity increased to a maximum at 50 °C. This led the authors to speculate that the active enzyme was bound more strongly to the cellulose surface at higher temperatures. Whatever the trends, 50 °C seems to be the optimum hydrolysis temperature at the pH optimum of 4.8.

Specific surface area was also found to be a major determinant in protein adsorption[9,31,35]. Lee and Fan[35], using Solka Floc as substrate, came to the following conclusions: (1) The hydrolysis rate is influenced little by mass transfer limitations of the enzyme; (2) The enzymatic portion of the soluble protein (cellulase preparations are inherently impure) is more readily adsorbed than the nonenzymatic portion; (3) The maximum hydrolysis rate and maximum extent of soluble protein adsorption is attained at the onset of hydrolysis; (4) The initial extent of soluble protein adsorption and the initial hydrolysis rate are linearly related to the initial enzyme concentration; (5) The extent of initial soluble protein adsorption increases approximately linearly as the specific surface area increases; and (6) The hydrolysis rate depends on: (a) the initial extent of protein adsorption, which depends on initial cellulose concentration, enzyme concentration and specific surface area; and (b) the effectiveness of the adsorbed protein to promote the hydrolysis, which is related to the crystallinity of the cellulose.

2.4 Properties of Lignocellulosics Which Limit Enzyme Accessibility

Several physical properties of lignocellulosics limit the accessibility of the cellulose to the cellulase complex[9]. These include specific surface area available to the enzyme, cellulase crystallinity, and lignin content. Researchers studying microcrystalline cellulose tend to place more emphasis on

crystallinity, while with lignocellulosics, their observations normally place more importance on surface area, and in a related fashion, distribution of lignin in the matrix. These points will be discussed in the following sections. Since hemicellulose is easily hydrolyzed and is normally removed in most pretreatments[9], it will not be discussed here.

2.4.1 Available Surface Area

Wood and cotton fibers contain two types of voids: (1) gross capillaries (0.02 to 10 μm diameter) including cell lumina and pit aperatures, and (2) cell-wall capillaries, which include spaces between amorphous regions in the cellulose and between microfibrils. The cell wall capillaries are closed off when the material is dry, but when fully saturated with water, they are often as large as 0.02 μm in diameter[18]. Removal of matrix components serves to increase this pore size.

Two models have been proposed for the microstructure of cellulose fibers. In the Fringed Micellar Model[36], the cellulose fibers are envisioned to have a crystalline core 50 × 100 Å in cross section, surrounded by an amorphous sheath containing regions of order and disorder along the length of the fiber (see Figure 2.1). It was postulated that these fibers associate into microfibrils which contain 15-40 cellulose chains and are segmented into crystalline and paracrystalline regions (Figure 2.2). The Folding Chain Model[37] proposes that the basic unit is the platellite, with a cross section of

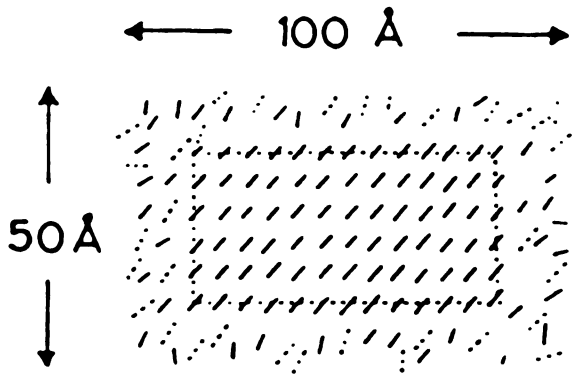


Figure 2.1: The crystalline core envisioned in the Fringed Micellar Model [36]. Figure is from Cowling and Kirk [18].

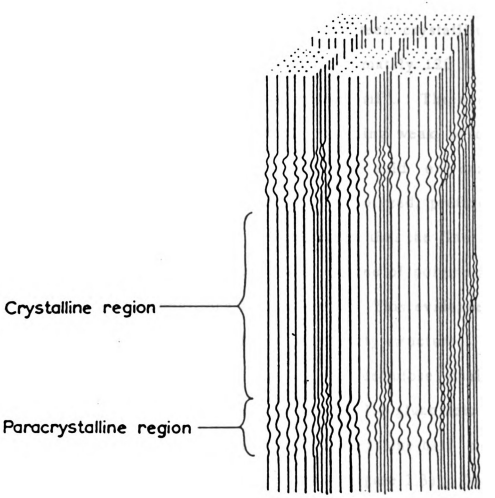


Figure 2.2: Packing arrangement of the cellulose cores into elementary microfibrils in the Fringed Micellar Model. Figure is from Cowling and Kirk [18].

36 × 6 Å and a degree of polymerization (DP) of up to 1000 glucose residues in each platellite. Several of these platellites pack into an elementary fibril, called a crystallite, and sixteen crystallites pack into a fibril, giving a cross section of approximately 120 Å square. Four fibrils pack together to give a microfibril[36]. Three or four glucose bonds are involved in each fold, providing weak, susceptible links for cellulase attack. During dilute acid or enzymatic hydrolysis, these microfibrils are partially hydrolyzed, preferentially in the amorphous regions. It has been observed that the final DP for a given substrate (called the "leveling-off" degree of polymerization, or LODP) at the same conditions is constant[37-39]. This evidence seems to support the folding-chain model with its orderly structure.

Regardless of the correct model, the cellulose chains are linearly aligned in microfibrils with the β-1,4-glucosidic bonds between the anhydroglucopyranose units buried one-half molecule deep on the surface of the microfibril[36] (see Figure 2.3). Thus, the number of glucosidic bonds accessible to the enzyme will be proportional to the surface area of the microfibrils that is accessible. As with most enzymes, the cellulase must come into direct contact with the glucosidic bond to catalyze its hydrolysis[18]. Thus, the rate of enzymatic hydrolysis should depend directly on the available surface area. Whether or not the dependence is linear will depend on the effects of other factors, such as enzyme concentration, crystallinity, and lignin content. If these effects are small or

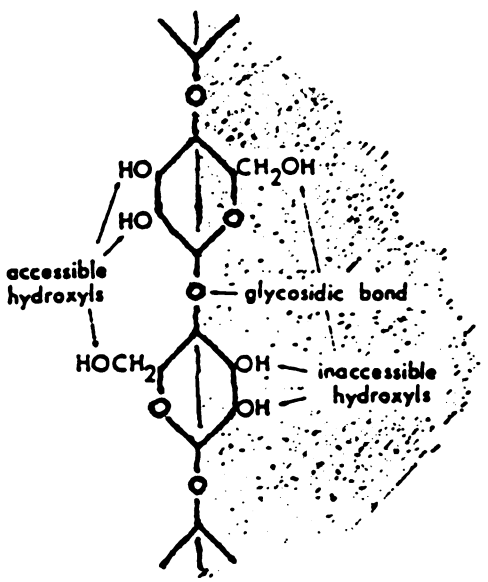


Figure 2.3: Arrangement of the glucosidic bonds on the surface of a microfibril [36].

negligible, the dependence will be essentially linear. Stone *et al.*[27] reported a linear relationship between the initial rate of enzymatic hydrolysis and available surface area in acid-swollen cotton. Grethlein[17] reported the same linearity in a study of dilute acid hydrolysis of various lignocellulosic materials. Grous *et al.*[40] also reported a linear relationship between initial rate of enzymatic hydrolysis and surface area in steam exploded poplar. In this study the same enzyme and activity used by Grethlein[17] were used; remarkably, the slopes of both correlations were nearly identical (0.375 and 0.326 %/m^2, respectively). These results suggest that surface area available to the enzyme is the initial limiting factor, although the correlations were not tested with delignified substrates.

Fan *et al.*[41,42] developed an empirical correlation for microcrystalline cellulose relating the relative extent of hydrolysis in eight hours (REH) to specific surface area (SSA) measured by nitrogen adsorption, and crystallinity index (CrI):

$$REH = 0.380\ (SSA)^{0.195}\ (100 - CrI)^{1.04} \qquad (2\text{-}1)$$

which indicates that SSA has only a small effect, while crystallinity is much more important, at least in the case of microcrystalline cellulose. For wheat straw, however, Gharpuray *et al.*[43] developed a similar correlation which showed a much greater dependence on surface area, while exhibiting a much smaller effect of crystallinity and a moderate lignin effect:

$$REH = 2.044\ (SSA)^{0.998}\ (100 - CrI)^{0.257}\ (Lignin)^{-0.388} \qquad (2\text{-}2)$$

These results clearly show that the enzymatic hydrolysis of microcrystalline cellulose and lignocellulosic materials are fundamentally different.

2.4.2 Cellulose Crystallinity

The close association of cellulose chains and the nature of its monomers, *i.e.* hydroxyl groups, creates an ideal situation for interchain hydrogen bonding in a highly oriented manner[9]. Crystallinity may be measured by a number of methods, which can be placed into two categories, those giving cotton a crystallinity of about 70 %, and those giving a cotton crystallinity of about 90 %[36]. Methods in the first category include X-Ray diffraction in its older interpretation, density, deuterium exchange, infrared absorption, by dielectric constant, water sorption, and heat of wetting[36]. Those in the second category include thallation, formylation, acid hydrolysis, and periodate oxidation[36]. In the past there has been considerable disagreement on which method is best, although the X-Ray diffraction method of Segal *et al.*[44] is widely accepted today. The structure of the unit cell of crystalline cellulose is depicted in Figure 2.4, with the lattice planes labeled. The 002 plane diffracts near $2\theta = 22.8°$, while completely amorphous cellulose gives a diffracted minimum near $2\theta = 18°$. The crystallinity index (CrI) of Segal *et al.*[44] is then defined as:

$$CrI = \{(I_1 - I_2)/I_1\} \times 100 \qquad (2\text{-}3)$$

where I_1 is the maximum diffracted X-Ray intensity (near $2\theta = 22.8°$)

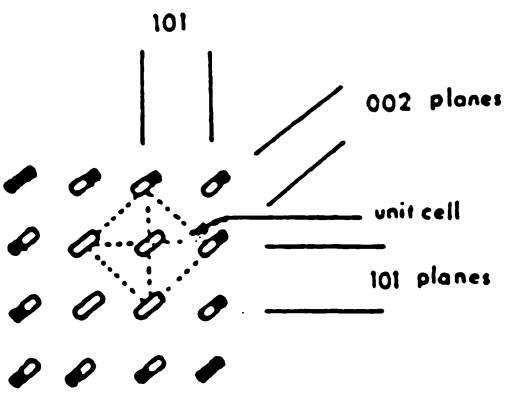


Figure 2.4: The unit cell of crystalline cellulose, showing the 002 plane. Figure is from Scallan [36].

and I_2 is the minimum diffracted X-Ray intensity (near $2\theta = 18°$).

Cellulases act very differently on crystalline and amorphous celluloses. Ryu *et al.*[45] found that the affinity constant of the enzyme for amorphous cellulose was one-tenth of that for crystalline cellulose, while the maximum rate of hydrolysis was 40 times that for crystalline cellulose. Fan *et al.*[41] showed an inverse linear relationship between CrI and glucose production from Solka Floc using *T. reesei* cellulase. Their later correlation[42], equation (2-1), has an exponent of 1.04 for the crystallinity term, indicating that crystallinity has a large effect on the enzymatic hydrolysis of a cellulosic substrate. Klesov and Sinitsyn[46] hydrolyzed cotton linters with *T. reesei* and *A. niger* cellulases and found that the initial rate of hydrolysis increased as CrI decreased and as surface area increased. Lee and Fan[35] reported that surface area affected protein adsorption, while the effect of crystallinity was to decrease the effectiveness of the adsorbed enzyme.

For lignocellulosic substrates, the dependence of the hydrolysis on CrI seems to be less pronounced. The small exponent of 0.257 in equation (2-2), reported by Gharpuray *et al.*[43] underlines the differences between microcrystalline cellulose hydrolysis and hydrolysis of lignocellulosics: surface area is the more important factor for lignocellulosics, while CrI dominates for microcrystalline cellulose. Fan and Lee[47], however, noted that although the initial surface area may be important in the initial stages of the hydrolysis,

as amorphous cellulose is removed, the crystallinity may become the overriding factor. Several groups[48,49] have reported that in the enzymatic hydrolysis of lignocellulosics, sometimes pretreated substrates with higher crystallinities than the untreated material gave higher rates and extents of hydrolysis. It was concluded[49] that an increase in crystallinity in the pretreated substrate was due to the removal of amorphous cellulose and that the CrI was probably not as important as previously thought.

2.4.3 Lignin Content

Lignin, a complex aromatic polymer based on phenylpropane units, contains few hydrolyzable bonds and thus is not very susceptible to hydrolytic enzymes. The various types of monomer units and inter-monomer bonds in spruce lignin are shown in Figure 2.5. Together, lignin, hemicellulose, and cellulose form a very resistant material, both through association in the matrix and through chemical linkages[50]. The resulting material is resistant to both chemical and biological attack[51]. The presence of hemicellulose also limits accessibility to the cellulose, although it is easily hydrolyzed and does not present as much of a barrier as does lignin[9]. Little is presently known about the role of hemicellulases in fungal wood degradation[52,53]. Blanchette *et al.*[54] has recently shown that hemicellulose is removed along with lignin during fungal degradation, suggesting that a similar accessibility problem to that for cellulases may exist for both hemicellulases and ligninases. Lignin and the

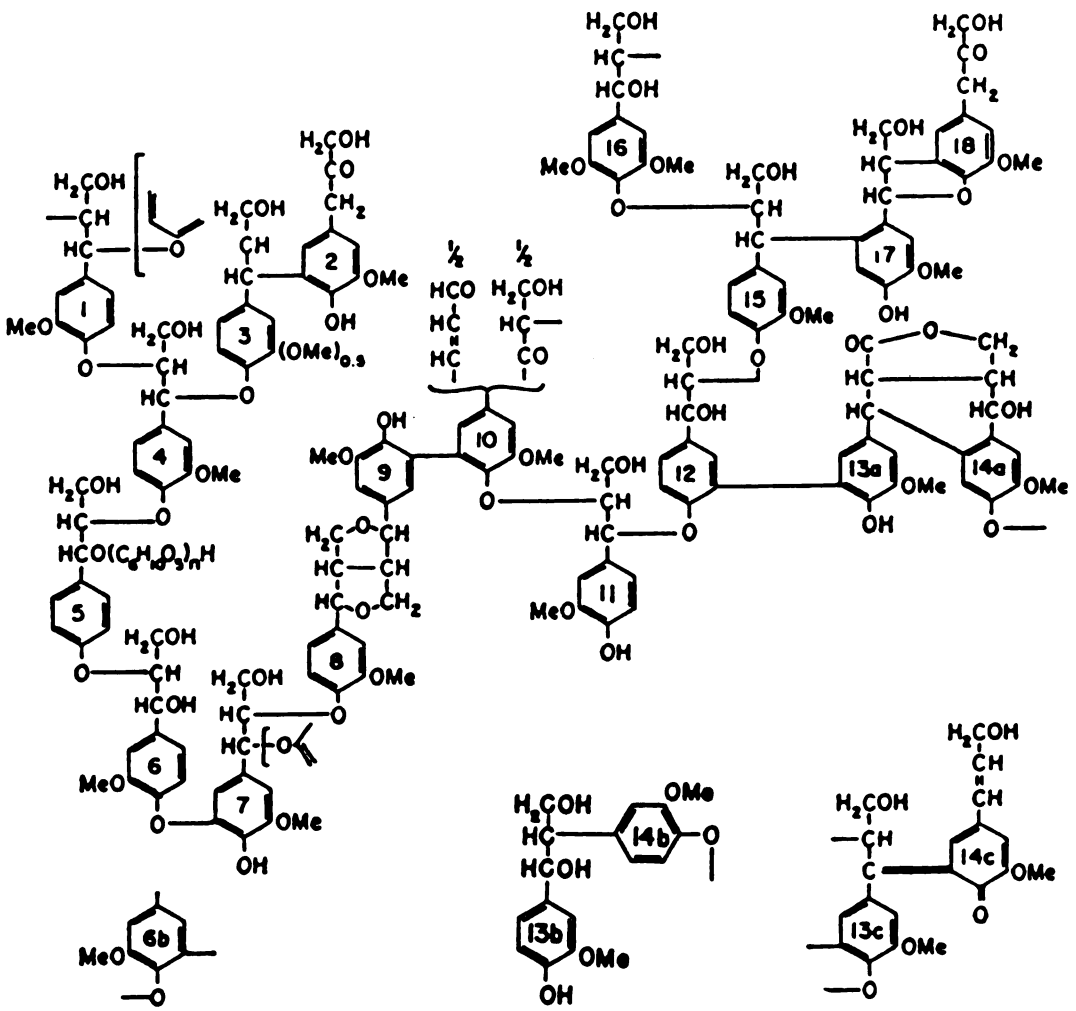


Figure 2.5: Typical monomer units and inter-monomer bonds found in spruce lignin [18].

compounds associated with it have been found to inhibit cellulase production, although no inhibition of *in vitro* cellulase activity was found[55].

Delignification enhances the enzymatic hydrolysis of softwoods more than for hardwoods[56]; this is possibly because softwoods are more heavily lignified than are hardwoods[57]. Sudo *et al.*[56] reported that the increase in the rate of cellulolytic hydrolysis in softwoods was small with initial removal of lignin, but increased greatly with increased delignification. Millet *et al.*[58] and Fan *et al.*[59], using alkali-treated hardwood and wheat straw pretreated by several methods, respectively, reported that cellulose hydrolysis increased sharply with initial delignification and leveled off after about half the lignin had been removed. Stranks[60] suggested that the physical relationship between lignin and the carbohydrates is responsible for the effect of lignin, and not the actual amount of lignin. Saddler *et al.*[61] agreed, stating that the effect of lignin on accessibility of cellulases is due to its distribution in the matrix rather than the lignin content itself. The complex association of lignin with the cellulose and hemicellulose polymers reported by Ruel *et al.*[51] would seem to support these suggestions.

2.5 Pretreatments

Pretreatments to enhance enzymatic hydrolysis can generally be placed into two major categories: physical and chemical. Physical pretreatments, which include various types of grinding, milling, and

irradiation pretreatments, aim to physically modify the crystallinity, specific surface area, or both. Chemical pretreatments can be classed into three major types: (1) those which solubilize the cellulose; (2) those which remove one or more matrix components to increase cellulase accessibility; and (3) those which swell the matrix. Some chemical pretreatments may fit into one or more of these classes, since often swelling of the matrix occurs when a matrix component is removed, as is the case with alkali treatments[62]. Physical pretreatments are generally more effective with cellulosics, while chemical pretreatments are generally better with lignocellulosics. This is expected since cellulosics are very nearly pure cellulose and have only small amounts of other components that can be removed by chemical pretreatments; a possible exception is with the swelling action of alkali treatments.

2.5.1 Physical Pretreatments

Ball milling, the most common form of physical pretreatment, decreases crystallinity and particle size, causing an increase in specific surface area and increasing water solubility[63]. Mandels *et al.*[64] found ball milling to be the most effective pretreatment for newsprint, although Wilke *et al.*[65] found it to be too expensive in a process design study. In a study of ball milled hardwoods and softwoods, Matsumura *et al.*[66] observed that the rate of hydrolysis increased as crystallinity and particle size decreased, although they concluded that it was the physical and chemical state of the lignin

that was the major factor inhibiting the hydrolysis. Millet *et al.*[67] ball milled cotton linters, red oak, and a variety of other lignocellulosics and noted that the CrI decreased to zero as the milling time was increased. Complete enzymatic conversion was attained in ten days with cotton linters milled for one hour, and 93 % conversion was attained with red oak milled for four hours. Rivers and Emert[68], in a comparative study of wet and dry ball attrition of lignocellulosics, found that the terminal CrI (after enzymatic hydrolysis) decreased after wet ball attrition, but increased after dry attrition; even though the particle size was smaller for the dry milled substrates than for the wet, there was not a significant difference in their enzymatic conversion. This is possibly because they were lignocellulosics, since lignocellulosics exhibit less dependence on particle size and CrI than do cellulosics. Surface area in cellulosics is mainly external since the lignin and hemicellulose matrix has already been removed, and the remaining cellulose collapses to form a crystalline material. Lignocellulosics, on the other hand, contain most of their surface area in the interior of the matrix, in pores. Since ball milling increases external surface area only, only a small effect is expected for lignocellulosics.

Another type of milling which is potentially useful in pretreating lignocellulosic is compression or roll milling. In one report, Tassinari and Macy[69] found that the extent of hydrolysis of wood chips increased 7 to 17 times after compression milling, while

the hydrolysis of newspaper was increased only modestly (2 times). The susceptibility of hardwoods and agricultural residues are increased more by compression milling than for softwoods[70,71], possibly because of plasticization of the softwoods due to their chemical content. Gharpuray *et al.*[43] found a more than three-fold increase in the extent of hydrolysis with compression milled wheat straw, which accounted for about 75 % of that attainable with ball milling.

Hammer milling does not disrupt the fiber structure, but chops the fibers into smaller pieces, creating only new ends. Thus, this type of milling has little or no effect on enzymatic hydrolysis[64,72].

Irradiation pretreatments are another class of physical pretreatment which shows potential utility. Kumakura and Kaetsu[73] pretreated rice straw, rice husks and cedar sawdust with electron beam irradiation at 2×10^6 rad/min and found that with a 5×10^8 rad exposure, 80-90 % yield of reducing sugars was attainable in all cases after 35 hours of enzymatic hydrolysis. Khan *et al.*[74] found that with spruce chips, electron beam irradiation produced a substrate approximately equivalent to untreated filter paper cellulose; 90 % conversion was observed in 24 hours using *T. reesei* Rut-C30 cellulase. Pigden *et al.*[75] pretreated forage crops with γ-ray irradiation and found that with 2.7×10^8 rad exposure, digestibility increased but that the dose was too small to solubilize the cellulose, while Han *et al.*[76] γ-irradiated various lignocellulosics in swelling

agents such as NaOH and ethylenediamine and found that mostly oligosaccharides and glucose degradation products were formed.

Microwave irradiation has also been studied extensively[15,77,78]. When the pretreatment is done in water, autohydrolysis occurs as acetyl groups are cleaved from the hemicellulose (so microwave treatments might also be termed acid treatments)[77]. Ooshima *et al.*[77] irradiated rice straw and bagasse of 84 to 94 % moisture content with microwaves and observed 1.6 and 3.2-fold increases, in enzymatic conversion, respectively. Azuma *et al.*[15,78] pretreated various lignocellulosics with microwaves and found that the maximum enhancement of enzymatic susceptibility occurred in the temperature range 223-229 °C, regardless of the lignocellulosic source. Yields of 77 to 84 % were obtained using bagasse, rice straw, and rice hulls, while yields ranged from 65.9 to 93 % for a softwood, a hardwood, and bamboo. A possible reason why they found no difference in the optimal enhancement temperature could be that the xylose was degraded to furfural, which then polymerized and precipitated, blocking the pores of the matrix. This is plausible, since they reported that both acidity and furfural production increased with increasing temperature[78].

Other types of physical pretreatments used with lignocellulosic materials include freeze-explosion[79,80] and cryomilling[81,82].

2.5.2 Chemical Pretreatments

Chemical pretreatments are designed to increase accessibility to cellulolytic enzymes by removing a matrix component, swelling the matrix, dissolving the cellulose, or by a combination of these. Many different chemicals have been used to pretreat lignocellulosics. Five general classes of chemicals may be conveniently grouped: (1) alkali pretreatments; (2) acid pretreatments, including dilute mineral acids and autohydrolysis; (3) delignification; (4) cellulose solvation treatments; and (5) pretreatment with living organisms or *in vitro* enzymatic treatments.

2.5.2.1 Alkali Pretreatments

Alkali pretreatments disrupt the structure of the lignocellulosic matrix by removing part of the lignin and hemicellulose, swelling the fibers and often decreasing the cellulose crystallinity[62]. NaOH is the most common alkali used[83-87], although aqueous or liquid NH_3 is often used[58,83,84,88]. Ammonia explosion[89] and supercritical ammonia[13,90] pretreatments have also been used. Other systems include $Ca(OH)_2$ and Na_2CO_3[14], and alkaline peroxide[5,91-93].

With NaOH treatments, concentrations will commonly vary between 0.5 and 5 %, and up to 1 g NaOH/g substrate. Temperatures vary from room temperature up to 160 °C, with reaction times as short as 30 minutes or as long as 2 to 3 days. Higher temperatures allow for lower NaOH concentrations and shorter reaction times while increasing hemicellulose removal, often more so than lignin removal[9].

The NaOH is normally removed by washing. NH_3 or NH_4OH treatments provide an economical alternative to NaOH pretreatment, since NH_3 can be recovered by a simple evaporation process; however, they are not nearly as effective pretreatments as NaOH[84]. Alkaline peroxide often gives increased enzymatic susceptibility over NaOH alone; in addition, room temperature is adequate and liquor recycle is possible[92]. Mild alkali (less than 10 % NaOH) cause intercrystalline swelling, while stronger alkalis (greater than 10 % NaOH) tend to cause intracrystalline swelling[9]. Hemicellulose is removed by solubilization, rather than by hydrolysis of its glycosidic bonds[9]. Lignin removal appears to increase with alkali concentration up to a maximum removal and then level off[94]. Alkalis have little effect on cellulose, with little or no removal at 5 % NaOH or less[9].

The major effect of alkali treatments is the hydrolysis of ester-type lignin-carbohydrate bonds, solubilizing the hemicellulose and swelling the matrix[9]. As an example, alkali treatment of a hardwood increased accessible protein size from a molecular weight of around 30,000 to 40,000-50,000[83]. The fiber saturation point, as measured using the nonsolvent water content technique[95], increased from 40 % to 70-80 %.

2.5.2.2 Acid Pretreatments

In a dilute acid pretreatment, hemicellulose is preferentially attacked, followed by amorphous cellulose[96]. In contrast to alkali treatments, during acid hydrolysis the hemicellulose is removed by

hydrolysis of its glycosidic bonds; often, nearly complete removal of the hemicellulose can be attained[97]. Critical parameters in acid pretreatments include temperature (and pressure), time, and acid to solids ratio if the solids concentration is above 10 %. The rate of hemicellulose degradation increases with temperature; however, monomer degradation is highly time dependent, so reactor designs tend to use higher temperatures and shorter times[98,99]. Actual conditions will vary with equipment design and the aim of the process[11,98,100-102]. Acid-treated cellulose is also susceptible to recrystallization[11]. During pretreatment, lignin is often depolymerized (from carbohydrate-lignin bond breakage) and then repolymerized[103,104]; some of this lignin can then be extracted with organic solvents[9].

Autohydrolysis, which is similar to dilute acid hydrolysis, occurs when the dry substrate is "cooked" under high temperature (high or low pressure) steam[16,80,105-107]. The catalyst in this case is acetic acid, which is formed when acetyl groups are cleaved from the hemicellulose at high temperature[3,106].

Steam explosion is carried out on dry substrates at high temperature and pressure, but unlike autohydrolysis, the mixture is explosively decompressed to atmospheric pressure, causing the fibers to explode. According to Delong[108], the beneficial effects of steam explosion include: (1) the reaction is rapidly quenched; (2) liquid is evaporated; (3) volatile by-products are flashed; (4) expansion of the

matrix and rendering of lignin separable by organic extraction. In the Iotech Process[108], low moisture substrates are cooked at 185-240 °C under steam, which plasticizes the hemicellulose and lignin; the cellulose is separated on explosive decompression. Reaction times are short, so little degradation of products occurs and high temperatures may be used[108]. Enhancement of enzymatic hydrolysis of substrates pretreated by the Iotech Process is reportedly high. Steam explosion pretreatments have been used for a variety of substrates, including wheat straw[109,110], sallow[111], sugarcane bagasse[112,113], and aspen[114,115]. Morjanoff and Gray[112] found that by adding 1 % H_2SO_4 to the reaction, they could get 83 % yield of glucose and 84 % xylose yield. Brownell *et al.*[115], using a similar procedure, reported nearly quantitative recovery of all components of aspen wood. They concluded that preimpregnation with dilute acid not only improves the solubility of the pentosans, but it also allows for the use of lower temperatures, which destroys less of the pentosans and gives a better substrate for enzymatic hydrolysis. In addition, they found that the explosive decompression had no effect on the accessibility of the pretreated aspen to enzymatic hydrolysis and was therefore unimportant to the process[115]. Cellulases are inhibited by impurities in steam exploded wood, but washing of the pretreated wood removes the impurities and thus alleviates the problem[116].

2.5.2.3 Delignification

Delignification pretreatments aim to remove part or all of the lignin, and sometimes alter the distribution of the remaining lignin in the matrix. Kraft and sulfite pulping are commonly used in the pulp and paper industry to remove lignin; however, the purpose of these treatments is to recover cellulose fibers to use in paper, not to disrupt these fibers. Two general classes may be defined for delignifying chemicals used for pretreatments to enhance enzymatic susceptibility: (1) oxidizing agents, which oxidize the lignin and often destroy the crystalline structure of the cellulose[3,117]; and (2) organic solvents, such as amines or alcohols, with a catalyst to degrade lignin and hemicellulose (so-called "organosolv" processes)[118]. Chemicals used as oxidizing agents include $NaClO_2$[3,119], NaOCl[3,120], H_2O_2 with a heavy metal ion such as Mn(IV), Mn(II), or Fe(II)[3,121-123], ozone[2,124,125], SO_2[3,86], and many others[3]. Very high extents of delignification can be achieved in addition to high removal of hemicellulose and little loss of cellulose using $NaClO_2$ on rice straw[119]. One oxidation pretreatment which shows considerable promise is pretreatment with ozone. Ozone is attractive since[124]: (1) no toxic, acidic, or basic residues remain after pretreatment; (2) residual ozone is easily removed by conversion to O_2; (3) ozone can be cheaply generated as necessary on-site; and (4) ambient temperature and pressure are possible in most cases. In addition, particle size need not be smaller than 0.5 mm, and reaction times are

short (10-30 minutes with excess ozone). Using ozone pretreatments on wheat straw resulted in large amounts of lignin and hemicellulose removal[86,117] and very high yields of glucose after enzymatic hydrolysis, on the order of 75 %[117,125]. Binder *et al.*[87] found that they could increase enzymatic glucose yields after ozone pretreatment of wheat straw to 99 % by adding a step using concentrated H_3PO_4 to dissolve the cellulose.

Organosolv reagents consist of a catalyst such as an acid or base to degrade hemicellulose and lignin, and an organic solvent to extract the degraded fragments[126]. Acids such as H_2SO_4[118,126,127] and acetic acid[127] are commonly used, but often NaOH[126,128] or other bases such as NH_3 are used[126]. Aliphatic and aromatic alcohols are often used as solvents[118,126-129], although organic amines such as ethylenediamine, ethanolamine, and n-butylamine are often used since they act as both catalyst and solvent[118,130,131]. Other catalytic solvents include NH_4OH, EDTA (ethylenediaminetetraacetic acid), and DMSO[130]. Lee *et al.*[118] found that at 95 °C, 97.4 % delignification of corn stover was possible using ethylenediamine, including significant removal of hemicellulose. Over 90 % delignification was also achieved using ethanolamine (93.2 %) and n-butanol with 0.5 % H_2SO_4 and at 160 °C (93.9 %). Aromatic alcohols are generally more effective lignin solvents than aliphatic alcohols[118], perhaps due to their similarities to the lignin monomer structures. High enzymatic conversions have been obtained with alkaline-ethanol pretreatment of

corn stover[128], and treatment of wheat straw with 50 % ethylenediamine[130]. Since ethylenediamine is very toxic, however, its commercial use has proven to be limited; in this respect, catalyst-alcohol systems offer the advantages that they are relatively non-toxic and could be removed by evaporation and washing.

2.5.2.4 Cellulose Solvation Pretreatments

According to Ladisch *et al.*[132], cellulose solvents can be grouped into four classes: (1) strong mineral acids; (2) quaternary ammonium bases; (3) aprotic solvents; and (4) metal complexes. Cadoxen[133], which consists of 25-30 % ethylenediamine, 70-75 % water, and 4.5-7 % cadmium oxide, has been found to be very effective in selectively solubilizing the cellulose. Less toxic analogs of cadoxen, such as CMCS, and aqueous solution of 17 % sodium tartrate. 6.6 % $FeCl_3$, 7.8 % NaOH, and 6 % sodium sulfite, show increased ease of recovery and often better enzymatic glucose yields than does cadoxen[38].

Strong mineral acids are also very effective cellulose solvents[123,125,134]. Binder *et al.*[125] pretreated wheat straw with concentrated H_3PO_4 and obtained 80 % conversion of the cellulose to glucose after enzymatic hydrolysis, and Szczodrak *et al.*[123] obtained a high conversion of oak sawdust using 85 % H_3PO_4 at 4 °C for four hours. Chen and Gong[134] dissolved avicel in $ZnCl_2$ and 0.5 % HCl at 145 °C and found that complete enzymatic hydrolysis was achieved in 72 hours.

Although cellulose solvation pretreatments are very effective, the costs of chemicals and their toxicity has prevented them from being used in practical industrial applications[9].

2.5.2.5 Biological Pretreatments

Biological pretreatments are plausible alternatives to physical and chemical pretreatments, since fungi and some bacteria possess the enzymes necessary to degrade lignin[3] and hemicellulose[53,54]. An excellent review of the lignin-degrading enzymes of *Phanerochaete chrysosporium* has been published recently by Kirk[7]. Pretreatment of bagasse with a mutant strain of *P. chrysosporium* lacking cellulase was recently found to yield pulps that were highly susceptibly to cellulases[135]. Fungal pretreatments have also been found to increase yields in paper pulping[136]. Recent evidence[53,54,137] suggests that the ligninases and hemicellulases also have accessibility problems; thus, if enzymes are used to pretreat the substrate, it is reasonable to expect that more effective pretreatments will use both types of enzymes. The field of biological pretreatments thus shows great promise for future applications, both for enhancing cellulolytic action and for the pulp and paper industry.

CHAPTER III: MATERIALS AND METHODS

3.1 Pretreatment Substrate

The substrate for all pretreatments was 60-mesh, air dried mixed hardwood (90 % birch, 10 % maple) Type 060 obtained from Wilner Wood Products (Norway, ME). This untreated (hereafter referred to as "Native") mixed hardwood was presumed to have a homogeneous particle size distribution.

3.2 Acid Hydrolysis and Autohydrolysis Pretreatments

Dilute acid hydrolysis (FRWA) and autohydrolysis (FR) pretreatment of Native mixed hardwood were performed in a high temperature plug flow reactor system[138] as described by Allen *et al.*[97].

A schematic diagram of the reactor system used (MBI High Temperature Flow Reactor, Michigan Biotechnology Institute, Lansing, MI) is shown in Figure 3.1. The reactor is fed from a 20 L feed tank by a Moyno pump (moving cavity positive displacement pump, Robbins-Myer, Springfield, OH) with 18 stages, which was designed to handle slurries at up to 1650 psig and flow rates from 1 to 8 L/min. The reactor is constructed of 0.5" o.d. stainless steel tubing with 0.048" wall thickness, with a total reactor volume of 190.1 mL. High temperatures are attained by the injection of steam through a series of drill holes near the inlet of the reactor. A 1 mm orifice is located near the end of the reactor at which the reaction mixture is flashed from reactor pressure and temperature to atmospheric pressure (and 100 °C). The remaining steam is then

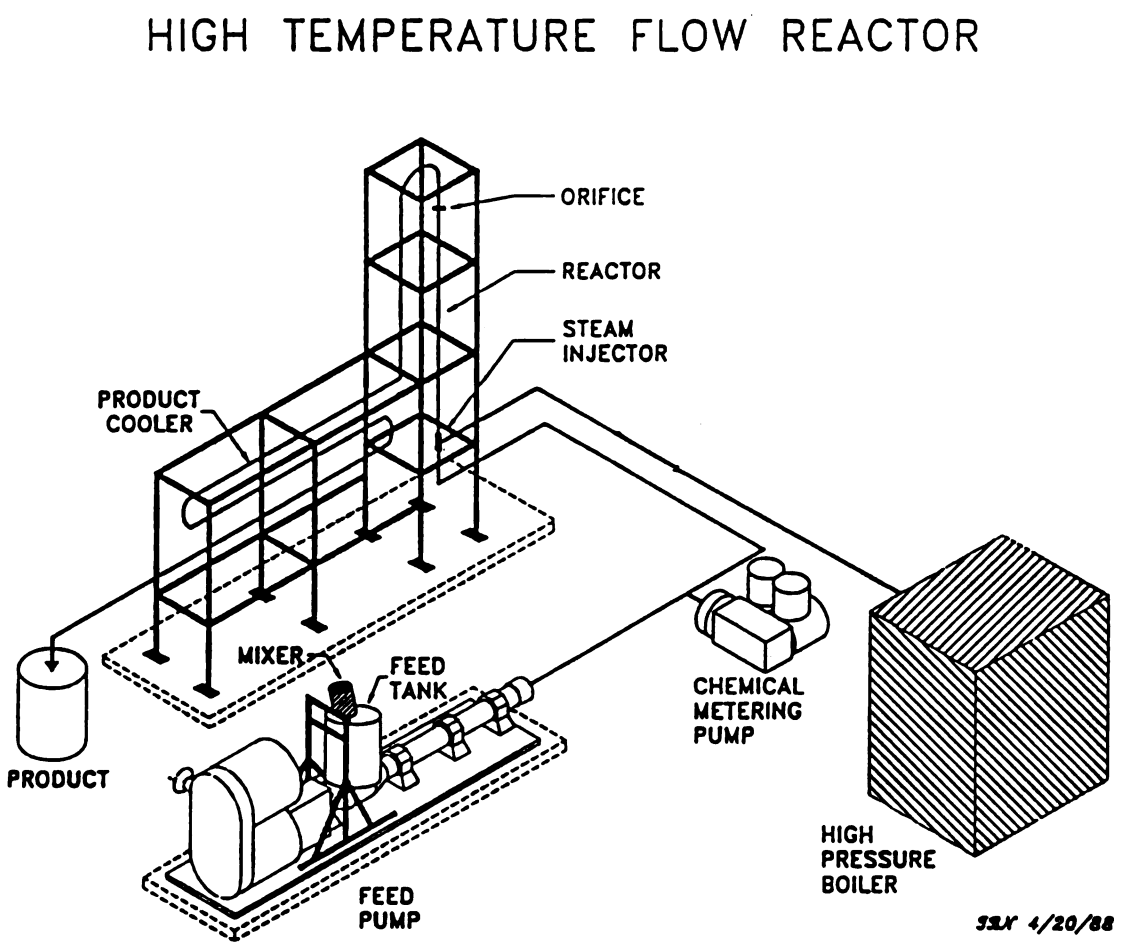


Figure 3.1: Schematic diagram of the MBI High Temperature Flow Reactor.

condensed and the pretreated slurry collected at the reactor outlet. Temperature is controlled by steam addition, and residence time is controlled by mass flow rate of the slurry in conjunction with steam addition. Operating pressure is typically about 50 psi above the steam saturation pressure at the desired reactor temperature.

Both pretreatments were carried out on 5.0 % (w/v) slurries, with 1.0 wt. % H_2SO_4 added for the FRWA treatment. Pretreatments were run at a nominal residence time of 7.3 seconds, although actual residence times were larger because higher flow rates of steam were needed to attain the higher temperatures. Run temperatures, slurry flow rates, and actual residence times for each pretreatment are given in Table 3.1. Each pretreatment was run at three temperatures to give varying degrees of treatment severity.

Recovery and washing steps included filtration on Whatman No. 41 filter paper and storage at pH 1-2 under distilled water at 4 °C to preserve the pore structure of the wood (drying of the wood collapses its pores, which then are again inaccessible). The pretreated wood in each case was washed with distilled water until the filtrate was clear and colorless.

3.3 Ethylenediamine Pretreatment

Organosolv pretreatment with ethylenediamine (EDA) was carried out using the conditions given by Lee *et al.*[118]. Reaction was carried out on 15 gram batches of air dried Native mixed hardwood, which were mixed together after recovery and washing. Three EDA

Table 3.1: Pretreatment run conditions for the FRWA and FR pretreatment of Native mixed hardwood.

Pretreatment	T (°C)	Flow Rate (kg/min)	Res. time τ (sec)
FRWA	180.0 ± 0.3	1.38	8.27
FRWA	200.0 ± 0.1	1.36	8.39
FRWA	220.0 ± 0.5	1.34	8.51
FR	240.0 ± 0.9	1.29	8.84
FR	260.0 + 2.1 - 1.0	1.21	9.43
FR	280.0 ± 0.5	0.90	12.7

concentrations were used, including 10 %, 50 %, and 70 % by volume of aqueous EDA, and all batches were run in a shaker bath at 90 ± 3 °C and 175 rpm.

Each batch was prepared for pretreatment by weighing (to the nearest 0.01 g) 15.00 g of Native wood into a 500 mL Erlenmeyer flask and adding 300 mL of a solution of the desired concentration of aqueous EDA. The flask was then stoppered, mixed thoroughly, and allowed to presoak for one hour (1.5 hours for the 70 % EDA batches) at room temperature with occasional mixing. After the presoak was completed, the flask was transferred to the shaker bath, which was set at the desired conditions, and the reaction was allowed to take place for one hour. Since the resulting reaction liquor (containing EDA, lignin and hemicellulose) was viscous and thus difficult to filter, the reaction mixture was then centrifuged for 15 minutes at 10,000 rpm and the supernatant was discarded.

After centrifugation, filtration was still difficult, so a rather lengthy washing and neutralization procedure was employed, in which the solids were washed cyclically with distilled water and 0.001 M HCl. On a per batch basis, this normally required a total water volume of about 5 L and about 1.5 L of 0.001 M HCl. The criteria for wash termination were that the filtrate be clear and colorless and that the final filtrate pH be in the range 6.5-7.5 (each batch separately). When all the batches at a condition met these criteria, they were thoroughly mixed together and stored under distilled water

at 4 °C (to preserve the pore structure). Later it was found that neutralization of the reaction mixture immediately after centrifugation and removal of the supernatant yielded a more easily filtered solid. Thus, the 10 % and 50 % EDA batches were first neutralized (initial pH was around 13) with 4 M HCl and then washed as described earlier, but with only 5 L of distilled water and no acid. In all cases, filtration was performed using Whatman GF/C glass fiber filter paper (pore size of about 1.2 μm).

3.4 Alkaline Peroxide Pretreatment

Alkaline peroxide pretreatment was performed according to the protocol established by Gould[5,92,122] and Gould and Freer[139]. Five percent (w/v) slurries were pretreated in 15 gram batches using H_2O_2 at a concentration of 2.5 % (w/v), or 0.5 g H_2O_2/g wood), 25 °C, pH 11.50, and 200 rpm in a shaker bath. Reaction times were varied to produce pretreated substrates with varying degrees of delignification and hemicellulose removal; the reaction times chosen were 0.5, 5, and 19 hours. Following pretreatment and washing, all batches at a particular reaction time were mixed together.

One batch run consisted of the following steps. Fifteen grams of Native wood were weighed, to the nearest 0.01 g, into a 500 mL Erlenmeyer flask. Next, 300 mL of a solution of aqueous 2.5 % (w/v) H_2O_2 was added and the flask was stoppered and thoroughly mixed. The pH of the resulting slurry was then adjusted to 11.50 with the addition of approximately 22 mL of 8 N NaOH.

This adjustment caused the mixture to foam extensively; thus, the mixture was shaken and vented to the atmosphere periodically over a 3-5 minute period. The flask was then placed in the shaker bath, which was set at the desired conditions, and the reaction was allowed to take place for the desired reaction time. Upon completion of the run, the mixture was centrifuged for 20 minutes at 10,000 rpm, and the supernatant was discarded. The solids were then resuspended in distilled water and the pH of the slurry adjusted to 3.4-3.5 with the addition of about 15 mL of 4 M HCl. This pH adjustment decreased the water-absorbing capabilities of the pretreated wood[140], which made filtration much easier. The slurry was then filtered on Whatman GF/D glass fiber filter paper (pore size approximately 2.7 μm). Because the fibers were still quite hydroscopic, filtration still proved difficult. Thus, large volumes of distilled water (total of about 4 L per batch) and 0.0002 M HCl (total of about 0.5 L per batch) were used in a cyclical washing and neutralization procedure (water, acid, water, acid, etc.) using 150 mL aliquots of acid and 1 L aliquots of distilled water. The final pH of the filtrate was in all cases between 6.5 and 7.5, and in all cases the filtrate was clear and colorless after washing. All batches for a particular reaction time were mixed together thoroughly and stored under distilled water at 4 °C (to preserve the pore structure).

3.5 Cellulose Crystallinity

The crystallinity of the cellulose in each sample was measured by X-Ray diffraction using the method of Segal *et al.*[44]. Wet samples were first suction dried on filter paper and broken up into small pieces with a spatula. The samples were then filled into a cell mount and scraped with a spatula to give a smooth, flat surface which was flush with the surface of the cell mount, and ideally consisted of randomly oriented fibers. The samples were then exposed to X-Rays and the fraction diffracting from $2\theta = 12°$ to $28°$ were measured and stored in a data acquisition system. Samples were measured using the horizontal wide-angle goniometer section (D2155R6) of the Rigaku D/Max-RBx Horizontal X-Ray Powder Diffractometer (Rigaku/USA, Danvers, MA). Settings were at 45 kV and 16 mA with a 12 kW rotating copper anode. Crystallinities were expressed as Crystallinity Indexes (CrI) as defined by Segal *et al.*[44] and as given in equation (2-3).

3.6 Substrate Composition

Extractives determinations were made according to the TAPPI Standard Method T-12 (1959) with ethanol-benzene in a Soxhlet extractor apparatus. Approximately 500.0 mg of ground, oven dried sample was weighed, to the nearest 0.1 mg into a clean, tared coarse porosity fritted glass extraction thimble and extracted with 100 mL of 2:1 benzene:ethanol by volume. Extraction was continued until no further change in sample mass occurred with further extraction. The

sample was then sucked reasonably dry under vacuum and dried at 105 °C to constant weight. The percent extractives was then calculated as:

$$\% \text{ Extractives} = \{(m_i - m_f)/m_i\} \times 100 \qquad (3\text{-}1)$$

where m_i is the initial sample mass and m_f is the final sample mass (both in grams).

Carbohydrate and lignin compositions were determined using the Quantitative Saccharification Method of Saeman *et al.*[141]. In this procedure, unextracted lignocellulosic samples were subjected first to hydrolysis with 72 % H_2SO_4 at 30 °C for one hour and subsequently with 4 % H_2SO_4 at 121 °C for one hour in an autoclave. The remaining insoluble portion was defined as Klasson lignin with extractives and ash. The liquor from the hydrolysis was measured for carbohydrates by HPLC, using a BioRad HPX-87P Carbohydrate column. The procedure was as follows.

To begin, 500.0 ± 50.0 mg of ground, oven-dried sample was weighed, to the nearest 0.1 mg, into an 18×150 mm test tube. Five milliliters of 72 % (w/w) H_2SO_4 were then added and the mixture was mixed thoroughly with a glass stir-rod (which was left in the tube). The sample was then placed in a 30 °C water bath for one hour and was mixed with the stir rod at 10, 20 and 40 minutes. At the end of one hour, the sample was transferred quantitatively to a 500 mL Erlenmeyer flask using 28 mL of distilled water per 1 mL of 72 % H_2SO_4 originally used (140 mL of distilled water). The flask was

then covered with aluminum foil and autoclaved for one hour at 121 °C. The acid insoluble portion was quantitatively collected by filtration on a tared 30 mL coarse porosity fritted gooch crucible to which a 1.2 µm porosity Whatman GF/C glass fiber filter paper disk had been added to improve the filtration rate and recovery of small particles. Subsequent drying of the insoluble portion to constant weight in a 105 °C oven, cooling in a desiccator and weighing yielded the mass of Klasson lignin with extractives and ash. The liquor from the hydrolysis was quantitatively diluted with distilled water to 200.00 ± 0.10 mL in a volumetric flask. Twenty milliliters of this solution were then stored in a leak-proof polyethylene bottle at 4 °C for further analysis.

Determinations of the carbohydrate concentrations in the diluted hydrolysis liquor were made by HPLC using a BioRad HPX-87P column. Degassed HPLC-grade water (from a Millipore deionizing system) was used as eluent. Initial conditions for the chromatography were 0.400 mL/min and 83 °C, but as column performance (degree of separation) decreased, these were lowered to 0.380 mL/min and 80 °C. For a description of the HPLC apparatus and its components, refer to Figure 3.3 (page 55). Note that in this application, the zero dead-volume fitting shown in Figure 3.3 is replaced with the carbohydrate column and its precolumn system. The pre-column system consisted of carbohydrate deashing system (BioRad, Holder Cat. No. 125-0139, Cartridges Cat. No. 125-0118)

and a GC620 Guard Column System (Interaction Chemicals) which contained a lead-based ion-exchange resin similar to that of the chromatography column. Samples were prepared by first neutralizing 3 mL with a scoop of $PbCO_3$ (powder form, Fisher Scientific), centrifuging for 3 min at 14,000 rpm and filtering the supernatant through a 0.45 μm syringe filter into an autoinjector vial.

Concentrations determined by HPLC were corrected for recovery losses incurred during the high temperature step by performing an additional experiment to determine the recoveries of the various wood sugars when subjected to this step in monomer form. In this recovery experiment, solid samples containing all five wood sugars were subjected to the autoclave step of the procedure and then treated as normal lignocellulosic samples. The fractional recoveries were then defined as:

$$R_i = m_{i,f}/m_{i,i} \qquad (3\text{-}2)$$

where R_i is the fractional recovery of sugar *i*, $m_{i,f}$ mass of sugar *i* recovered from the experiment (measured by HPLC), and $m_{i,i}$ is the mass of sugar *i* initially present in the sample.

In addition, in their polymer forms, wood carbohydrates are present in anhydro- forms, so to complete the material balance, the mass of each sugar must be multiplied by a "polymerization factor", as defined below:

$$F_i = (MW_i - MW_w)/MW_i \qquad (3\text{-}3)$$

where F_i is the polymerization factor for sugar *i*, MW_i is the

molecular weight of sugar *i*, and MW_w is the molecular weight of water.

For any wood sugar, then, the potential amount in the original sample, expressed as as percent, is:

$$P_i = \{20\ C_i\}/\{W\ R_i\} \qquad (3\text{-}4)$$

where P_i is the percent potential sugar *i*, C_i is the diluted liquor concentration (g/L) of sugar *i*, and W is the mass of original lignocellulosic sample (g). R_i is used as defined in equation (3-2); note that R_i must be between 0 and 1. It should also be noted that since the P_i values are based on the masses of their free sugars and not their anhydro- forms, the material balance need not sum to 100 % or less. The carbohydrate contents, expressed in their anhydro- forms (for example, glucan instead of glucose), are then:

$$P_{M,i} = P_i\ F_i \qquad (3\text{-}5)$$

where $P_{M,i}$ is the percent potential sugar monomer *i*, and F_i and P_i are as defined in equations (3-3) and (3-4), respectively. The values of $P_{M,i}$, then, must sum to 100 % or less. When combined with the percent lignin with extractives and ash, calculated by dividing the mass of the acid insoluble fraction by the initial sample mass and multiplying by 100 %, these values complete the material balance (neglecting uronic acid content and recovery errors).

3.7 Solute Exclusion Technique

Measurements of the water in pores inaccessible to solutes of various molecular sizes were made according to the solute exclusion

technique of Stone and Scallan[27,28,142]. In this technique, a known mass of aqueous solute solution is added to a wet presscake and allowed to diffuse into accessible pores. The change in concentration is then measured and the inaccessible water is determined by a solute balance before and after contacting the wet pulp to give:

$$\delta_i \; p = (w + q) - w \; (C_i/C_f) \qquad (3\text{-}6)$$

where δ_i is the specific mass of inaccessible water for solute of size *i* (g inaccessible water/g wood), w is the mass of solute solution added (g), q is the mass of water in the wet presscake (g), p is the dry weight of presscake (g), C_i is the initial solute solution concentration (%), and C_f is the final solute (diluted) concentration, corrected for minor water soluble compounds in the presscake (%). In all cases the density of water at ambient conditions was assumed to be 1 g/mL and thus the volume of water inaccessible to a solute of size *i* is:

$$d_i = \delta_i \qquad (3\text{-}7)$$

Dextrans ranging in molecular weight from 6000 to 2×10^6 and having equivalent spherical diameters in solution[28] from 38 to 560 Å were used as solutes. In addition, four low molecular weight carbohydrates (see Table 3.2) ranging in diameter from 8 to 12 Å were used to supplement the dextran size range. Solute concentrations were 0.5 % or 2 %, prepared by dissolving 0.5 or 2 g of the anhydrous solute in 100 mL of distilled water (without heating) and storing overnight at 4 °C. Solute solutions were made

Table 3.2: Molecular probes used in the solute exclusion technique.

Solute	Vendor	M_w[a]	M_w/M_n[a]	Estimated Diameter[c] (Å)
Glucose	Sigma	180	1.0	8
Fructose	Sigma	180	1.0	8
Maltose	Fisher	342	1.0	10
Raffinose	Sigma	504	1.0	12
Dextran 6K	Fluka	6,000	N.A.[b]	38
Dextran T10	Pharmacia	10,500	1.74	51
Dextran 15-20K	Polysciences	17,500*	N.A.	61
Dextran T40	Pharmacia	38,800	1.60	90
Dextran T70	Pharmacia	72,200	1.88	118
Dextran 200-300K	Polysciences	2.50×10^{5}*	N.A.	204
Dextran T500	Pharmacia	5.07×10^{5}	2.16	270
Dextran T2000	Pharmacia	2.0×10^{6}	N.A.	560

a Obtained from vendor's lot analysis except where indicated by (*), where M_w is calculated as the midpoint of the molecular weight range reported.

b N.A. = Not Available

c Probe diameters estimated using the values given in the original work by Stone and Scallan [28] and from Weimer and Weston [71].

fresh before each experiment.

Next, wet pulp samples sufficient to represent one gram dry weight of fibers were weighed, to the nearest 0.0001 g, into 20 mL polyethylene scintillation vials with leak-proof caps (Baxter Scientific, Cat. No. R2555-3). Approximately 20 mL of solute solution was then quantitatively added (by mass) and the bottle was tightly capped. The bottles needed only to be air-dried, since no difference in mass was observed between bottles before and after handling. Also, since the solute solutions were at 4 °C at the time of addition to the bottles, the bottles (with samples and solutes) were allowed to come to room temperature before being weighed. Three replicates (bottles) were used per solute per sample. Three bottles containing only distilled water and wet pulp were also prepared as "blanks," controls used to subtract out the minor contributions of water soluble extractives from the wood to the final concentration measurements. The validity of this method was proven with a preliminary experiment which showed that refractive indexes (when taken relative to water) and optical rotations are additive. The final concentration measurements were made first by refractive index and later by optical rotation. After equilibration overnight with occasional vigorous shaking, the wet presscake from each bottle was collected quantitatively on a tared 30 mL coarse porosity fritted gooch crucible as shown in Figure 3.2. The liquid fraction was collected in a test tube, which was then removed from the vacuum

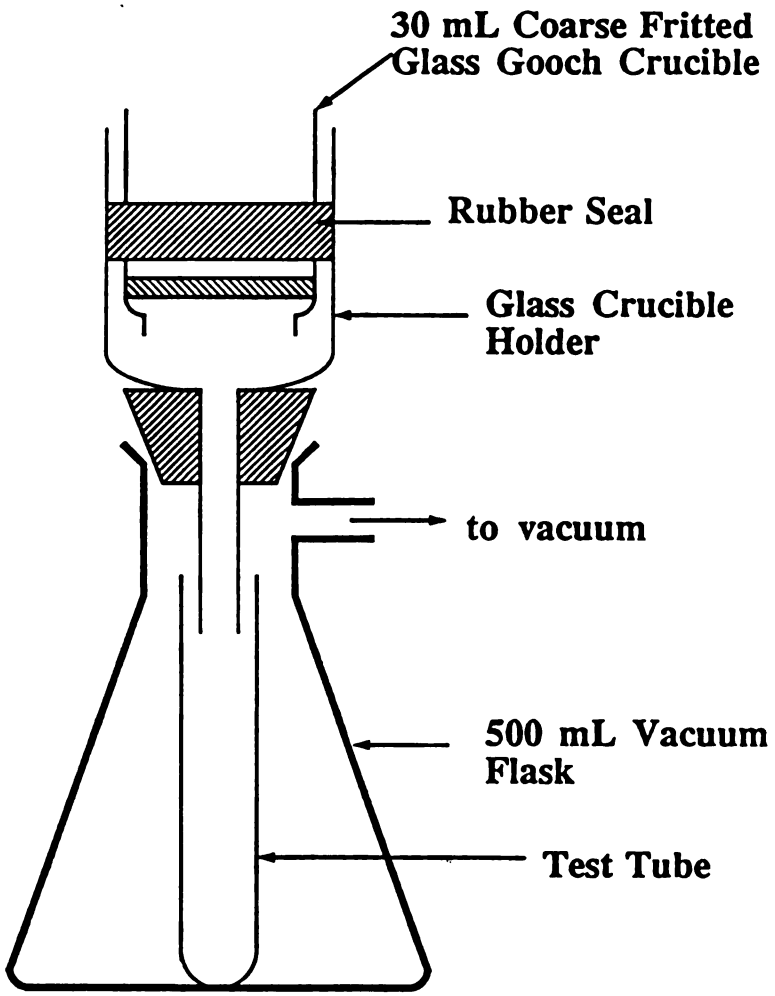


Figure 3.2: Separation apparatus for pulp and liquid in the solute exclusion technique.

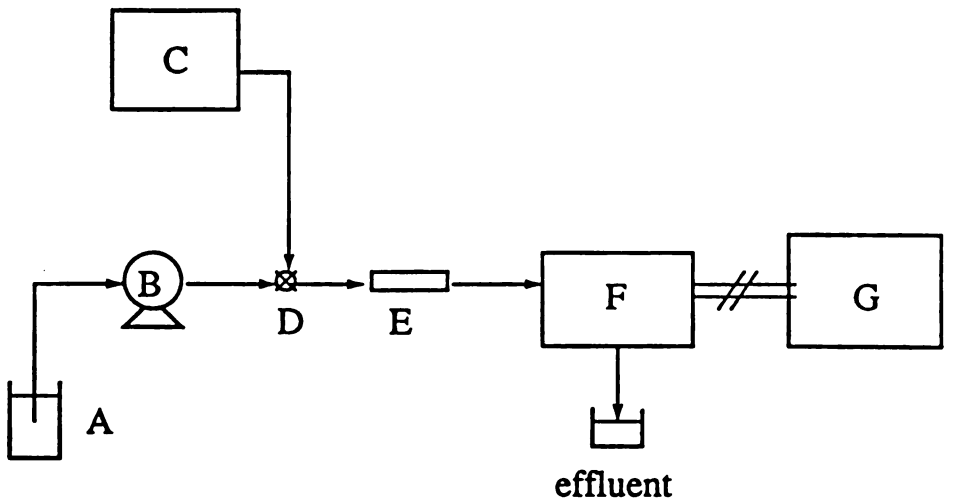
flask. Care was taken to collect liquid samples in 15 seconds or less to prevent evaporation of the sample under vacuum[144]. The presscake in the gooch crucible was then washed free of solutes and dried to constant weight at 105 °C. In cases where filtration was very slow (5 mL/min or less), the solids were not washed, but dried immediately and their dry weights corrected for solutes by the equation:

$$p = \{p' - wC_i(1 - [\alpha_f/\alpha_i])\}/\{1 + (wC_i/\alpha_i)\ (\alpha_f/p)_{blank}\} \qquad (3\text{-}8)$$

where p' is the dry weight of presscake and residual solute (g), α_f is the optical rotation of the final (diluted) solute solution (°), α_i is the optical rotation of initial solute solution (°), and $(\alpha_f/p)_{blank}$ is the average of (α_f/p) for the blanks (°/g), and p, w, C_i , and C_f are as defined earlier. This equation is derived in Appendix A.

The solute concentrations before and after the procedure were measured initially by refractive index as described by Lin *et al.*[145,146]; however, the variances in readings obtained with this method were large. The measurement apparatus is shown in Figure 3.3. Since it is a flow system, the high viscosities of the 2 % dextran solutions necessitated the use of 0.5 % solutes, but even this did not improve reproducibility of readings. Thus, optical rotations and 2 % solutes were used in later experiments. These were measured with a precision polarimeter (Carl Zeiss, West Germany) which was accurate to ± 0.01°. A sodium lamp (589.1 nm) was used.

Final concentrations were corrected for water soluble extractives by subtracting out the specific reading per unit mass of



A. Water Reservoir
B. Model 2350 HPLC Pump (Isco, Inc., Lincoln, NE)
C. Autoinjector AN-728 (Anspec, Inc., Ann Arbor, MI)
D. VICI Injection Valve (Valco Instruments, Inc., Houston, TX)
E. Zero dead-volume fitting
F. ERC-7522 Differential Refractive Index Detector (Erma, Inc., Tokyo, Japan)
G. Waters 745 Data Module (integrator), (Millipore Corp., Waters Chromatography Div., Milford, MA)

Figure 3.3: Schematic diagram of the modified HPLC apparatus used to measure concentrations of solute solutions.

wood from the blanks:

$$R_{f,c} = R_f - p\ (R_f/p)_{blank} \qquad (3\text{-}9)$$

where $R_{f,c}$ is the corrected final solution reading (refractive index or optical rotation), (units), R_f is the actual final solution reading (units), p is the dry weight of the sample presscake, and $(R_f/p)_{blank}$ is the averaged correction from the blanks, (units/g). The solutes (dextrans) were assumed to be spheres in solution[147] and thus they represented pore diameters when compared with their inaccessible water values. The resulting pore volume distributions were fit, using the program EUREKA (Borland International, Scotts Valley, CA), to the equation[148]:

$$d(t) = K\ [1 - e^{-At}\ \{\cosh(Bt) + C\ \sinh(Bt)\}] \qquad (3\text{-}10)$$

$$A = \zeta/\tau \qquad (3\text{-}11)$$

$$B = (\zeta^2 - 1)^{0.5}/\tau \qquad (3\text{-}12)$$

$$C = \zeta/(\zeta^2 - 1)^{0.5} \qquad (3\text{-}13)$$

$$t = \log_{10}D - \log_{10}4 \qquad (3\text{-}14)$$

where D is the molecular diameter (pore diameter, Å), d is the inaccessible water (mL/g wood), and K, ζ, and τ are constants. This equation describes the response of a second order overdamped ($\zeta > 1$) system to a unit impulse input and has the desired properties; namely, a sigmoidal curve which increases monotonically. Since the pore size distributions represent increases in pore volume with increasing pore diameter, the curves must monotonically increase. Water, with a diameter of 4 Å is assumed to be completely accessible

to all pores; thus, d = 0 at D = 4 Å.

Assuming that the pore geometry is adequately described by the parallel plate model[142], which is represented in Figure 3.4, the surface area in the pores can be calculated by the following equation[142]:

$$\Delta A_{i\text{-}j} = (2\ \Delta V_{i\text{-}j})/w_{i\text{-}j} \qquad (3\text{-}15)$$

where $\Delta A_{i\text{-}j}$ is the specific surface area in pores in the size range *i-j* (m^2/g), $\Delta V_{i\text{-}j}$ is the increase in pore volume over the pore size range *i-j* (m^3/g), and $w_{i\text{-}j}$ is the average pore width over the pore size range *i-j* (m). As defined, $w_{i\text{-}j}$ is the average of w_i and w_j, the pore widths at sizes *i* and *j*, respectively (equal to *i* and *j*). By summing the values of ΔA over a range of pore widths, the surface area in pores within that size range can be calculated. A molecule of diameter 560 Å is assumed to be completely inaccessible[142], so the sum of ΔA over 4 to 560 Å represents the total surface area. Likewise, summing from 51 to 560 Å gives the surface area in pores larger than 51 Å.

3.8 Enzymatic Hydrolysis

Enzymatic hydrolysis of all pulp samples was carried out in stoppered 250 mL Erlenmeyer flasks with crude cellulase powder from *Trichoderma reesei* (Rutgers C30, U.S. Army Natick Research and Development Center, Natick, MA) with a reported cellulase activity of 0.38 FPU/mg enzyme powder (0.408 FPU/mg dry enzyme powder). In addition, the cellulase, which has very low levels of β-glucosidase[9], was supplemented with Novozym TN 188, a liquid β-glucosidase from *Aspergillus niger* (Novo Industries, Denmark) with

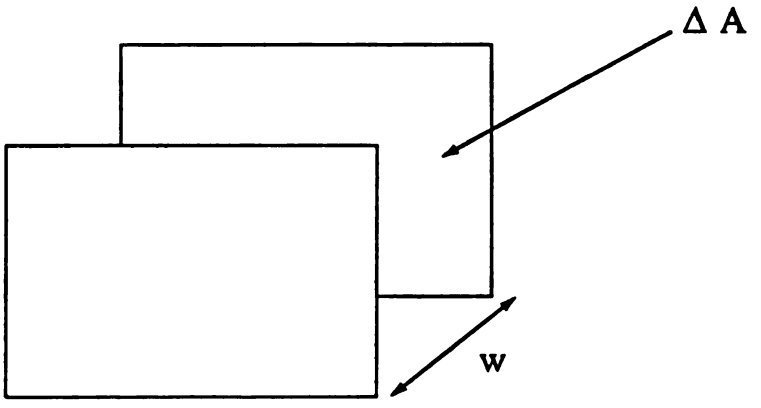


Figure 3.4: Parallel plate pore geometry, where the plates are microfibrils in the lamella of the cell wall [142].

a β-glucosidase activity of 588 Units/mL. This was done to minimize cellobiose accumulation, which not only lowers the measured glucose levels, but also inhibits the cellobiohydrolase activity of the *T. reesei* cellulase complex[9]. Hydrolysis was performed at the optimum[9] conditions of 50.0 °C and pH 4.80. The reaction mixture, preheated to 50 °C by the time of enzyme addition, consisted of citrate buffer (50 mM, pH 4.80, 3.08 mM in sodium azide to inhibit bacterial growth), Rutgers C30 cellulase (40.8 FPU/g substrate), Novozym TN 188 β-glucosidase (40.8 Units/g substrate), and 2 % (w/v) oven-dried equivalent of wet substrate. The high enzyme to substrate ratio ensured that there was no rate limitation due to lack of enzyme, and was determined by a series of experiments with the most accessible substrate (FRWA 220 °C) which were designed to find the maximum enzymatic activity (defined at μ_{max}, the maximum initial rate). The value of 40.8 FPU/g substrate used was above the maximum amount of enzyme necessary to achieve maximum enzymatic activity. The cellulase used in these experiments was Novo CCN 3000 (Novo Industries, Denmark), a liquid cellulase from *T. reesei* with a reported cellulase activity of 52.1 FPU/mL. A comparison between the CCN 3000 cellulase at 49.6 FPU/g wood and the C30 cellulase at 38.8 FPU/g wood gave nearly identical initial rates and thus 1 unit of C30 is equivalent to 1.28 units of CCN 3000.

Reaction was carried out at 200 rpm in a shaker bath. Homogeneous 2 mL samples were withdrawn at time intervals of 0.5,

1, 2, 4, 12, and 24 hours and quenched by adding 10 μL of 72 % H_2SO_4 (this stops the reaction by precipitating the enzyme). The samples were then neutralized with a scoop of $BaCO_3$ (powder, Mallinckrodt) and analyzed for glucose with a YSI enzymatic glucose analyzer (YSI Model 27, Yellow Springs Instrument Co., Yellow Springs, OH). Cellobiose was measured by HPLC with a BioRad HPX-87H column using 0.008 N H_2SO_4 as eluent. With equivalent activities of CCN 3000 and Novozym TN 188, it was reported[149] that no cellobiose was formed in the hydrolysis of Sigmacell 20; since equivalent activities of cellulase and β-glucosidase were used in this study (in terms of equivalent C30), cellobiose was measured only in selected early samples. No detectable cellobiose was found in any of the samples from this study.

Glucose yield from the enzymatic hydrolysis was calculated as the percent of potential glucose in the original sample which was formed as a result of the hydrolysis, or (glu = glucose):

$$\% \text{ Yield} = (\text{g glu formed})/(\text{g potential glu}) \times 100 \qquad (3\text{-}16)$$

CHAPTER IV: RESULTS

4.1 Pretreatments

Pretreatment with alkaline peroxide resulted in substrates which appeared to have markedly reduced particle sizes, as evidenced by the passage of large amounts of small particles through the filter paper (maximum mean pore diameter of 2.7 μm). Native wood, when washed, passed no particles through the filter paper (Whatman GF/D). In addition, the water absorbancy of the alkaline H_2O_2 pretreated wood increased markedly. This point will be made more clear when the fiber saturation points for all the pretreated substrates are shown. Because of this increase in water absorbancy, filtration of the pretreated wood was extremely difficult; the wood tended to load the filter paper rapidly. Only adjustment of the slurry pH from around 13.5 to a slightly acidic pH helped increase the speed of filtration. Alkaline peroxide pretreated wood was whitish in color due to the bleaching effect of peroxide (a similar process is used in the pulp and paper industry to bleach paper pulp). Degree of bleaching generally increased with pretreatment time (see Figure 4.1(a)).

Ethylenediamine (EDA) pretreated wood was reddish-brown in color (Figure 4.1(b)), and reaction liquors were nearly black. This pretreatment produced substrates which were difficult to filter due to the EDA remaining in the pores of the pretreated wood (EDA is relatively viscous), and also because the slurries were at a very high pH (about 14). Removal of the EDA by centrifugation and

(a)

(b)

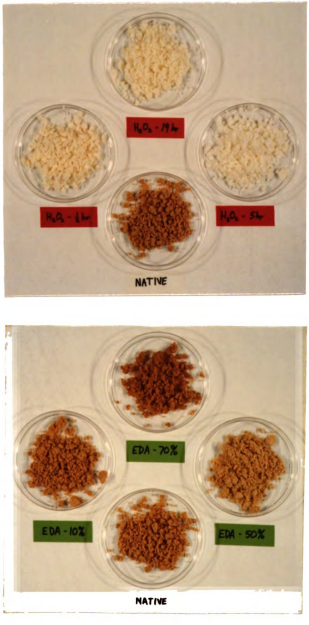


Figure 4.1: Comparison of the physical appearances of pretreated mixed hardwood (wet) with Native wood:

(a) Alkaline H_2O_2, 2.5 %, 25 °C, 200 rpm,
(b) EDA, 90 °C, 1 hr, 175 rpm.

subsequent neutralization of the slurry resolved the filtration problems. With 50 % and 70 % EDA concentrations, excess EDA was present, since EDA vapors were observed in the head space of the reaction flasks after reaction was completed. With 10 % EDA, however, no EDA vapors were observed, suggesting that the EDA had completely reacted; since EDA is both the reactant and the solvent, this brings into question whether the lowered molecular weight lignin molecules formed in the latter stages of the reaction were extracted or just redistributed.

Autohydrolysis pretreatment in the plug flow reactor (FR) formed dark brown substrates, with darker color as temperature increased (Figure 4.2(a)). Lignin has a softening temperature below 240 °C; since this was the lowest autohydrolysis temperature, lignin was melted and separated from the matrix at all three temperatures. This separate lignin-rich fraction was easily separated by its preferential loading on the surface of the filter paper. Filtration was quite easy with Whatman No. 41 filter paper once this fraction, which appeared to be less dense than the remaining lignocellulosic matrix, was removed.

Dilute acid hydrolysis in the plug flow reactor (FRWA) produced substrates ranging in color from reddish (180 °C) to very dark brown (220 °C) with increasing temperature (Figure 4.2(b)). As temperature was increased, the pretreated slurries formed emulsions which were very difficult to filter. The solids pretreated at 200 °C

(a)

(b)

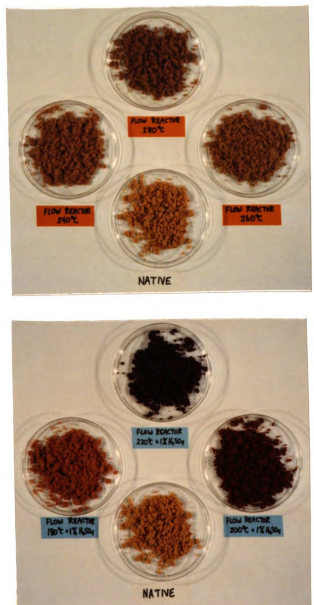


Figure 4.2: Comparison of the physical appearances of pretreated mixed hardwood (wet) with Native wood:

(a) Autohydrolysis,
(b) Acid hydrolysis, 1 % H_2SO_4.

and 220 °C resembled a thick, muddy substance.

4.2 Substrate Chemical Compositions

Results of the carbohydrate recovery experiment are tabulated in Table 4.1, including polymerization factors for each wood sugar. These values were used with equations (3-3) and (3-4) to calculate carbohydrate compositions in their anhydro- and free sugar forms from measured concentrations as described in Section 3.6.

The carbohydrate, lignin and extractives contents of Native mixed hardwood are given in Table 4.2. All components were present in amounts that are typical for this type of hardwood[17]. The mass balance accounts for only 85.1 % of the total 100 % dry weight of mixed hardwood; the remaining 14.9 % is attributed to unknown glucuronic and galacturonic acid contents (from the hemicellulose) and to recovery errors in the technique. The mass balance, however, is comparable to other published data[17].

The compositions of all pretreated substrates are listed in Tables 4.3(a) through 4.3(d). The ratios of glucan/lignin (G/L) and glucan/hemicellulose (G/H; hemicellulose was calculated as the sum of the compositions of sugars other than glucose) ratios for all pretreatments are given in Table 4.4. For the alkaline peroxide pretreatment (Table 4.3(a)), glucan content increased steadily with pretreatment time, while hemicellulose decreased (increasing G/H) slowly at short treatment time, then faster as treatment time was increased. Lignin content decreased (increasing G/L) more quickly in

Table 4.1: Fractional recoveries (R_i) and polymerization factors (F_i) for the wood carbohydrates.

Wood Sugar (*i*)	R_i	F_i
Glucose	0.927	0.90
Xylose	0.788	0.88
Mannose	0.904	0.90
Galactose	0.882	0.90
Arabinose	0.888	0.88

Table 4.2: Composition of Wilner 060 Native mixed hardwood.

Potential Component	Composition[a] (%)
Carbohydrates	
Glucan	38.6 ± 0.4
Xylan	15.5 ± 0.1
Mannan	1.9 ± 0.0
Galactan	1.1 ± 0.1
Arabinan	0.6 ± 0.1
Other	
Extractives	2.5 ± 0.1
Klasson lignin with ash	24.9 ± 0.2
SUM	85.1 ± 0.5[b]

a Based on 100 % dry weight of material.

b Remaining fraction attributed to unknown uronic acid content and to recovery errors in analysis techniques.

Table 4.3(a): Compositional analyses of alkaline peroxide pretreated wood.

Potential Component	Composition[a] (%) 1/2 hr	5 hr	19 hr
Glucan	41.6 ± 0.4	46.6 ± 0.2	49.7 ± 0.7
Xylan	16.8 ± 1.4	16.6 ± 0.3	12.7 ± 0.3
Mannan	2.0 ± 0.1	1.9 ± 0.1	0.9 ± 0.0
Galactan	1.0 ± 0.0	0.9 ± 0.1	0.9 ± 0.0
Arabinan	0.6 ± 0.1	0.5 ± 0.0	0.5 ± 0.1
Extractives	N.A.[b]	N.A.	0.5 ± 0.1
Lignin with ash & extractives	25.6 ± 0.4	24.3 ± 0.4	24.5 ± 0.1
SUM[c]	87.6 ± 1.5	90.8 ± 0.6	90.1 ± 1.2

Table 4.3(b): Compositional analyses of EDA pretreated wood.

Potential Component	Composition[a] (%) 10% EDA	50% EDA	70% EDA
Glucan	43.3 ± 0.6	48.2 ± 1.1	51.8 ± 2.5
Xylan	16.3 ± 0.2	12.0 ± 0.4	9.0 ± 0.7
Mannan	1.9 ± 0.1	1.9 ± 0.2	2.0 ± 0.2
Galactan	1.4 ± 0.1	0.8 ± 0.5	1.4 ± 0.1
Arabinan	0.5 ± 0.2	0.4 ± 0.0	0.8 ± 0.0
Extractives	N.A.[b]	N.A.	0.4 ± 0.3
Lignin with ash & extractives	24.9 ± 0.3	24.4 ± 0.2	24.9 ± 0.1
SUM[c]	88.3 ± 0.7	87.7 ± 1.3	89.9 ± 2.6

a Based on 100 % dry weight.
b Extractives not determined.
c Remaining fraction attributed to unknown uronic acid content and to recovery errors in analysis techniques.

Table 4.3(c): Compositional analyses of autohydrolysis (FR) pretreated wood.

Potential Component	Composition[a] (%) 240 °C	260 °C	280 °C
Glucan	43.8 ± 1.9	60.0 ± 0.3	66.3 ± 3.0
Xylan	12.7 ± 0.7	7.9 ± 0.0	3.9 ± 0.1
Mannan	1.4 ± 0.1	1.4 ± 0.1	0.9 ± 0.1
Galactan	1.2 ± 0.5	1.5 ± 0.1	0.7 ± 0.2
Arabinan	0.4 ± 0.0	0.5 ± 0.1	0.4 ± 0.0
Extractives	N.A.[b]	N.A.	4.8 ± 0.5
Lignin with ash & extractives	26.0 ± 0.2	22.8 ± 0.1	19.2 ± 0.1
SUM[c]	85.5 ± 2.1	94.1 ± 0.4	91.4 ± 3.0

Table 4.3(d): Compositional analyses of acid hydrolysis (FRWA) pretreated wood (1 % H_2SO_4).

Potential Component	Composition[a] (%) 180 °C	200 °C	220 °C
Glucan	46.8 ± 1.1	52.5 ± 2.0	57.6 ± 2.5
Xylan	9.8 ± 0.4	3.1 ± 0.3	1.8 ± 0.4
Mannan	0.8 ± 0.2	0.6 ± 0.1	0.4 ± 0.0
Galactan	1.0 ± 0.1	1.3 ± 0.2	0.6 ± 0.2
Arabinan	0.4 ± 0.0	0.2 ± 0.1	0.2 ± 0.0
Extractives	N.A.[b]	N.A.	13.9 ± 0.2
Lignin with ash & extractives	28.0 ± 0.3	29.7 ± 0.3	33.0 ± 0.2
SUM[c]	86.8 ± 1.2	87.4 ± 2.1	93.6 ± 2.5

a Based on 100 % dry weight.
b Extractives not determined.
c Remaining fraction attributed to unknown uronic acid content and to recovery errors in analysis techniques.

Table 4.4: Relative amounts of lignin and hemicellulose in each pretreated substrate after pretreatment.

Pretreatment		G/H[a]	G/L[b]
Native		2.02	1.55[c]
H_2O_2 :	0.5 hr	2.04	1.63
	5 hr	2.34	1.92
	19 hr	3.13	2.03
EDA :	10 %	2.15	1.74
	50 %	3.19	1.98
	70 %	3.92	2.08
FR :	240 °C	2.79	1.68
	260 °C	5.31	2.63
	280 °C	11.2	3.45
FRWA:	180 °C	3.90	1.67
	200 °C	10.1	1.77
	220 °C	19.2	1.75

a G/H ≡ Glucan/Hemicellulose ratio.
b G/L ≡ Glucan/Lignin ratio.
c Extractives have been subtracted out for Native. All others include extractives.

the initial stages of treatment, slowing at longer treatment times. For the EDA pretreatment (Table 4.3(b)), glucan increased with EDA concentration, while hemicellulose removal increased steadily with increasing EDA, and lignin removal increased up to 50-70 % EDA and appeared to level off around 70 %.

Autohydrolysis (Table 4.3(c)) produced the substrates with the highest glucan content, probably due to the large amounts of hemicellulose and lignin removed; G/H was nearly four times those for peroxide and EDA at the most harsh conditions, while G/L was 1.5 times higher at the same conditions. Acid hydrolysis (Table 4.3(d)) removed nearly all the hemicellulose at 220 °C; this removal decreased with temperature. Only a little lignin was removed in the acid hydrolysis pretreatments. Extractives were determined only for the most harsh level of each pretreatment; the highest amounts of extractives were for FR (280 °C) and FRWA (220 °C), but the increase in extractives was attributed to the production of smaller molecular weight, organic solvent extractable lignin and was thus ignored in the acid insoluble portion from the quantitative saccharification procedure (which represents lignin with extractives and ash). Ash content was also ignored, since very little ash is generally present in hardwoods. Extractives in the EDA and peroxide pretreatments were very small (Tables 4.3(a) and (b)), and thus were ignored.

Values for potential glucose, which will be used later, have

been tabulated for all substrates in Table 4.5. Recall from Section 3.6 that these are the free sugar forms, so when combined with the other compositions, they need not add to 100 %.

4.3 Substrate Crystallinities

The X-Ray diffractogram for Native mixed hardwood (wet cake) is shown in Figure 4.3. The intensities I_1 and I_2 are labeled, and are measured as the absolute intensities at the points shown. The CrI for Native wood is thus 71.6. Values for CrI for all pretreated substrates and Native are given in Table 4.6. The trends for all substrates with increasing pretreatment harshness were to increase CrI, with the exceptions of EDA (70 %), H_2O_2 (0.5 hr) and H_2O_2 (5 hr), which were all slightly lower than the CrI for Native (69.0, 68.4, and 69.0, respectively). The highest CrI values were for FR (260 °C) and FR (280 °C), at 85.2 and 85.8, respectively, and closely followed by FRWA (200 °C) and FRWA (220 °C), at 82.0 and 82.3, respectively. This was expected because of the nature of acid pretreated cellulose being more susceptible to recrystallization. Compared to the crystallility of Native wood, however, these represent only modest increases in CrI.

4.4 Pore Size Distributions and Surface Areas

The pore size distribution for Native mixed hardwood, determined as described in Section 3.7, is shown in Figure 4.4. All data points are shown; results from both refractive index measurements and optical rotation measurements (different

Table 4.5: Potential glucose amounts in each pretreated substrate.

Pretreatment		Potential Glucose (%)
Native		42.9 ± 0.4
H_2O_2 :	0.5 hr	46.2 ± 0.4
	5 hr	51.8 ± 0.2
	19 hr	55.2 ± 0.8
EDA :	10 %	48.1 ± 0.7
	50 %	53.6 ± 1.2
	70 %	57.6 ± 2.8
FR :	240 °C	48.7 ± 2.1
	260 °C	66.7 ± 0.3
	280 °C	73.4 ± 3.3
FRWA:	180 °C	52.0 ± 1.2
	200 °C	58.3 ± 2.2
	220 °C	64.0 ± 2.8

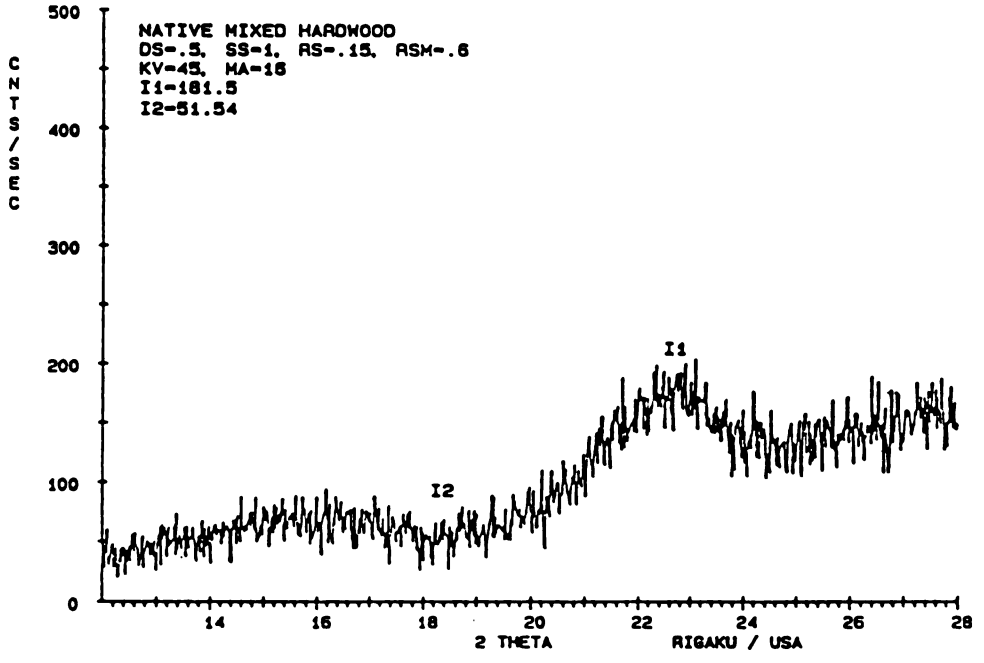


Figure 4.3: X-Ray diffractogram for Native mixed hardwood.

Table 4.6: Measured values of CrI for all substrates.

Pretreatment		I_1 (22.8°)	I_2 (18°)	CrI[a] (%)
Native		181.5	51.54	71.6
H_2O_2 :	0.5 hr	231.5	73.08	68.4
	5 hr	255.4	79.23	69.0
	19 hr	219.2	50.00	77.2
EDA :	10 %	238.5	65.38	72.6
	50 %	294.6	73.08	75.2
	70 %	161.5	50.00	69.0
FR :	240 °C	217.7	47.69	78.1
	260 °C	296.9	43.85	85.2
	280 °C	336.9	47.69	85.8
FRWA:	180 °C	251.5	53.08	78.9
	200 °C	243.1	43.85	82.0
	220 °C	316.9	56.15	82.3

a $CrI = \{(I_1 - I_2)/I_1\} \times 100$

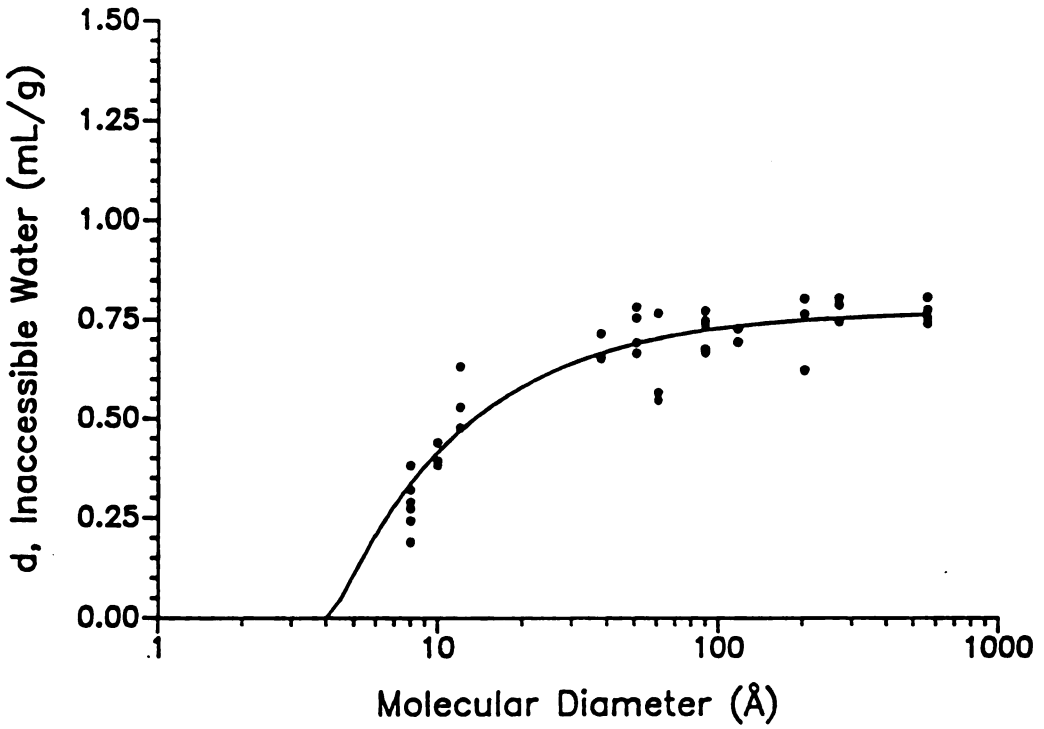


Figure 4.4: Pore size distribution for Native mixed hardwood.

experiments) were used. The curve shown is the best fit curve according to equations (3-10) through (3-14). The constants from equation (3-10) for each substrate, as well as their respective mean square residual errors, are given in Table 4.7. In all cases the residual errors were small. The exceptions were FRWA (200 °C) and FRWA (220 °C), in which the data showed too long of a lag for the type of curve used to fit the data for the other pretreatments. In these two cases, the curves were hand-drawn and the mean square residual errors calculated as before. The residual errors for the hand-drawn curves were in the range of those for the best fits, so the curves were presumed to be adequate.

A solute of 560 Å was assumed in Section 3.7 to be completely inaccessible to any of the pores. Thus the curve in Figure 4.4 represents a cumulative pore volume distribution; the total pore volume (found at 560 Å) is 0.764 mL/g wood. It is evident that the majority of the pore volume in Native mixed hardwood is in small pores. Since it is the surface area in these pores that the enzyme must get to in order to catalyze the reaction, it is reasonable to expect that any pretreatment which increases the accessibility of this surface area to the enzyme must increase the pore size. This effect was observed for all of the different pretreatments, as shown in the following figures.

In Figure 4.5, the pore volume distributions for the three levels of alkaline H_2O_2 pretreatment are shown. The effect of early

Table 4.7: Best fit curve parameters and mean square residual errors for all substrates.

Pretreatment		K	ζ	τ	MSE[a] (×10²)
Native		0.774	2.300	0.111	0.516
H_2O_2 :	0.5 hr	0.883	1.004	0.319	0.513
	5 hr	1.491	1.002	0.432	1.769
	19 hr	1.490	1.005	0.461	0.949
EDA :	10 %	1.106	1.191	0.276	1.542
	50 %	1.046	1.147	0.432	1.034
	70 %	1.694	1.002	0.611	0.883
FR :	240 °C	0.873	1.034	0.389	0.538
	260 °C	1.066	1.779	0.241	0.498
	280 °C	1.193	1.251	0.366	0.655
FRWA:	180 °C	0.785	1.029	0.343	0.490
	200 °C	N.A.[b]	N.A.	N.A.	1.130
	220 °C	N.A.	N.A.	N.A.	0.771

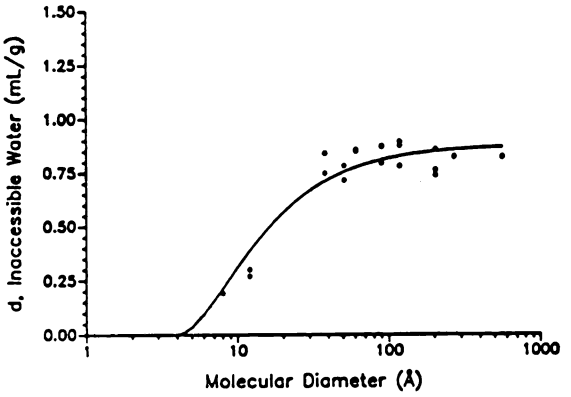
a MSE ≡ Mean Square Residual Error, and is defined as:

$$MSE = [\Sigma(y' - y_i)^2]/[n - p]$$

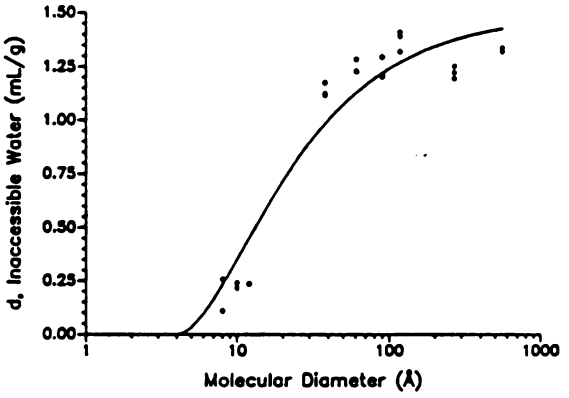
where y' is the predicted response, the y_i are the data points, n is the number of points and p is the number of constants in the model.

b Not applicable since these curves were hand-drawn.

(a)



(b)



(c)

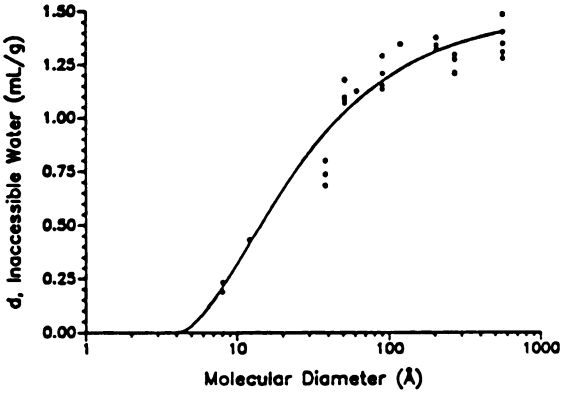


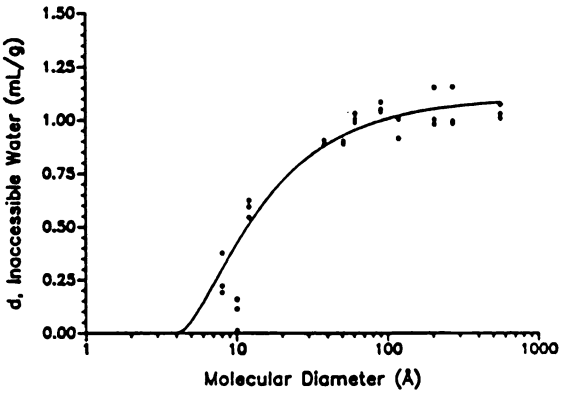
Figure 4.5: Pore size distributions for the alkaline H_2O_2 pretreatments. (a) 0.5 hr, (b) 5 hr, (c) 19 hr.

delignification from 0.5 hours of reaction to 5 hours is seen as a large increase in pore volume, especially above 12 Å pore diameter. Not much change occurs in the hemicellulose removal portion of the reaction (5 to 19 hours). Thus there is not much difference in the pore volume distributions of 5 hour and 19 hour alkaline H_2O_2 pretreatments, except for a slight decrease in the amount of pore volume in the smaller pores.

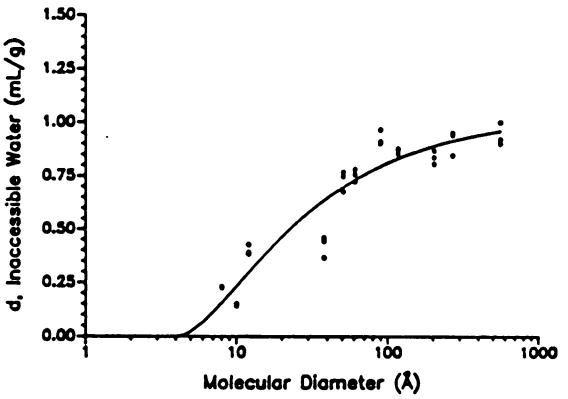
Figure 4.6 shows the pore volume distributions for the three levels of EDA pretreatment. Trends in the pore volume distributions for the EDA pretreatments are not very similar to those for alkaline H_2O_2. Pretreatment with 10 % EDA, which resulted in removal of both lignin and hemicellulose in comparable amounts (Table 4.4) increased pore volume markedly. However, pretreatment with 50 % EDA (greater hemicellulose removal) actually *decreased* total pore volume while increasing the volume in larger pores relative to the 10 % EDA treatment. With 70 % EDA, total pore volume was increased to a great extent, and the majority of pore volume was then in large pores.

For the FR (autohydrolysis) pretreatments, Figure 4.7, pore volume did not increase as much as would have been expected, considering the amounts of hemicellulose and lignin removed, and in light of the results for the alkaline H_2O_2 and EDA pretreatments. A steady increase in pore volume is seen at all pore diameters with increasing temperature of pretreatment, but not to the degree of that

(a)



(b)



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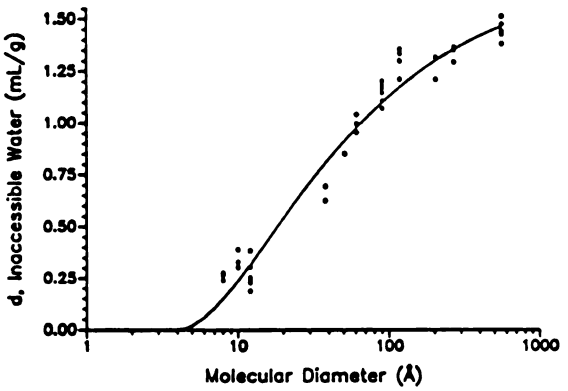
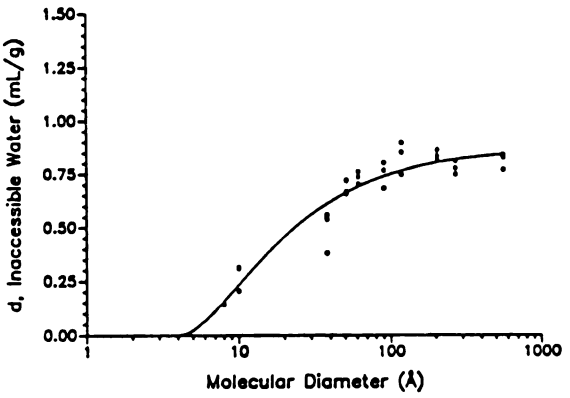
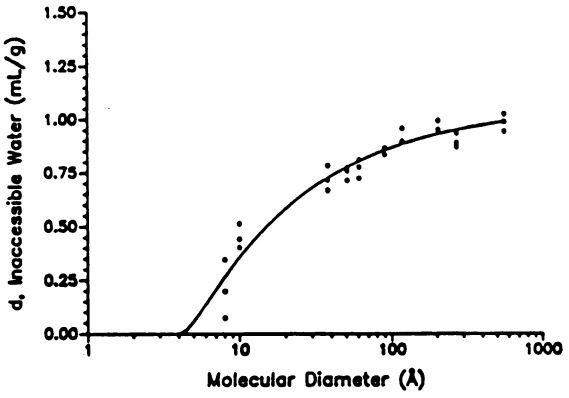


Figure 4.6: Pore size distributions for the EDA pretreatments. (a) 10 %, (b) 50 %, (c) 70 %.

(a)



(b)



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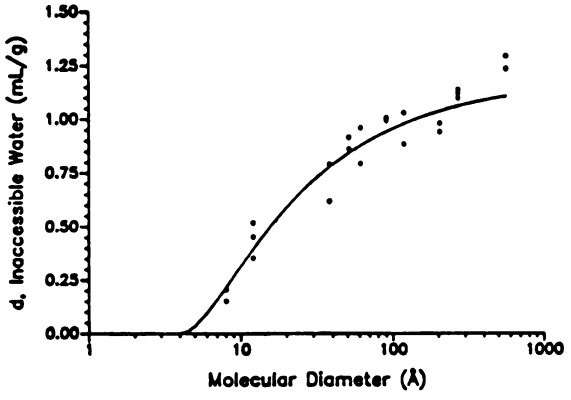


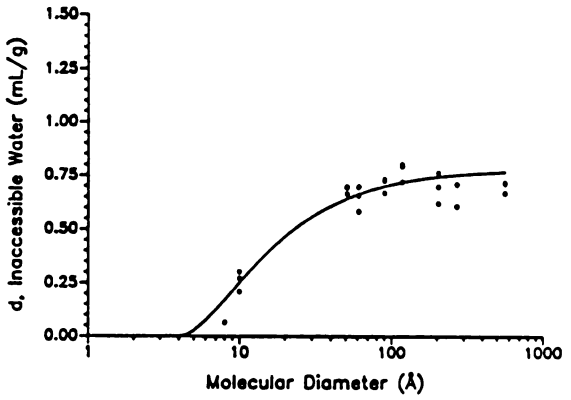
Figure 4.7: Pore volume distributions for the autohydrolysis (FR) pretreatments. (a) 240 °C, (b) 260 °C, (c) 280 °C.

observed for the previous cases. Perhaps the removal of both components in such large amounts (Table 4.4) caused the collapse of the remaining matrix, or the swelling action of the alkali in the H_2O_2 and EDA pretreatments (EDA is strongly basic) was enough to increase the pore volumes to those observed.

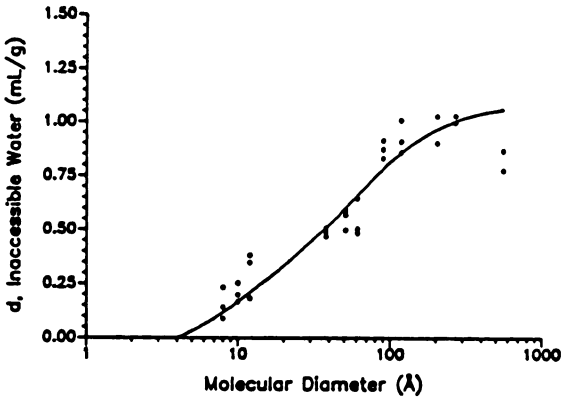
Pretreatments with dilute H_2SO_4 in the flow reactor resulted in substrates with the pore volume distributions shown in Figure 4.8. At 180 °C, pore volume was not increased much over that for Native wood. At 200 and 220 °C, however, a dramatic increase in pore volume in pores larger than around 12 Å was observed. In both cases, the great majority of pore volume is in large pores and thus more accessible to the enzyme. Practically all of these increases were due to hemicellulose removal alone; FRWA 220 °C had nearly complete hemicellulose removal.

Total surface areas calculated using parallel plate geometry are given in Table 4.8, along with total pore volumes for each (fiber saturation point, at 560 Å). Since the cellulase size range extends from 24 to 77 Å, surface areas in pores larger than these nominal diameters were calculated and are given in Table 4.9. Steady increases in surface area are seen in pores larger than each assumed enzyme diameter with increasing pretreatment severity for all pretreatments. As expected, the FRWA pretreatments at 200 and 220 °C provide large increases in surface area in larger pores, while total surface area is actually decreased. EDA 70 % also markedly

(a)



(b)



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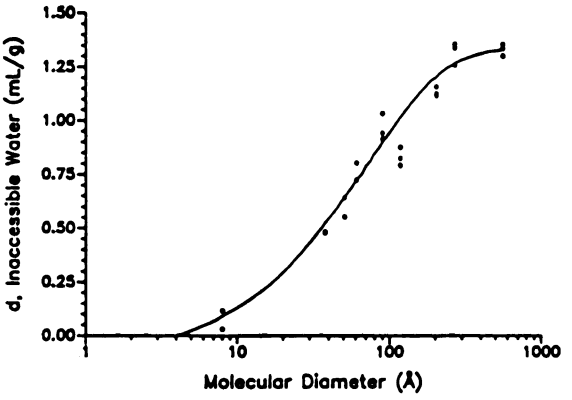

Figure 4.8: Pore volume distributions for the acid hydrolysis (FRWA) pretreatments. (a) 180 °C, (b) 200 °C, (c) 220 °C.

Table 4.8: Fiber saturation points (FSP) and total specific surface areas (SSA) for all substrates.

Pretreatment	FSP (mL/g)	Total SSA (m^2/g)
Native	0.764	1577
H_2O_2 : 0.5 hr	0.874	1374
5 hr	1.428	1758
19 hr	1.407	1636
EDA : 10 %	1.083	1781
50 %	0.960	1149
70 %	1.465	1366
FR : 240 °C	0.843	1118
260 °C	0.989	1514
280 °C	1.108	1466
FRWA1:80 °C	0.771	1134
200 °C	1.059	944
220 °C	1.331	923

Table 4.9: Specific surface area (SSA) in pores larger than various enzyme sizes over the range 24 to 77 Å.

Pretreatment		SSA (m^2/g) in pores larger than: 24 Å	38 Å	51 Å	61 Å	77 Å
Native		61.9	26.1	14.8	10.4	6.65
H_2O_2 :	0.5 hr	107	44.4	24.5	16.8	10.4
	5 hr	231	110	66.7	48.6	33.7
	19 hr	232	115	70.8	52.2	35.4
EDA :	10 %	124	53.4	30.7	21.7	14.1
	50 %	152	75.5	47.3	35.2	24.2
	70 %	265	144	94.5	72.4	51.3
FR :	240 °C	125	58.2	34.7	25.0	16.5
	260 °C	124	60.9	38.0	28.3	19.5
	280 °C	160	78.0	48.4	35.8	24.5
FRWA:	180 °C	103	45.0	25.8	18.1	11.6
	200 °C	211	128	85.7	65.7	44.9
	220 °C	295	183	128	100	72.0

increased the surface area in large pores. The increases in surface area for the FR pretreatments were small, in keeping with their small increases in pore volume. Alkaline H_2O_2 pretreatment did not provide much of an increase in surface area in large pores after 5 hours of pretreatment. Also, the water absorbing capabilities spoken of in Section 4.1 now become clear; the fiber saturation points for 5 and 19 hours of pretreatment are quite high, signifying a large potential for absorbing water.

4.5 Enzymatic Hydrolysis

4.5.1 Choice of Enzyme Level

A series of experiments were performed with the most accessible pretreated substrate, FRWA 220 °C, to determine the amount of cellulase needed to completely saturate the active sites. This "saturation" level corresponds to the maximum cellulase activity needed to ensure that no enzyme limitations were present. Because only a limited supply of Rutgers C30 cellulase was available, Novo CCN 3000 cellulase (also from *T. reesei*) was used. The variation of initial glucose yield with specific enzyme activity is shown in Figure 4.9; saturation is achieved by 50 FPU/g wood. To convert this activity to units of C30 enzyme, FRWA 220 °C wood was subjected to C30 hydrolysis at various concentrations. It was found that the initial yields were practically identical for CCN 3000 at 49.6 FPU/g wood and C30 at 38.8 FPU/g wood, as shown in Figure 4.10. Thus an activity of 40.8 FPU/g wood (slightly larger than 38.8) was used for

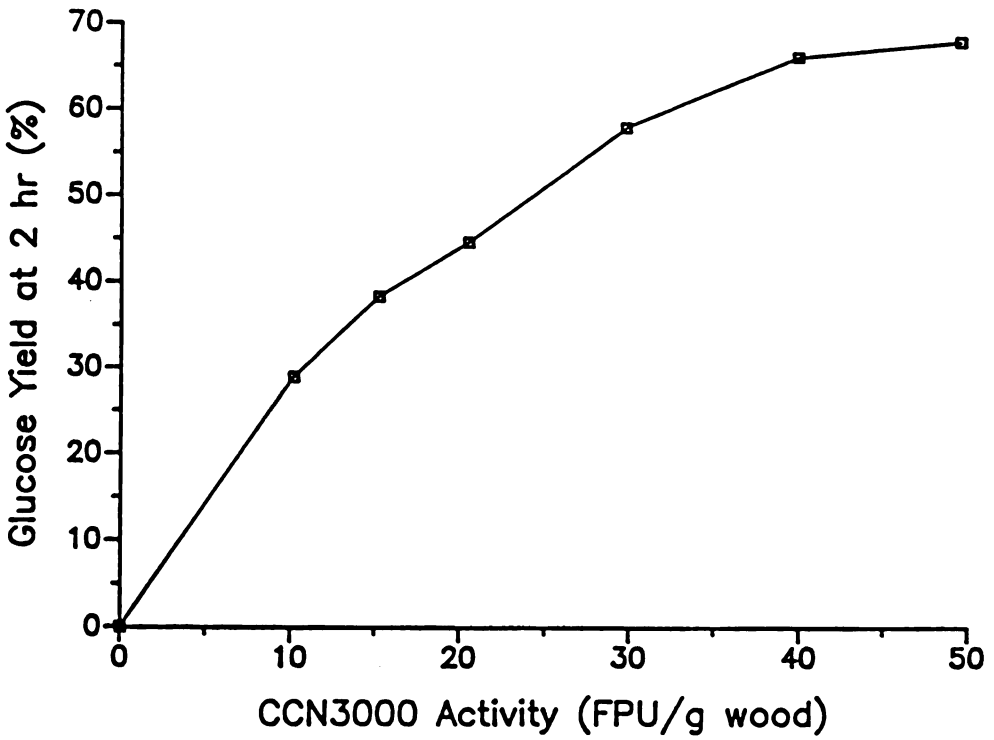

Figure 4.9: Determination of maximum CCN 3000 activity for FRWA 220 °C wood.

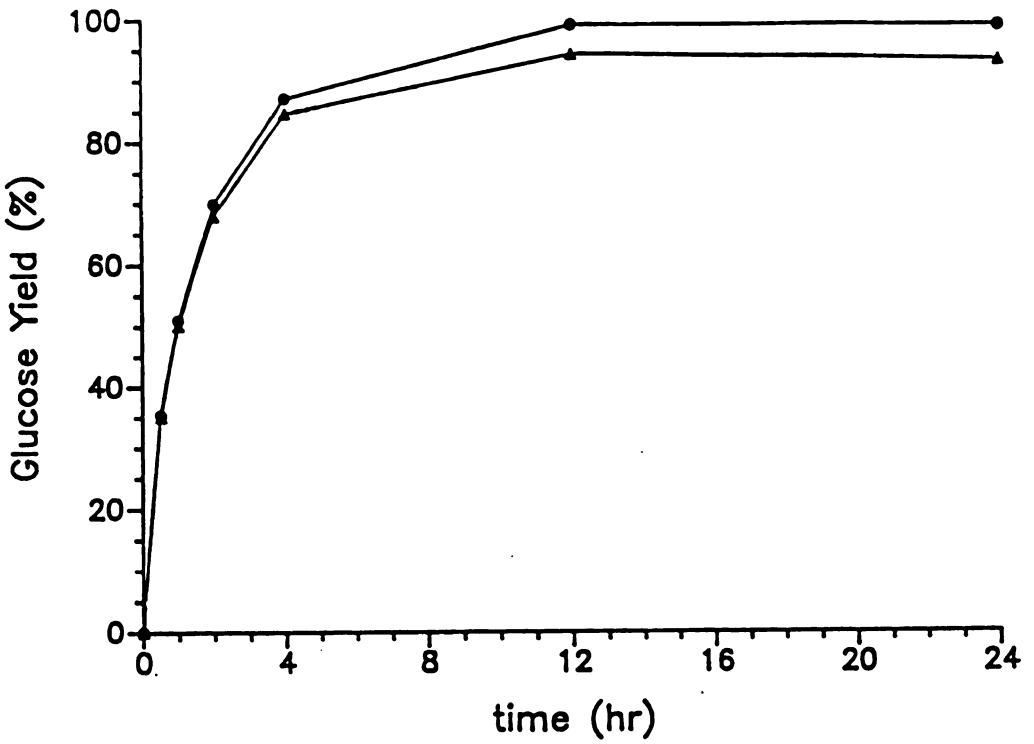

Figure 4.10: Comparison of the time course of enzymatic hydrolysis of FRWA 220 °C wood with (▲) Novo CCN 3000 cellulase and (●) Rutgers C30 cellulase.

all substrates.

4.5.2 Enzymatic Hydrolysis of Pretreated Wood

The time courses of the enzymatic hydrolysis of wood pretreated at the three levels of alkaline H_2O_2 are shown in Figure 4.11. Glucose yields at 24 hr are increased markedly over that for Native wood, from about 14.7 % for Native to 47.1, 59.9 and 68.2 % for 0.5, 5, and 19 hours of pretreatment, respectively. In the initial period of hydrolysis, however, the differences are not so large; the 5 and 19 hour pretreatments are nearly indistinguishable from one another within the first hour of enzymatic hydrolysis. Considering the results of the surface area analysis of the last section, this is not unexpected.

The enzymatic hydrolysis of EDA pretreated wood fractions are shown in Figure 4.12. Again, increasing pretreatment severity increases glucose yields at 24 hr quite markedly. In the case of EDA, however, initial glucose yields are quite different for the different levels of pretreatment. For EDA 70 %, 77 % of the 24 hr yield was obtained in only 2 hours of enzymatic hydrolysis. This is quite an improvement over Native wood, in which the hydrolysis was, for all practical purposes, finished at 4 hours of hydrolysis with only 10.5 % glucose yield.

For the FR (autohydrolysis) pretreatments, the enzymatic hydrolysis time profiles (Figure 4.13) appear much different from those for alkaline H_2O_2 and EDA. The initial increase in glucose

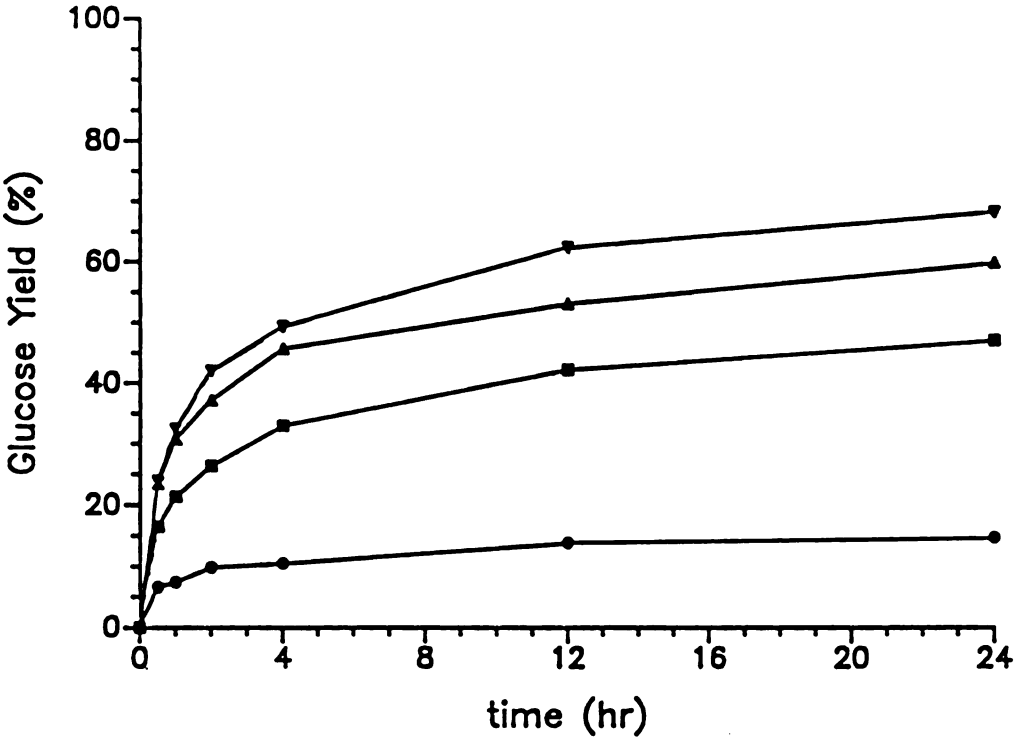

Figure 4.11: Time course of the enzymatic hydrolysis of Native (●), and alkaline H_2O_2 pretreated wood; (■) 0.5 hr, (▲) 5 hr, and (▼) 19 hr.

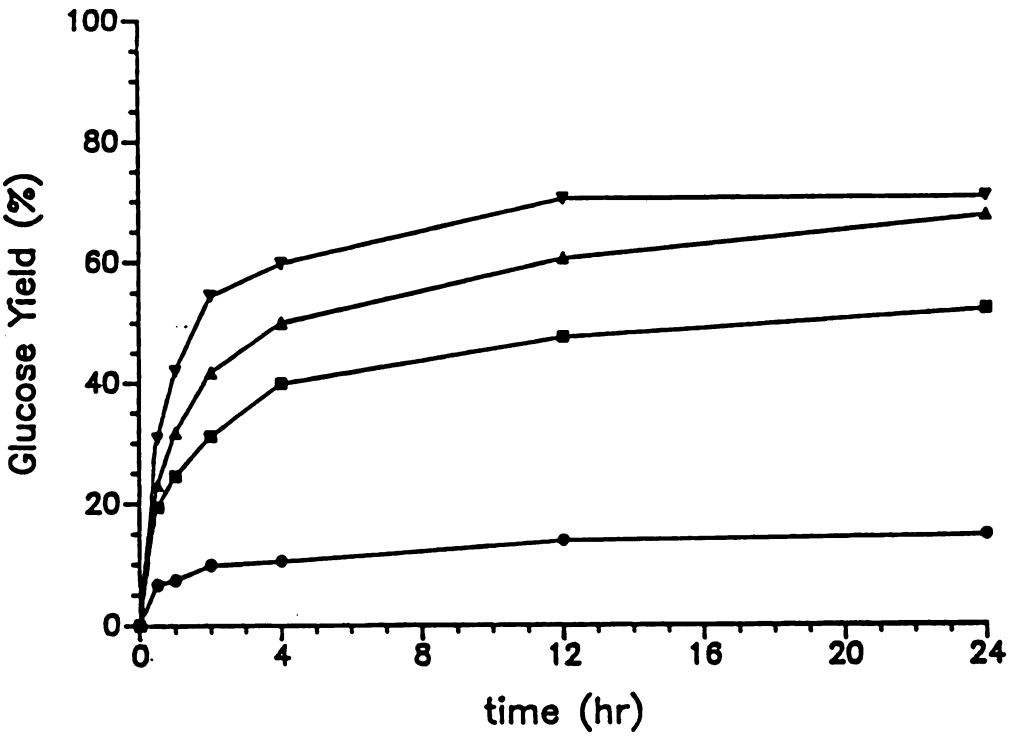

Figure 4.12: Time course of the enzymatic hydrolysis of Native (●), and EDA pretreated wood; (■) 10 %, (▲) 50 %, and (▼) 70 %.

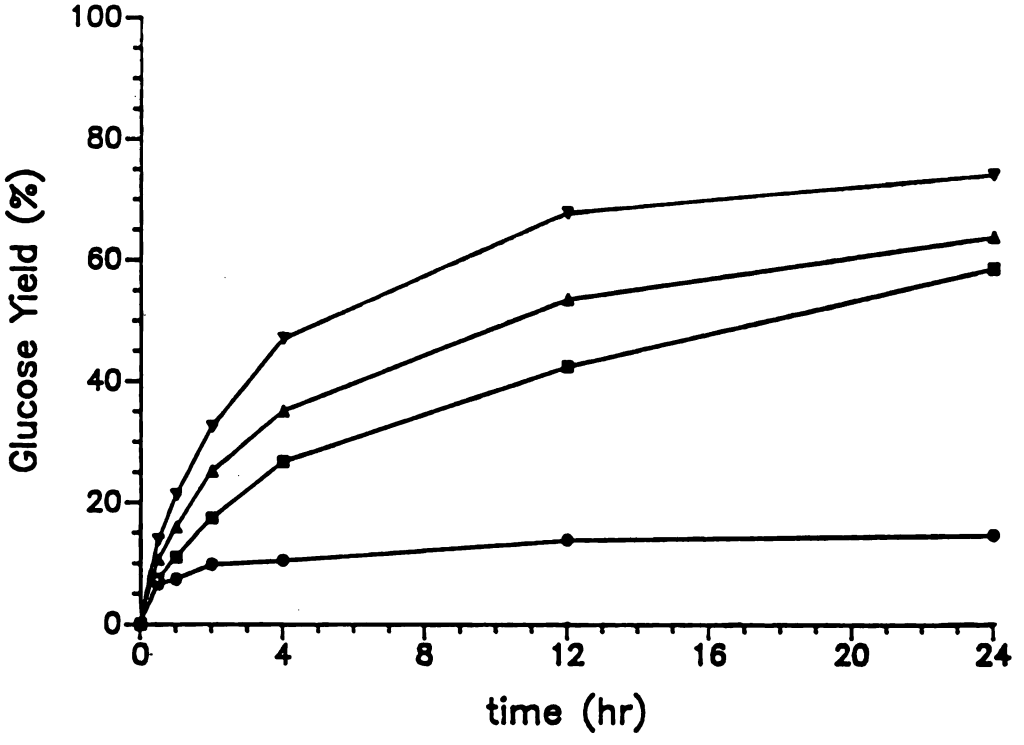

Figure 4.13: Time course of the enzymatic hydrolysis of Native (●), and FR (autohydrolysis) pretreated wood; (■) 240 °C, (▲) 260 °C, and (▼) 280 °C.

yield is much slower, and the curves do not level off completely by 24 hr, as with the others. Perhaps this is the effect of crystallinity in the latter stages of the enzymatic hydrolysis. As before, increasing pretreatment severity increased 24 hr glucose yields; although their initial rates were lower than those for alkaline H_2O_2 and EDA, the 24 hr yields for the FR pretreatments were as good or better than those for peroxide and EDA.

As shown in Figure 4.14, dilute acid hydrolysis (FRWA) produced the greatest increases in enzymatic glucose yields of any of the pretreatments. The lone exception was FRWA 180 °C, which had the lowest final yield, with about three times that for Native wood. FRWA 200 °C and FRWA 220 °C pretreatments provided final glucose yields at 24 hr of 92.1 and 97.7 %, respectively. Their initial rates (yields) were also very high, at 51.1 and 71.0 % at 2 hours, respectively. With only four hours of enzymatic hydrolysis, these two pretreated substrates had already equalled or surpassed the 24 hr yields of all the other pretreated substrates.

Defining a hydrolysis time for the average initial rate is difficult; thus, up to 4 hours of hydrolysis was considered to represent an average initial rate (for example, average initial rate at 0.5 hr, 1 hr, etc.). The yields at these times are given in Table 4.10, and are used separately as measures of the average initial rates.

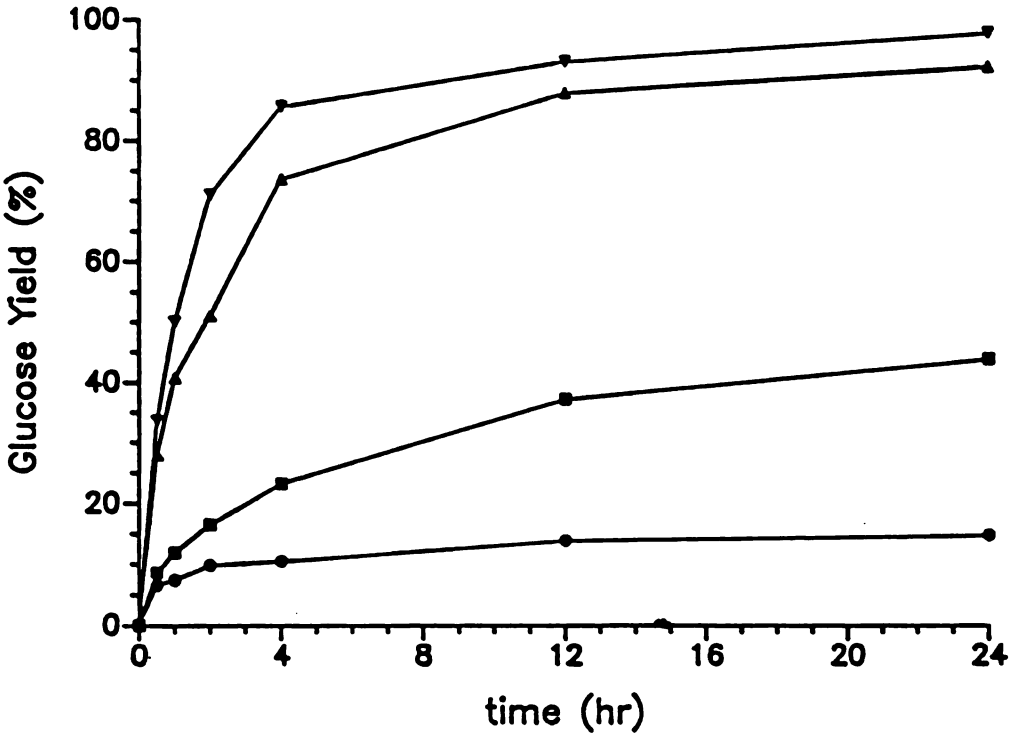

Figure 4.14: Time course of the enzymatic hydrolysis of Native (●), and FRWA (acid hydrolysis) pretreated wood; (■) 180 °C, (▲) 200 °C, and (▼) 220 °C.

Table 4.10: Glucose yields from enzymatic hydrolysis at times ranging from 0.5 to 4 hours.

Pretreatment		Glucose Yield (%) at : 1/2 hr	1 hr	2 hr	4 hr
Native		6.59	7.42	9.83	10.47
H_2O_2 :	0.5 hr	16.50	21.24	26.35	32.93
	5 hr	23.48	30.76	37.20	45.66
	19 hr	23.87	32.43	41.98	49.33
EDA :	10 %	19.47	24.55	31.10	39.90
	50 %	23.08	31.72	41.77	49.99
	70 %	30.77	42.01	54.48	59.77
FR :	240 °C	7.45	10.95	17.45	26.77
	260 °C	10.57	15.92	25.21	35.10
	280 °C	13.78	21.27	32.51	47.01
FRWA:	180 °C	8.58	11.87	16.49	23.23
	200 °C	27.95	40.74	51.05	73.58
	220 °C	33.59	50.03	70.99	85.56

4.6 Surface Area Correlations

The values for surface area available to solutes ranging in size from 24 to 77 Å (Table 4.9) were combined with the initial rate (glucose yield) data from Table 4.10 to derive linear relationships between glucose yield (at a certain hydrolysis time) and surface area available to solutes of sizes ranging from 24 to 77 Å. The correlation for the 2 hour glucose yield and the surface area available to a solute of 51 Å diameter is shown in Figure 4.15. The slope of this correlation is 0.605 %/m^2 and its correlation coefficient (r^2) is 0.843; the correlation, therefore, is fairly good (r^2 of 1 is a perfect correlation and an r^2 of 0 shows no correlation). The slopes and correlation coefficients for the other fits are given in Tables 4.11.

It can be seen from Table 4.11(b) that fairly good correlations exist for a fairly wide range of assumed enzyme sizes. The correlation coefficients, however, should be higher at the true (or "average") enzyme size. Figure 4.16 shows the variation of the correlation coefficients with minimum accessible pore diameter. The best range of correlations occurs with the 2 hour yield, so this is probably the correct time to use for the average initial rate. For all but the 0.5 hour series, a maximum r^2 occurs at (or around) 38 Å, suggesting that the average size of the component enzymes of the *T. reesei* cellulase complex may be near 38 Å. In any case, the existence of a linear correlation between glucose yield and available surface area tends to suggest that surface area is the major limiting

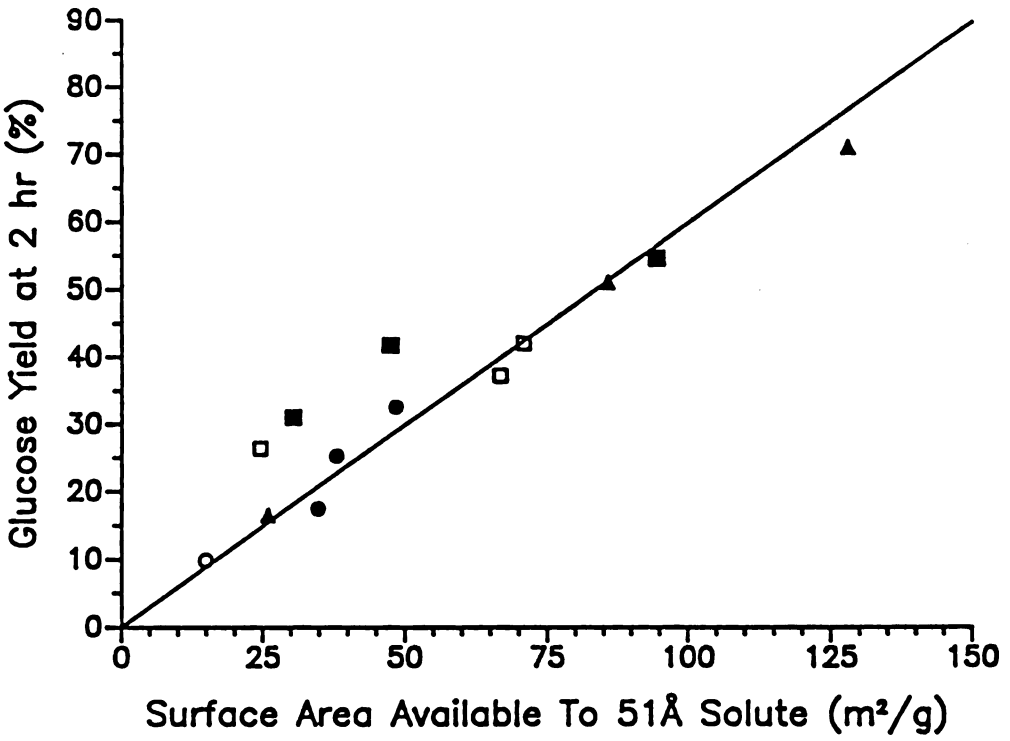

Figure 4.15: Linear correlation between glucose yield at 2 hr and specific surface area available to a solute of 51 Å diameter: Native (○), alkaline H_2O_2 (□), EDA (■), FR (●), and FRWA (▲).

Table 4.11(a): Slopes of the correlations derived for glucose yields at various times and surface areas available to solutes of various sizes.

Minimum Pore Diameter (Å)	Correlation Slope (%/m²) for Yield at: 0.5 hr	1 hr	2 hr	4 hr
24	0.112	0.157	0.210	0.263
38	0.210	0.296	0.395	0.494
51	0.321	0.452	0.605	0.756
61	0.421	0.594	0.796	0.993
77	0.598	0.846	1.134	1.413

Table 4.11(b): Correlation coefficients for the correlations given in Table 4.11(a).

Minimum Pore Diameter (Å)	Correlation Coefficient (r^2): 0.5 hr	1 hr	2 hr	4 hr
24	0.800	0.829	0.842	0.770
38	0.765	0.849	0.881	0.807
51	0.683	0.798	0.843	0.753
61	0.629	0.759	0.808	0.629
77	0.568	0.710	0.764	0.643

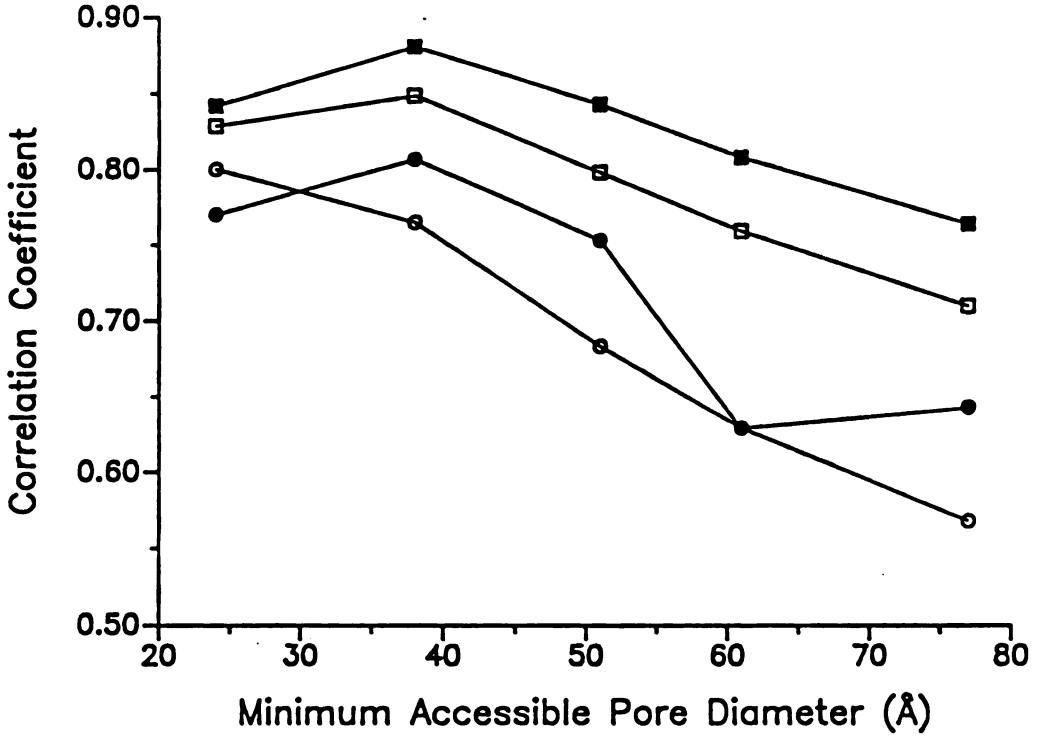

Figure 4.16: Variation of the correlation coefficients from the linear correlations in Table 4.11(a): (○) 0.5 hr yields, (□) 1 hr yields, (■) 2 hr yields, and (●) 4 hr yields.

factor in the hydrolysis.

4.7 Empirical Correlations

Empirical correlations similar to those presented in Section 2.4.1 (equations (2-1) and (2-2)) were derived to determine whether crystallinity and/or lignin content has any effect on the initial glucose yield. Since 2 hours was determined to be the best time for measurement of the initial rate, these correlations were derived only for 2 hour yields. The correlations were of the form:

$$GY_2 = a\ (100 - CrI)^b\ (G/L)^c\ (SSA_i)^d \qquad (4\text{-}1)$$

where GY_2 is the glucose yield at 2 hours, CrI is the crystallinity index, G/L is the glucan to lignin ratio, SSA_i is the surface area in pores larger than size *i*, and a, b, c, and d are constants. Equation (4-1) was linearized to the form:

$$\ln(GY_2) = \ln(a) + b\ \ln(100 - CrI) + c\ \ln(G/L) + d\ \ln(SSA_i) \qquad (4\text{-}2)$$

Linear regression was then performed and the constants a, b, c, and d determined. The values for the constants using the glucan/lignin ratios given in Table 4.4, the CrI given in Table 4.6, and the available surface areas given in Table 4.9 are given in Table 4.12. The correlation for each was tested by performing linear regression on the actual versus predicted yields. The slopes and correlation coefficients for these tests are given in Table 4.13. If the correlations are "good," the best fit lines should have slopes of 1. For all the correlations, the slope of the best fit was nearly one. In addition, the predicted yields are plotted in Figure 4.17 against the

Table 4.12: Constants a, b, c, and d determined by linear regression for the empirical fits to equation (4-2).

Minimum Pore Diameter (Å)	Empirical Constants:* a	b	c	d
24	0.1080	-0.00806	0.03378	1.124
38	0.2637	0.2171	0.2108	0.9142
51	0.4437	0.2932	0.2470	0.8270
61	0.6113	0.3213	0.2487	0.7840
77	0.9798	0.3217	0.2174	0.7387

* The empirical correlations are of the form:

$$\text{Yield (\%)} = a\,(100 - \text{CrI})^b\,(\text{G/L})^c\,(\text{SSA})^d$$

Table 4.13: Slopes and correlation coefficients for the correlations derived for actual glucose yield at 2 hr vs predicted yields from the empirical correlations.

Minimum Pore Diameter (Å)	Best Fit Slope**	r^2
24	1.005	0.842
38	0.999	0.870
51	0.996	0.875
61	0.996	0.875
77	0.996	0.872

** A perfect empirical fit would have a slope of 1 and an r^2 of 1.

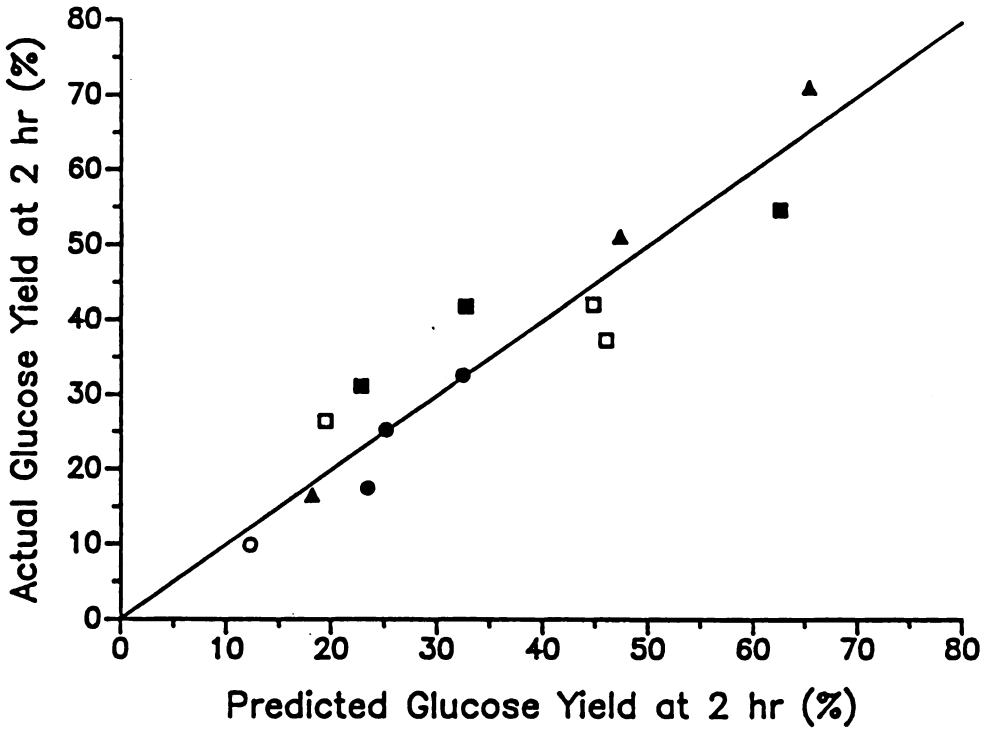

Figure 4.17: Linear correlation between actual glucose yield at 2 hr and predicted glucose yield from the empirical correlation using surface area available to a solute of 51 Å diameter: (○) Native, (□) alkaline H_2O_2, (■) EDA, (●) FR, and (▲) FRWA.

actual data for the correlation using surface area available to a 51 Å solute. The best prediction of glucose yields at 2 hr is that using the surface area available to a 51 Å solute. In all cases, the effects of CrI and G/L are small because the numbers themselves are small, in addition to their small exponents. Surface area, on the other hand, is the dominating factor in all cases, as evidenced by the large exponents for the surface area terms (the values for surface area are larger than those for CrI or G/L). Thus, the empirical correlatons tell pretty much the same story as the linear correlations between glucose yield and surface area alone: surface area is the dominating factor in the early stages of enzymatic hydrolysis.

CHAPTER V: DISCUSSION

5.1 Significance

The results of this study are significant because this is the first time that pretreatments have been compared on an equal basis. Past comparisons of pretreatment effectiveness have invariably been based on results from different substrates, different enzymes, and different analytical methods, some of which were more reliable than others. The linear correlations developed by Stone *et al.*[27] Grethlein[17], and Grous *et al.*[40] have been tested with a common substrate which was modified by four different methods to produce a range of compositions of each wood component. The correlations were proven again, showing that surface area is definitely the controlling factor in the initial stages of enzymatic hydrolysis. The similarities of the resulting empirical correlations to those of Gharpuray *et al.*[43] again shows that the enzymatic hydrolysis of lignocellulosics is fundamentally different than for cellulosics. Studies to determine the effects of various factors on any property of lignocellulosic materials should therefore be performed on lignocellulosic materials; the cellulosic materials differ from lignocellulosics so much in structure and chemical reactivity that they do not behave similarly at all.

5.2 Pretreatment Effectiveness

The major point of this study was to prove or disprove the universality of the linear surface area correlation. The results, however, can also be used to compare the effectiveness of various

types of pretreatments. For all pretreatments, enzyme accessibility increased with pretreatment severity, but to different degrees, as evidenced by the trends in the accessible surface areas produced. A point to consider here is how the removal of the various components serves to increase this surface area. Alkaline peroxide pretreatment swells the cellulose matrix and removes moderate amounts of hemicellulose and lignin. EDA, which is also alkaline, swells the cellulose and also removes similar amounts of hemicellulose and lignin (slightly higher hemicellulose removal). Perhaps the reason that EDA at 70 % produced a substrate that was much more accessible than the 19 hr alkaline pretreatment was because of the relative concentrations of NaOH (about 22 wt. %) and EDA (70 vol. %), although swelling of the cellulose is not likely to completely account for the differences.

The autohydrolysis (FR) pretreatment removed more lignin than any of the other pretreatments, and also more hemicellulose than either alkaline peroxide or EDA, yet its surface areas were smaller. This was probably due to a collapse of the matrix because so much of its supporting material (lignin and hemicellulose) was removed. The FRWA pretreatments, which removed mainly hemicellulose, had the highest yields at all times, and had very high yields in as little as 2 hr of enzymatic hydrolysis. Clearly this is the best pretreatment, since economics dictate that reaction times must be kept as short as possible or costs go up. In addition, the hydrolysis was nearly

completed in four hours for FRWA 200 °C and FRWA 220 °C, and 92-98 % yields of glucose were obtained in 24 hr.

5.3 Cellulase size

The fact that the correlations for initial glucose yield versus available surface area are fairly linear over a wide range of assumed enzyme sizes (24 to 77 Å) suggests that the endo- and exoglucanase components are distributed in size over this range. Since the hydrolysis of cellulose completely to glucose cannot occur without both of these activities, it stands to reason that if the rate is proportional to surface area at both 24 and 77 Å, endo- and exoglucanases with sizes near these values must be present. The fact that the highest correlation occurs at an enzyme size of 38 Å suggests that the average cellulase complex component size is probably near 38 Å. Cowling and Kirk[6] reported that *T. reesei* cellulases were near the upper limits of the 24 to 77 Å size range; it would be interesting to collect gel filtration data and/or determine the molecular weight distribution of purified C30 cellulase to see if the suggestions from this study are in fact true.

5.4 Future Directions

An important point should be checked concerning the nature of the surface area in the pores of the lignocellulosic matrix. The assumption was that the surface area in the pores represented *cellulose* surface area -- the solute exclusion technique has no way of determining whether this assumption is true or not. A way of

determining the effect of lignin on the reactive surface area might be to treat the wood with a mixture of enzymes that degrade hemicellulose (a hemicellulase) to degrade the hemicellulose while leaving the lignin intact. Most pretreatments which remove only hemicellulose use temperatures above the glass transition temperature of lignin, so even though the lignin is not removed, its spacial arrangement is modified. Once this substrate (hemicellulose removed, with lignin intact) is prepared, the surface area correlations could be used with enzymatic hydrolysis data to see if the lignin matrix, when intact, has any effect on the correlation prediction (I expect that it would). One problem with this method is that hemicellulases and ligninases have been shown to have accessibility problems similar to those for cellulases[54].

An excellent test of the suggestion of this study that the average size of *T. reesei* cellulase complex components is near 38 Å would be to purify each separate component enzyme (including each endo- and exoglucanase), determine their individual sizes, and hydrolyze a lignocellulosic substrate (pretreated to get a large range of pore sizes) with mixtures of various components of the complex to see how the rate of hydrolysis varies with their individual sizes. If the suggestion of this study is in fact true, then the average of these values should be around 38 Å. It is important to keep in mind that since the complex acts synergistically to degrade cellulose to glucose, both endo- and exoglucanases as well as β-glucosidases (these act on

soluble cellobiose and thus do not have an accessibility problem) must be included in the experiment. Otherwise a range of glucose oligomers, some soluble and some not, would have to be measured.

Another area which merits attention is, in fact, the question of how ligninases and hemicellulases are limited by the matrix structure. Recent evidence[53,54,137] has suggested that the enzymes penetrate the matrix in a synergistic manner; that is, neither enzyme would be very accessible without the action of the other.

Finally, it would be interesting to hydrolyze the pretreated substrates from this study with the enzyme level used by Grethlein[17] and Grous *et al.*[40] (16.2 FPU/g wood) to see if the slope of the new correlation would be the same as their slopes (0.375 and 0.326 %/m^2, respectively).

APPENDIX

Appendix A

Derivation of equation (3-8)

A.1 Derivation

Equation (3-8) was used to correct dry weights from solute exclusion experiments in which the filtration was too slow to wash the solutes from the wet presscake in a reasonable length of time. The form of equation (3-8) is:

$$p = \{p' - wC_i(1 - [\alpha_f/\alpha_i])\}/\{1 + (wC_i/\alpha_i)\ (\alpha_f/p)_{blank}\} \qquad (3\text{-}8)$$

where p' is the dry weight of presscake and residual solute (g), p is the dry weight of the wet presscake (g), α_f is the optical rotation of the final (diluted) solution (°), α_i is the optical rotation of the initial solution (°), $(\alpha_f/p)_{blank}$ is the average of (α_f/p) (correction factors for the blanks, °/g), w is the mass of solute solution added (g), and C_i is the initial solute solution concentration (%).

Additional parameters needed are: $w_{D,f,l}$, the mass of dextran left in the pulp (g); $w_{D,i}$, the mass of dextran initially in the bottle (g); $w_{D,f}$, the mass of dextran in solution after equilibration (g); and $\alpha_{f,corr}$, the optical rotation of the final solution, corrected for contributions of solubles (°).

The actual dry weight of pulp which contains residual dextrans is:

$$p' = p + w_{D,f,i} \qquad \text{(A-1)}$$

Now, the mass of dextran initially in the bottle is:

$$w_{D,i} = w\ C_i \qquad \text{(A-2)}$$

In addition, the ratio of the initial and final optical rotations is equal to the ratio of the initial and final concentrations, or

$$\alpha_i/\alpha_{f,corr} = C_i/C_f \qquad \text{(A-3)}$$

Similarly,

$$w_{D,f}/w_{D,i} = C_f/C_i \qquad \text{(A-4)}$$

Combining equations (A-2), (A-3) and (A-4) gives:

$$w_{D,f} = w\ C_i\ [\alpha_{f,corr}/\alpha_i] \qquad \text{(A-5)}$$

Now, the mass of dextran in the pulp is:

$$w_{D,f,i} = w_{D,i} - w_{D,f} \qquad \text{(A-6)}$$

or

$$w_{D,f,i} = w\ C_i - w\ C_i\ [\alpha_{f,corr}/\alpha_i] \qquad \text{(A-7)}$$

Substitution of equation (A-7) into (A-1) gives:

$$p' = p + w\ C_i\ \{1 - [\alpha_{f,corr}/\alpha_i]\} \qquad \text{(A-8)}$$

Now, from equation (3-9), we know that

$$\alpha_{f,corr} = \alpha_f - p\ (\alpha_f/p)_{blank} \qquad \text{(A-9)}$$

Then we have

$$p' = p\ \{1 + w\ C_i\ (\alpha_f/p)_{blank}/\alpha_i\} + w\ C_i\ \{1 - (\alpha_f/\alpha_i)\} \qquad \text{(A-10)}$$

Solving for p then gives equation (3-8), or:

$$p = \{p' - wC_i(1 - [\alpha_f/\alpha_i])\}/\{1 + (wC_i/\alpha_i)\ (\alpha_f/p)_{blank}\} \qquad \text{(3-8)}$$

A.2 Limitations

A limitation of the above correction method is that not all of the solutes in the interstices between the particles are collected with the liquid sample (only 80 to 90 % of the interstitial liquid is collected). However, a little washing must occur to get the residual wood left in the bottle onto the gooch crucible. Thus, the assumption was made that this washing (about 10 to 20 mL of water) was enough to remove the interstitial solutes while leaving the solutes trapped in the pores in place. Actual corrections were found to range from 2 to 5 % of the dry weight of the wet pulp, which is within the range determined by a preliminary calculation to determine corrections for the ideal case (1 to 5 % for the range of inaccessible water values determined for the substrates used). Thus, the correction method used seems to have accurately predicted the dry weights of the wet pulp containing dextrans.

Appendix B

Tabulated Data

Table B2.1: Tabulated data for the pore size distribution for Native mixed hardwood (refer to Figure 4.4).

Solute	Molecular Diameter (Å)	Measurement Method[a]	d (mL/g wood)
Glucose	8	RI	0.2419
		RI	0.2890
		RI	0.2726
		RI	0.3197
Fructose	8	P	0.1885
		P	0.3807
Maltose	10	RI	0.3819
		RI	0.3925
		P	0.4383
Raffinose	12	RI	0.4760
		P	0.5285
		P	0.6310
Dextran 6K	38	P	0.7140
		P	0.6546
		P	0.6518
Dextran T10	51	RI	0.6650
		P	0.7542
		P	0.6913
		P	0.7813
Dextran 15-20K	61	RI	0.7661
		P	0.5464
		P	0.5651
		P	0.5650
Dextran T40	90	RI	0.7359
		RI	0.6666
		RI	0.7472
		P	0.6750
		P	0.7717
		P	0.7446
Dextran T70	118	RI	0.6930
		RI	0.7255
		RI	0.7259
Dextran 200-300K	204	P	0.8022
		P	0.7629
		P	0.6214
Dextran T500	270	RI	0.7864
		RI	0.8033
		RI	0.7440
Dextran T2000	560	RI	0.8053
		RI	0.7393
		RI	0.7712
		P	0.8056
		P	0.7746
		P	0.7533

a RI = Refractive Index, P = Polarimeter

Table B2.2: Tabulated data for the pore size distribution for alkaline H_2O_2 (0.5 hr) pretreated wood (refer to Figure 4.5(a)).

Solute	Molecular Diameter (Å)	Measurement Method[a]	d (mL/g wood)
Fructose	8	P	0.1925
Raffinose	12	P	0.2721
		P	0.3026
Dextran 6K	38	P	0.8431
		P	0.7494
Dextran T10	51	P	0.7165
		P	0.7848
Dextran 15-20K	61	P	0.8599
		P	0.8530
Dextran T40	90	P	0.7973
		P	0.8755
Dextran T70	118	P	0.8976
		P	0.8786
		P	0.7830
Dextran 200-300K	204	P	0.8617
		P	0.7647
		P	0.7403
Dextran T500	270	P	0.8275
		P	0.8256
		P	0.9086
Dextran T2000	560	P	0.8241
		P	0.8261

a RI = Refractive Index, P = Polarimeter

Table B2.3: Tabulated data for the pore size distribution for alkaline H_2O_2 (5 hr) pretreated wood (refer to Figure 4.5(b)).

Solute	Molecular Diameter (Å)	Measurement Method[a]	d (mL/g wood)
Fructose	8	P	0.2568
		P	0.1085
Maltose	10	P	0.2417
		P	0.2172
Raffinose	12	P	0.2364
Dextran 6K	38	P	1.125
		P	1.175
		P	1.116
Dextran 15-20K	61	P	1.285
		P	1.285
		P	1.229
Dextran T40	90	P	1.296
		P	1.214
		P	1.202
Dextran T70	118	P	1.321
		P	1.411
		P	1.391
Dextran T500	270	P	1.223
		P	1.252
		P	1.194
Dextran T2000	560	P	1.322
		P	1.339

a RI = Refractive Index, P = Polarimeter

Table B2.4: Tabulated data for the pore size distribution for alkaline H_2O_2 (19 hr) pretreated wood (refer to Figure 4.5(c)).

Solute	Molecular Diameter (Å)	Measurement Method[a]	d (mL/g wood)
Fructose	8	P	0.1880
		P	0.2325
Raffinose	12	P	0.4323
Dextran 6K	38	P	0.6848
		P	0.7371
		P	0.8005
Dextran T10	51	RI	1.070
		RI	1.081
		P	1.097
		P	1.180
Dextran 15-20K	61	P	1.126
Dextran T40	90	RI	1.292
		P	1.137
		P	1.155
		P	1.209
Dextran T70	118	RI	1.347
Dextran 200-300K	204	P	1.322
		P	1.343
		P	1.379
Dextran T500	270	RI	1.213
		P	1.210
		P	1.298
		P	1.274
Dextran T2000	560	RI	1.310
		RI	1.486
		P	1.280
		P	1.405
		P	1.350

a RI = Refractive Index, P = Polarimeter

Table B2.5: Tabulated data for the pore size distribution for EDA (10 %) pretreated wood (refer to Figure 4.6(a)).

Solute	Molecular Diameter (Å)	Measurement Method[a]	d (mL/g wood)
Fructose	8	P	0.3761
		P	0.2209
		P	0.1901
Maltose	10	P	0.0109
		P	0.1131
		P	0.1579
Raffinose	12	P	0.5946
		P	0.6239
		P	0.5439
Dextran 6K	38	P	0.9033
		P	0.9038
		P	0.8823
Dextran T10	51	P	0.8871
		P	0.8972
		P	0.9019
Dextran 15-20K	61	P	1.030
		P	1.004
		P	0.9878
Dextran T40	90	P	1.049
		P	1.037
		P	1.082
Dextran T70	118	P	0.9122
		P	1.003
		P	1.009
Dextran 200-300K	204	P	1.001
		P	0.9787
		P	1.151
Dextran T500	270	P	0.9924
		P	0.9829
		P	1.154
Dextran T2000	560	P	1.006
		P	1.027
		P	1.070

a RI = Refractive Index, P = Polarimeter

Table B2.6: Tabulated data for the pore size distribution for EDA (50 %) pretreated wood (refer to Figure 4.6(b)).

Solute	Molecular Diameter (Å)	Measurement Method[a]	d (mL/g wood)
Fructose	8	P	0.2242
		P	0.2283
Maltose	10	P	0.1486
		P	0.1391
		P	0.1397
Raffinose	12	P	0.3895
		P	0.4268
		P	0.3819
Dextran 6K	38	P	0.4427
		P	0.4584
		P	0.3646
Dextran T10	51	P	0.6761
		P	0.7472
		P	0.7679
Dextran 15-20K	61	P	0.7547
		P	0.7805
		P	0.7233
Dextran T40	90	P	0.9003
		P	0.9645
		P	0.9092
Dextran T70	118	P	0.8610
		P	0.8513
		P	0.8752
Dextran 200-300K	204	P	0.8664
		P	0.8053
		P	0.8353
Dextran T500	270	P	0.9385
		P	0.8444
		P	0.9501
Dextran T2000	560	P	0.9991
		P	0.8992
		P	0.9194

a RI = Refractive Index, P = Polarimeter

Table B2.7: Tabulated data for the pore size distribution for EDA (70 %) pretreated wood (refer to Figure 4.6(c)).

Solute	Molecular Diameter (Å)	Measurement Method[a]	d (mL/g wood)
Glucose	8	RI	0.2393
		RI	0.2650
Fructose	8	P	0.2754
Maltose	10	RI	0.3893
		RI	0.3028
		RI	0.3294
Raffinose	12	RI	0.3050
		RI	0.2302
		RI	0.2521
		P	0.1857
		P	0.2281
		P	0.3827
Dextran 6K	38	P	0.6919
		P	0.6936
		P	0.6238
Dextran T10	51	P	0.8487
		P	0.8515
Dextran 15-20K	61	RI	1.040
		P	0.9953
		P	0.9533
Dextran T40	90	RI	1.201
		RI	1.148
		RI	1.0700
		P	1.179
		P	1.166
		P	1.105
Dextran T70	118	RI	1.299
		RI	1.356
		RI	1.333
		P	1.338
		P	1.336
		P	1.210
Dextran 200-300K	204	P	1.316
		P	1.209
Dextran T500	270	RI	1.352
		RI	1.365
		P	1.292
		P	1.350
		P	1.354
Dextran T2000	560	RI	1.474
		RI	1.512
		P	1.441
		P	1.380
		P	1.426

a RI = Refractive Index, P = Polarimeter

Table B2.8: Tabulated data for the pore size distribution for FR (240 °C) pretreated wood (refer to Figure 4.7(a)).

Solute	Molecular Diameter (Å)	Measurement Method[a]	d (mL/g wood)
Fructose	8	P	0.1424
Maltose	10	P	0.2056
		P	0.3145
		P	0.3096
Dextran 6K	38	P	0.5394
		P	0.3803
		P	0.5585
Dextran T10	51	P	0.6572
		P	0.7195
		P	0.6664
Dextran 15-20K	61	P	0.7354
		P	0.7030
		P	0.7602
Dextran T40	90	P	0.6816
		P	0.8014
		P	0.7654
Dextran T70	118	P	0.7462
		P	0.8959
		P	0.8504
Dextran 200-300K	204	P	0.8603
		P	0.8325
		P	0.8180
Dextran T500	270	P	0.7468
		P	0.8084
		P	0.7756
Dextran T2000	560	P	0.8252
		P	0.8389
		P	0.7684

a RI = Refractive Index, P = Polarimeter

Table B2.9: Tabulated data for the pore size distribution for FR (260 °C) pretreated wood (refer to Figure 4.7(b)).

Solute	Molecular Diameter (Å)	Measurement Method[a]	d (mL/g wood)
Fructose	8	P	0.1971
		P	0.3456
		P	0.0724
Maltose	10	P	0.4417
		P	0.4020
		P	0.5131
Dextran 6K	38	P	0.6675
		P	0.7829
		P	0.7159
Dextran T10	51	P	0.7140
		P	0.7736
		P	0.7579
Dextran 15-20K	61	P	0.7240
		P	0.7762
		P	0.8090
Dextran T40	90	P	0.8341
		P	0.8620
		P	0.8647
Dextran T70	118	P	0.8967
		P	0.9571
Dextran 200-300K	204	P	0.9925
		P	0.9496
Dextran T500	270	P	0.9337
		P	0.8691
		P	0.8885
Dextran T2000	560	P	0.9866
		P	0.9418
		P	1.023

a RI = Refractive Index, P = Polarimeter

Table B2.10: Tabulated data for the pore size distribution for FR (280 °C) pretreated wood (refer to Figure 4.7(c)).

Solute	Molecular Diameter (Å)	Measurement Method[a]	d (mL/g wood)
Fructose	8	P	0.2035
		P	0.1499
Raffinose	12	P	0.5166
		P	0.4509
		P	0.3517
Dextran 6K	38	P	0.7908
		P	0.6174
Dextran T10	51	P	0.8613
		P	0.9155
Dextran 15-20K	61	P	0.7924
		P	0.9599
Dextran T40	90	P	0.9924
		P	1.006
Dextran T70	118	P	1.029
		P	0.8825
Dextran 200-300K	204	P	0.9802
		P	0.9405
Dextran T500	270	P	1.098
		P	1.137
		P	1.118
Dextran T2000	560	P	1.235
		P	1.232

a RI = Refractive Index, P = Polarimeter

Table B2.11: Tabulated data for the pore size distribution for FRWA (180 °C) pretreated wood (refer to Figure 4.8(a)).

Solute	Molecular Diameter (Å)	Measurement Method[a]	d (mL/g wood)
Fructose	8	P	0.0629
Maltose	10	P	0.2088
		P	0.2709
		P	0.3012
Dextran T10	51	P	0.6943
		P	0.6962
		P	0.6640
Dextran 15-20K	61	P	0.6564
		P	0.6962
		P	0.5824
Dextran T40	90	P	0.7328
		P	0.7238
		P	0.6685
Dextran T70	118	P	0.8013
		P	0.7197
		P	0.7927
Dextran 200-300K	204	P	0.6210
		P	0.6986
		P	0.7629
Dextran T500	270	P	0.6078
		P	0.7101
Dextran T2000	560	P	0.6699
		P	0.7179
		P	0.7148

a RI = Refractive Index, P = Polarimeter

Table B2.12: Tabulated data for the pore size distribution for FRWA (200 °C) pretreated wood (refer to Figure 4.8(b)).

Solute	Molecular Diameter (Å)	Measurement Method[a]	d (mL/g wood)
Fructose	8	P	0.2310
		P	0.0878
		P	0.1403
Maltose	10	P	0.2518
		P	0.1975
		P	0.1657
Raffinose	12	P	0.3799
		P	0.1803
		P	0.3459
Dextran 6K	38	P	0.4681
		P	0.4901
		P	0.5076
Dextran T10	51	P	0.4967
		P	0.5663
		P	0.5908
Dextran 15-20K	61	P	0.4827
		P	0.5029
		P	0.6449
Dextran T40	90	P	0.8708
		P	0.9118
		P	0.8295
Dextran T70	118	P	1.006
		P	0.9069
		P	0.8566
Dextran 200-300K	204	P	1.016
		P	0.8992
		P	1.024
Dextran T500	270	P	1.026
		P	0.9953
Dextran T2000	560	P	0.8636

a RI = Refractive Index, P = Polarimeter

Table B2.13: Tabulated data for the pore size distribution for FRWA (220 °C) pretreated wood (refer to Figure 4.8(c)).

Solute	Molecular Diameter (Å)	Measurement Method[a]	d (mL/g wood)
Fructose	8	P	0.1150
		P	0.0294
Dextran 6K	38	P	0.4815
		P	0.4771
Dextran T10	51	P	0.5529
		P	0.6427
Dextran 15-20K	61	P	0.7230
		P	0.8031
		P	0.8122
Dextran T40	90	P	1.033
		P	0.9406
		P	0.9140
Dextran T70	118	P	0.7926
		P	0.8763
		P	0.8249
Dextran 200-300K	204	P	1.117
		P	1.158
		P	1.126
Dextran T500	270	P	1.357
		P	1.339
		P	1.259
Dextran T2000	560	P	1.302
		P	1.356
		P	1.337

a RI = Refractive Index, P = Polarimeter

Table B2.14: Tabulated data for the determination of maximum CCN 3000 activity for FRWA 220 °C wood (refer to Figure 4.9).

CCN 3000 Activity (FPU/g wood)	Glucose Yield at 2 hr (%)
0.0	0.00
10.2	28.59
15.2	37.97
20.5	44.20
29.8	57.34
39.9	65.49
49.6	67.31

Table B2.15: Tabulated data for the comparison of the time courses of enzymatic hydrolysis of FRWA 220 °C wood with CCN 3000 and Rutgers C30 cellulases (refer to Figure 4.10).

Hydrolysis Time (hr)	Glucose Yield (%) using: CCN 3000 at 49.6 FPU/g wood	Rutgers C30 at 38.8 FPU/g wood
0.0	0.00	0.00
0.5	34.78	34.97
1.0	49.73	50.48
2.0	67.31	69.23
4.0	83.89	86.25
12.0	93.30	98.09
24.0	92.16	97.81

Figure B2.16: Tabulated data for the time courses of enzymatic hydrolysis of Native and alkaline H_2O_2 pretreated wood (refer to Figure 4.11).

Hydrolysis Time (hr)	Glucose Yield (%) For Wood Treated With: None (Native)	Alkaline H_2O_2 for: 0.5 hr	5 hr	19 hr
0.0	0.00	0.00	0.00	0.00
0.5	6.59	16.50	23.48	23.87
1.0	7.42	21.34	30.76	32.43
2.0	9.83	26.35	37.20	41.98
4.0	10.47	32.93	45.66	49.33
12.0	13.85	42.15	53.09	62.35
24.0	14.70	47.06	59.86	68.24

Figure B2.17: Tabulated data for the time courses of enzymatic hydrolysis of Native and EDA pretreated wood (refer to Figure 4.12).

Hydrolysis Time (hr)	Glucose Yield (%) For Wood Treated With: None (Native)	Aqueous EDA at: 10 %	50 %	70 %
0.0	0.00	0.00	0.00	0.00
0.5	6.59	19.47	23.08	30.77
1.0	7.42	24.55	31.72	42.01
2.0	9.83	31.10	41.77	54.48
4.0	10.47	39.90	49.99	59.77
12.0	13.85	47.53	60.51	70.38
24.0	14.70	52.20	67.53	70.63

Figure B2.18: Tabulated data for the time courses of enzymatic hydrolysis of Native and autohydrolysis (FR) pretreated wood (refer to Figure 4.13).

Hydrolysis Time (hr)	Glucose Yield (%) For Wood Treated With: None (Native)	Auitohydrolysis at: 240 °C	260 °C	280 °C
0.0	0.00	0.00	0.00	0.00
0.5	6.59	7.45	10.57	13.78
1.0	7.42	10.95	15.92	21.27
2.0	9.83	17.45	25.21	32.51
4.0	10.47	26.77	35.10	47.01
12.0	13.85	42.39	53.56	67.83
24.0	14.70	58.66	63.88	74.15

Figure B2.19: Tabulated data for the time courses of enzymatic hydrolysis of Native and acid hydrolysis (FRWA) pretreated wood (refer to Figure 4.14).

Hydrolysis Time (hr)	Glucose Yield (%) For Wood Treated With: None (Native)	Aqueous EDA at: 180 °C	200 °C	220 °C
0.0	0.00	0.00	0.00	0.00
0.5	6.59	8.58	27.95	33.59
1.0	7.42	11.87	40.74	50.03
2.0	9.83	16.49	51.05	70.99
4.0	10.47	23.23	73.58	85.56
12.0	13.85	37.11	87.77	92.93
24.0	14.70	43.78	92.12	97.66

Table B2.20: Tabulated data for the correlation of glucose yield at 2 hr and specific surface area available to a 51 Å solute (refer to Figure 4.15).

Pretreatment		Surface Area (m^2/g wood)	Glucose Yield at 2 hr (%)
Native		14.8	9.83
H_2O_2 :	0.5 hr	24.5	26.35
	5 hr	66.7	37.20
	19 hr	70.8	41.98
EDA :	10 %	30.7	31.10
	50 %	47.3	41.77
	70 %	94.5	54.48
FR :	240 °C	34.7	17.45
	260 °C	38.0	25.21
	280 °C	48.4	32.51
FRWA:	180 °C	25.8	16.49
	200 °C	85.7	51.05
	220 °C	128	70.99

Table B2.21: Tabulated data for the empirical correlation of measured glucose yield at 2 hr with glucose yields predicted by the empirical correlation using SSA in pores 51 Å and larger, CrI, and G/L ratios. (refer to Figure 4.16).

Pretreatment		Measured Glucose Yield at 2 hr (%)	Predicted* Glucose Yield at 2 hr (%)
Native		9.83	12.25
H_2O_2 :	0.5 hr	26.35	19.41
	5 hr	37.20	46.02
	19 hr	41.98	44.79
EDA :	10 %	31.10	22.80
	50 %	41.77	32.69
	70 %	54.48	62.61
FR :	240 °C	17.45	23.42
	260 °C	25.21	25.15
	280 °C	32.51	32.45
FRWA:	180 °C	16.49	18.11
	200 °C	51.05	47.32
	220 °C	70.99	65.43

* Empirical correlation for this case is:

$$\text{Yield (\%)} = 0.4437(100 - \text{CrI})^{0.2932}\ (\text{G/L})^{0.2470}\ (\text{SSA}_{51\,\text{Å}})^{0.8270}$$

BIBLIOGRAPHY

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1. Tsao, G.T., M.R. Ladisch and H.R. Bungay, "Biomass Refining," in **Advanced Biochemical Engineering**, H.R. Bungay and G. Belfort (eds.), Wiley Interscience, N.Y., pp. 79-101, 1987.

2. Wright, J.D., "Ethanol from biomass by enzymatic hydrolysis," **Chem. Eng. Prog.**, **84**(8):62, 1988.

3. Fan, L.T., Y.-H. Lee and M.M. Gharpuray, "The nature of lignocellulosics and their pretreatments for enzymatic hydrolysis," in **Adv. Biochem. Eng.**, Springer-Verlag, N.Y., **23**:157, 1982.

4. Hanselmann, K.W., "Lignochemicals," **Experientia**, **38**:176, 1982.

5. Gould, J.M., "Enhanced polysaccharide recovery from agricultural residues and perennial grasses treated with alkaline hydrogen peroxide," **Biotech. Bioeng.**, **27**:893, 1985.

6. Cowling, E.B., "Physical and chemical constraints in the hydrolysis of cellulose and lignocellulosic materials," **Biotech. Bioeng. Symp.**, **5**:163, 1975.

7. Kirk, T.K., "Biochemistry of lignin degradation by *Phanerochaete chrysosporium*," in **Biochemistry and Genetics of Cellulose Degradation**, J.P. Aubert *et al.* (eds.), Academic Press, Ltd., pp. 315-332, 1988.

8. Tsao, G.T., "Bacterial hydrolysis: A review," in **Anaerobic Digestion and Carbohydrate Hydrolysis of Waste**, G.L. Ferrero *et al.* (eds.), Elsevier Appl. Sci. Publ., London, pp. 83-99, 1984.

9. Marsden, W.L. and P.P. Gray, "Enzymatic hydrolysis of cellulose in lignocellulosic materials," **Crit. Rev. Biotech.**, G.G. Stewart and I. Russel (eds.), CRC Press, Boca Raton, Fla., **3**:(3):235-276, 1986.

10. Walseth, C.S., "Influence of the fine structure of cellulose on the action of cellulases," **Tappi**, **35**:228, 1952.

11. Knappert, D., H. Grethlein and A. Converse, "Partial acid hydrolysis of cellulosic materials as a pretreatment for enzymatic hydrolysis," **Biotech. Bioeng., 22**:1449, 1980.

12. Goldstein, I.S., H. Pereira, J.L. Pittman, B.A. Strause, and F.P. Scaringelli, "The hydrolysis of cellulose with superconcentrated hydrochloric acid," **Biotech. Bioeng. Symp, 13**:17, 1983.

13. Weimer, P.J., Y.-C.T. Chou, W.M. Weston, and D.B. Chase, "Effect of supercritical ammonia on the physical and chemical structure of ground wood," **Biotech. Bioeng. Symp., 17**:5, 1986.

14. Playne, M.J., "Increased digestibility of bagasse by pretreatment with alkalis and steam explosion," **Biotech. Bioeng., 26**:426, 1984.

15. Azuma, Jun-ichi, F. Tanaka, and T. Koshijima, "Microwave irradiation of lignocellulosic materials. I. Enzymatic susceptibility of microwave-irradiated woody plants," **Mokuzai Gakkaishi, 30**(6):501, 1984.

16. Schwald, W., M. Chan, H.H. Brownell, and J.N. Saddler, "Influence of hemicellulose and lignin on the enzymatic hydrolysis of wood," in **Biochemistry and Genetics of Cellulose Degradation**, J.P. Aubert *et al.*, (eds.), Academic Press, Limited, pp. 285-302, 1988.

17. Grethlein, H.E., "The effect of pore size distribution on the rate of enzymatic hydrolysis of cellulosic substrates," **Bio/Technology**, pp. 155-160, Feb. 1985.

18. Cowling, E.B. and T.K. Kirk, "Properties of cellulose and lignocellulosic materials as substrates for enzymatic conversion processes," **Biotech. Bioeng. Symp., 6**:95, 1976.

19. Converse, A.O., I.K. Kwarteng, H.E. Grethlein, and H. Ooshima, "Kinetics of thermochemical pretreatment of lignocellulosic materials," **Appl. Biochem. Biotech., 20/21**:63, 1989.

20. Chen, H.-C., and H.E. Grethlein, "Effect of cellulase size reduction on activity and accessibility," **Biotech. Letters, 10**(12):913, 1988.

21. Ooshima, H., M. Sakata, and Y. Harano, "Enhancement of enzymatic hydrolysis of cellulose by surfactant," **Biotech. Bioeng., 28**:1727, 1986.

22. Mandels, M. and J. Weber, "The production of cellulases," **Adv. Chem. Ser., 95**:391, 1969.

23. Mandels, M., "Cellulases," **Annu. Rep. Ferment. Processes, 5**:35, 1982.

24. Montenecourt, B.S., and D.E. Eveleigh, "Selective screening methods for the isolation of high yielding cellulase mutants of *T. reesei*," **Adv. Chem. Ser., 181**:289, 1979.

25. Beltrame, P.L., P. Carniti, B. Focher, A. Marzetti, and V. Sarto, "Cellobiose hydrolysis of crude cellobiase. Kinetics and mechanism," **La Chimica E L'Industria, 65**(6):398, 1983.

26. Van Dyke, Jr., B.H., "Enzymatic hydrolysis of cellulose - A kinetic study," Ph.D. Dissertation, Massachusetts Institute of Technology, 1972.

27. Stone, J.E., A.M. Scallan, E. Donefer, and E. Ahlgren, "Digestibility as a simple function of a molecule of similar size to a cellulase enzyme," **Adv. in Chem. Ser., 95**:219, 1969.

28. Stone, J.E., and A.M. Scallan, "The effect of component removal upon the porous structure of the cell wass of wood," **Pulp and Paper Mag. Can., 69**:288, 1968.

29. Hayn, M., H. Esterbauer and B. Leonhard, "Characterization of cellulase from *Trichoderma reesei*," paper presented at the IEA/FE/CPD-2 Symposium, Graz, Austria, June 23-27, 1986.

30. Reese, E.T., "Degradation of polymeric carbohydrates by microbial enzymes," **Rec. Adv. Phytochem., 11**:311, 1977.

31. Kim, D.W.,J.H. Yang, and Y.K. Jeong, "Adsorption of cellulase from *Trichoderma viride* on microcrystalline cellulose" **Appl. Microbiol. Biotech., 28**:148, 1988.

32. Mandels, M., J. Kostick, and R. Parizek, "The use of adsorbed cellulase in the continuous conversion of cellulose to glucose," **J. Polym. Sci (C), 36**:445, 1977.

33. Ryu, D.D.Y, C. Kim, and M. Mandels, "Competitive adsorption of cellulase components and its signficance in a synergistic mechanism," **Biotech. Bioeng., 26**:488, 1984.

34. Bisaria, V.S, and T.K. Ghose, "Sorption characteristics of cellulase on cellulosic substrates," in **Bioconversion of Cellulosic Substances into Energy, Chemicals, and Microbial Protein**, Symposium Proceedings, T.K. Ghose (ed.), IIT, New Delhi, p. 155, 1978.

35. Lee, Y.-H., L.T. Fan, "Kinetic studies of enzymatic hydrolysis of insoluble cellulose: Analysis of the initial rates," **Biotech. Bioeng.**, 24:2383, 1982.

36. Scallan. A.M., "A quantative picture of the fringed micellar model of cellulose," **Textile Res. J.**, 41(8):647, 1971.

37. Chang, M., "Folding chain model and annealing of cellulose" **J. Polym. Sci. (C)**, 36:343, 1971.

38. Tsao, G.T., M. Ladisch, C. Ladisch, T.A. Hsu, B. Dale, and T. Chou, "Fermentation substrates from cellulosic materials: Production of fermentable sugars from cellulosic materials," **Annu. Rep. Ferment. Processes**, 2:1, 1978.

39. Chang, M.M., T.Y.C. Chou, and G.T. Tsao, "Structure, pre-treatment and hydrolysis of cellulose," in **Adv. Biochem. Eng.**, Springer-Verlag, N.Y., 20:15, 1981.

40. Grous, W.R., A.O. Converse, H.E. Grethlein, "Effect of steam explosion pretreatment on pore size and enzymatic hydrolysis of poplar," **Enzym. Microbiol. Technol.**, 8:274, 1986.

41. Fan, L.T.,Y.-H. Lee, and D.H. Beardmore, "Mechanism of the enzymatic hydrolysis of cellulose: Effects of major structural features of cellulose on enzymatic hydrolysis," **Biotech. Bioeng.**, 22:177, 1980.

42. Fan, L.T., Y.H. Lee, and D.H. Beardmore, "The influence of major structural features of cellulose on the rate of enzymatic hydrolysis," **Biotech. Bioeng.**, 23:419, 1981.

43. Gharpuray, M.M., Y.-H. Lee, and L.T. Fan, "Structural modification of lignocellulosics by pretreatments to enhance enzymatic hydrolysis," **Biotech. Bioeng.**, 25:157, 1983.

44. Segal, L., J.J. Creely, A.E. Martin, Jr., and C.M. Conrad, "An empirical method for estimating the degree of crystallinity of native cellulose using the X-ray diffractometer," **Textile Res. J.**, 29:788, 1959.

45. Ryu, D., S.B. Lee, and T. Tassinari, "Effect of crystallinity of cellulose on enzymatic hydrolysis kinetics," **Abstract Pap. Am. Chem. Soc.**, 182, Micr. 58, 1982.

46. Klesov, A.A., and A.P. Sinitsin, "Enzymatic hydrolysis of cellulose. IV. Effect of major physicochemical and structural features of the substrate," **Bioorg. Khim.**, 7:1801, 1981.

47. Lee, Y.-H., and L.T. Fan, "Kinetic studies of enzymatic hydrolysis of insoluble cellulose: Derivation of a mechanistic kinetic model," **Biotech. Bioeng.**, **25**:2707, 1983.

48. Marsden, W.L., N.W. Dunn, and P.P. Gray, "Action of *Trichoderma reesei* QM9414 and C30 cellulase on substrates with varying crystallinity," **Enzym. Microbiol. Technol.**, **5**:345, 1983.

49. Puri, V.P., "Effect of crystallinity and degree of polymerisation on enzymatic saccharification," **Biotech. Bioeng.**, **26**:1219, 1984.

50. Iverson, T., "Lignin-carbohydrate bonds in a lignin-carbohydrate complex isolated from spruce," **Wood Sci. Technol.**, **19**:243, 1985.

51. Ruel, K., F. Barnoud, and K.E. Eriksson, "Ultrastructural aspects of wood degradation by *Sporotrichum pulverulentum*," **Holzforshung**, **38**:61, 1984.

52. Eriksson, K.E., "Fungal degradation of wood components," **Pure Appl. Chem.**, **52**:1305, 1981.

53. Blanchette, R.A., and A.R. Abad, "Ultrastructural localization of hemicellulose in birch wood (*Betula papyrifera*) decayed by brown and white-rot fungi," **Holzforschung**, **42**:393, 1988.

54. Blanchette, R.A., A.R. Abad, R.L. Farrell, and T.D. Leathers, "Detection of lignin peroxidase and xylanase by immunocytochemical labeling in wood decayed by basidiomycetes," **Appl. Environ. Microbiol.**, **55**(6):1457, 1989.

55. Vohra, R.M., C.K. Shirkot, S. Dhawan, and K.G. Gupta, "Effect of lignin and some of its components on the production and activity of cellulases by *Trichoderma reesei*," **Biotech. Bioeng.**, **22**:1497, 1980.

56. Sudo, K., Y. Matsumura, and K. Shimizu, "Enzymatic hydrolysis of woods. I. Effect of deligninfication on hydrolysis of woods by *Trichoderma viride* cellulase," **Mokuzai Gakkaishi**, **22**:670, 1976.

57. Ladisch, M.R., K.W. Lin, M. Voloch, and G.T. Tsao, "Process considerations in the enzymatic hydrolysis of biomass," **Enzyme Microbiol. Technol.**, **5**(2):82, 1983.

58. Millett, M.A., A.J. Baker, and L.D. Satter, "Physical and chemical pretreatments for enhancing cellulose saccharification," **Biotech. Bioeng. Symp.**, **5**:193, 1975.

59. Fan, L.T., M.M. Gharpuray, and Y.-H. Lee, "Evaluation of pretreatments for enzymatic conversion of agricultural residues," **Biotech. Bioeng. Symp., 11**:29, 1981.

60. Stranks, D.W., "Utilisation of aspen wood residues," **For. Prod. J., 11**:288, 1961.

61. Saddler, J.N., H.H. Brownell, L.P. Clermont, and N. Levitin, "Enzymatic hydrolysis of cellulose and various pretreated wood fractions," **Biotech. Bioeng., 24**:1389, 1982.

62. Wilson, P.N., and T. Brigstocke, "The commercial straw process," **Process Biochem., 12**(7):17, 1977.

63. Andren, R.K., M. Mandels, and J.E. Medeiros, "Production of sugars from waste cellulose by enzymatic hydrolysis: Primary evaluation of substrates," **Process Biochem., 11**(8):2, 1976.

64. Mandels, M., L. Hontz, and J. Nystrom, "Enzymatic hydrolysis of waste cellulose," **Biotech. Bioeng., 16**:1471, 1974.

65. Wilke, C.R., U.V. Stockar, and R.D. Yang, "Process design basis for enzymatic hydrolysis of newsprint," **AIChE Symp. Ser., 72**:104, 1976.

66. Matsumura, Y., K. Sudo, and K. Shimizu, "Enzymatic hydrolysis of woods. II. Effect of grinding and alkali treatment on hydrolysis of woods by *Trichoderma viride* cellulase," **Mokuzai Gakkaishi, 23**:562, 1977.

67. Millet, M.A., M.J. Effland, and D.F. Caulfield, "Influence of fine grinding on the hydrolysis of cellulosic materials - acid vs. enzymatic," **Adv. Chem. Ser., 181**:71, 1979.

68. Rivers, D.B., and G.H. Emert, "Lignocellulose pretreatment: A comparison of wet and dry ball attrition," **Biotech. Letters, 9**(5):365, 1987.

69. Tassinari, T., and C. Macy, "Differential two speed roll mill pretreatment for cellulosic materials for enzymatic hydrolysis," **Biotech. Bioeng., 19**:1321, 1977.

70. Tassinari, T., C. Macy, L. Spano, and D.D.Y. Ryu, "Energy requirements and process design considerations in compression-milling pretreatment of cellulosic wastes for enzymatic hydrolysis," **Biotech. Bioeng., 22**:1689, 1980.

71. Tangnu, S.K., "Process development for ethanol production based on enzymatic hydrolysis of cellulosic biomass," **Process Biochem.**, **17**(3):36, 1982.

72. Horton, G.L., D.B. Rivers, and G.H. Emert, "Preparation of cellulosics for enzymatic conversion," **Ind. Eng. Chem. Prod. Res. Dev.**, **19**:422, 1980.

73. Kumakura, M., and I. Kaetsu, "Radiation-induced decomposition and enzymatic hydrolysis of cellulose," **Biotech. Bioeng.**, **20**:1309, 1978.

74. Khan, A.W., J.-P. Labrie, and J. McKeown, "Effect of electron-beam irradiation pretreatment on the enzymatic hydrolysis of softwood," **Biotech. Bioeng.**, **28**:1449, 1986.

75. Pigden, W.J., G.I. Pritchard, and D.P. Heaney, Proceedings of the International Grassland Congress, Paper No. 11, 1966.

76. Han, Y.W., J. Timpa, A. Ciegler, J. Courtney, W.F. Curry, and E.N. Lambremont, "γ-ray-induced degradation of lignocellulosic materials," **Biotech. Bioeng.**, **23**:2525, 1981.

77. Ooshima, H. K. Aso, Y. Harano, T. Yamamoto, "Microwave treatment of cellulosic materials for their enzymatic hydrolysis," **Biotech. Letters**, **6**(5):289, 1984.

78. Azuma, J.-I., F. Tanaka, and T. Koshijima, "Enhancement of enzymatic susceptibility of lignocellulosic wastes by microwave irradiation," **J. Ferm. Technol.**, **62**(4):377, 1984.

79. Dale, B.E., and M.J. Moreira, "A freeze-explosion technique for increasing cellulose hydrolysis," **Biotech. Bioeng. Symp.**, **12**:31, 1982.

80. Mes-Hartree, M., B.E. Dale, and W.K. Craig, "Comparison of steam and ammonia pretreatment for enzymatic hydrolysis of cellulose," **Appl. Microbiol. Biotechnol.**, **29**:462, 1988.

81. Sasaki, T., Y. Sato, T. Kobayashi, and K. Kainuma, "Enzymatic conversion of cryomilled waste cellulose to glucose," **Nippon Shokuhin Kogyo Gakkaishi**, **27**:270, 1980.

82. Sasaki, T., Y. Sato, S. Nakagawa, M. Shiraishi, and K. Kainuma, "Crystallinity and enzymatic hydrolysis of cryomilled rice hull cellulose," **Nippon Shakuhin Kogyo Gakkaishi**, **26**:523, 1979.

83. Tarkow, H., and W.C. Feist, "A mechanism for improving the digestibility of lignocellulosic materials with dilute alkali and liquid ammonia," **Adv. Chem. Ser.**, **95**:197, 1969.

84. Millet, M.A., A.J. Baker, and L.D. Satter, "Pretreatments for enhancing cellulose saccharification," **Biotech. Bioeng. Symp.**, **6**:125, 1976.

85. Macdonald, D.G., N.H. Bakhshi, J.F. Mathews, A. Roychowdhury, P. Bajpai, and M. Moo-Young, "Alkali treatment of corn stover to improve sugar production by enzymatic hydrolysis," **Biotech. Bioeng.**, **25**:2067, 1983.

86. Miron, J., and D. Ben-Ghedalia, "Effect of hydrolysing and oxidizing agents on the composition and degradation of wheat straw monosaccharides," **Eur. J. Appl. Microbiol. Biotechnol.**, **15**:83, 1982.

87. Ben-Ghedalia, D., G. Shefet, J. Miron, and Y. Dror, "Effect of ozone and sodium hydroxide treatments on some chemical characteristics of cotton straw," **J. Food Sci. Agric.**, **33**:1213, 1982.

88. Lewin, M., and L.G. Roldan, "The effect of liquid anhydrous ammonia in the structure and morphology of cotton cellulose," **J. Polym. Sci. (C)**, **36**:213, 1971.

89. O'Connor, J.J., "Ammonia explosion pulping: A new fiber separation process," **Tappi**, **55**(3):353, 1972.

90. Chou, Y.-C.T., "Supercritical ammonia pretreatment of lignocellulosic materials, " **Biotech. Bioeng. Symp.**, **17**:19, 1986.

91. Lachenal, D., C. De Choudens, and P. Monzie, "Hydrogen peroxide as a delignifying agent," **Tappi**, **63**(4):119, 1980.

92. Gould, J.M., "Alkaline peroxide delignification of agricultural residues to enhance enzymatic saccharification," **Biotech. Bioeng.**, **26**:46, 1984.

93. Wei, C.-J., and C.-Y. Cheng, "Effect of hydrogen peroxide pretreatment on the structural features and the enzymatic hydrolysis of rice straw," **Biotech. Bioeng.**, **27**:1418, 1985.

94. Feist, W.C., A.J. Baker, and H. Tarkow, "Alkali requirements for improving digestibility of hardwoods by rumen micro-organisms," **J. Anim. Sci.**, **30**:832, 1970.

95. Flory, P.J., in "Principles of polymer chemistry," Cornell Univ. Press, Ithaca, NY, p. 519, 1953.

96. du Toit, P.J., S.P. Olivier, and P.L. van Biljon, "Sugar cane bagasse as a possible source of fermentable carbohydrates. I. Characterization of bagasse with regard to monosaccharide, hemicellulose, and amino acid composition," **Biotech. Bioeng.**, **26**:1071, 1984.

97. Allen, D.C., H.E. Grethlein, and A.O. Converse, "Process studies for enzymatic hydrolysis using high solids slurries of acid pretreated mixed hardwood," **Biotech. Bioeng. Symp.**, **13**:99, 1983.

98. Knappert, D., H. Grethlein, and A. Converse, "Partial acid hydrolysis of poplar wood as a pretreatment for enzymatic hydrolysis," **Biotech. Bioeng. Symp.**, **11**:67, 1981.

99. Saddler, J.N., H.H. Brownell, L.P. Clermont, and N. Levitin, "Enzymatic hydrolysis of cellulose and various pretreated wood fractions," **Biotech. Bioeng.**, **24**:1389, 1982.

100. Nesse, N., J. Wallick, and J.M. Harper, "Pretreatment of cellulosic wastes to increase enzyme reactivity," **Biotech. Bioeng.**, **19**:323, 1977.

101. Maloney, M.T., T.W. Chapman, and A.J. Baker, "Dilute acid hydrolysis of paper birch: Kinetic studies of xylan and acetyl-group hydrolysis," **Biotech. Bioeng.**, **27**:355, 1985.

102. Tran, A.V., and R.P. Chambers, "Behaviors of southern red oak hemicelluloses and lignin in a mild sulfuric acid hydrolysis," **Biotech. Bioeng.**, **28**:811, 1986.

103. Lora, J.H., and M. Wayman, "Delignification of hardwoods by autohydrolysis and extraction," **Tappi**, **61**(6):47, 1978.

104. Wayman, M., and J.H. Lora, "Delignification of wood by auto-hydrolysis and extraction," **Tappi**, **62**(9):113, 1979.

105. Lamptey, J., C.W. Robinson, and M. Moo-Young, "Enhanced enzymatic hydrolysis of lignocellulosic biomass pretreated by low-pressure steam autohydrolysis," **Biotech. Letters**, 7(7):531, 1985.

106. Taylor, J.D., "Continuous autohydrolysis, a key step in the economic conversion of forest and crop residues into ethanol," in **Energy From Biomass**, 1st E.C. Conf., W. Palz (ed.), Applied Science Publishers, London, pp. 330-336, 1981.

107. Dietrichs, H.H., M. Sinner, and J. Puls, "Potential of steaming hardwoods and straw for feed and food production," **Holzforschung, 32**(6):193, 1978.

108. Delong, E.A., Australian Patent, AU-A1-37454, 1978.

109. Vallander, L., and K.-E. Eriksson, "Enzymic saccharification of pretreated wheat straw," **Biotech. Bioeng., 27**:650, 1985.

110. Longsworth, L.G., "Diffusion measurements, at 1°, of aqueous solutions of amino acids, peptides and sugars," **J. Am. Chem. Soc., 74**:4155, 1952.

111. Galbe, M., and G. Zacchi, "Pretreatment of sallow prior to enzymatic hydrolysis," **Biotech. Bioeng. Symp., 17**:97, 1986.

112. Morjanoff, P.J., and P.P. Gray, "Optimization of steam explosion as a method for increasing susceptibility of sugarcane bagasse to enzymatic saccharification," **Biotech. Bioeng., 29**:733, 1987.

113. Kling, S.H., C. Carvalho Neto, M.A. Ferrara, J.C.R. Torves, D.B. Magalhaes, and D.D.Y. Ryu, "Enhancement of enzymatic hydrolysis of sugar cane bagasse by steam explosion pretreatment," **Biotech. Bioeng., 29**:1035, 1987.

114. San Martin, R., H.W. Blanch, C.R. Wilke, and A.F. Sciamanna, "Production of cellulase enzymes and hydrolysis of steam-exploded wood," **Biotech. Bioeng., 28**:564, 1986.

115. Brownell, H.H., E.K.C. Yu, and J.N. Saddler, "Steam-explosion pretreatment of wood: Effect of chip size, acid moisture content and pressure drop," **Biotech. Bioeng., 28**:792, 1986.

116. Sinitsyn, A.P., L.S. Clesceri, and H.R. Bungay, "Inhibition of cellulases by impuriteis in steam-exploded wood," **Appl. Biochem. Biotechnol., 7**:455, 1982.

117. Binder, A., L. Pelloni, and A. Fiechter, "Delignification of straw with ozone to enhance biodegradability," **Eur. J. Appl. Microbiol. Biotechnol., 11**:1, 1980.

118. Lee, Y.-H., C.W. Robinson, and M. Moo-Young, "Evaluation of organosolv processes for the fractionation and modification of corn stover for bioconversion," **Biotech. Bioeng., 29**:572, 1987.

119. Taniguchi, M., M. Tanaka, R. Matsuno, and T. Kamikubo, "Evaluation of chemical pretreatment for enzymatic solubilization of rice straw," **Eur. J. Appl. Microbiol. Biotechnol., 14**:35, 1982.

120. David, C., R. Fornasier, C. Greindl-Fallon, and N. Vanlautem, "Enzymatic hydrolysis and bacterian hydrolysis - Fermentation of eucalyptus wood pretreated with sodium hypochlorite," **Biotech. Bioeng.**, **27**:1591, 1985.

121. Takagi, M., "Pretreatment of lignocellulosic materials with hydrogen peroxide in prescence of manganese compounds," **Biotech. Bioeng.**, **29**:165, 1987.

122. Gould, J.M., "Studies on the mechanism of alkaline peroxide delignification of agricultural residues," **Biotech. Bioeng.**, **27**:225, 1985.

123. Szczodrak, J., Z. Ilczuk, J. Rogalski, and A. Leonowicz, "Intensification of oak sawdust enzymatic hydrolysis by chemical or hydrothermal pretreatment," **Biotech. Bioeng.**, **28**:504, 1986.

124. Neely, W.C., "Factors affecting the pretreatment of biomass with gaseous ozone," **Biotech. Bioeng.**, **26**:59, 1984.

125. Binder, A., Th. Haltmeir, and A. Fiechter, "Pretreatment of straw," (Proceedings of Bioconversion and Biochemical Engineering Symposium, Vol. I, March 3-6, 1980, Indian Institute of Technology, Hauz Khas, New Delhi 110016, India), T.K. Ghose (ed.), pp. 315-332.

126. Holtzapple, M.T., and A.E. Humphrey, "The effect of organosolv pretreatment on the enzymatic hydrolysis of poplar," **Biotech. Bioeng.**, **26**:670, 1984.

127. Neilson, M.J., F. Shafizadeh, S. Aziz, and K.V. Sarkanen, "Evaluation of organosolv pulp as a suitable substrate for rapid enzymatic hydrolysis," **Biotech. Bioeng.**, **25**:609, 1983.

128. Avgerinos, G.C., and D.I.C. Wang, "Selective solvent delignification for fermentation enhancement," **Biotech. Bioeng.**, **25**:67, 1983.

129. Aravamuthan, R., W.-Y. Chen, K. Zargarian, and G. April, "Ethanol from southern hardwoods: The role of presulfonation in the acid hydrolysis process," **Biotech. Bioeng. Symp.**, **17**:107, 1986.

130. Detroy, R.W., L.A. Lindenfelser, G. St. Julian, Jr., and W.L. Orton, "Saccharification of wheat-straw cellulose by enzymatic hydrolysis following fermentative and chemical pretreatment," **Biotech. Bioeng. Symp.**, **10**:135, 1980.

131. Tanaka, M., C.W. Robinson, and M. Moo-Young, "Chemical and enzyme pretreatment of corn stover to produce soluble fermentation substrates," **Biotech. Bioeng.**, 27:362, 1985.

132. Ladisch, M.R., C.M. Ladisch, and G.T. Tsao, "Cellulose to sugars: New path gives quantitative yield," **Science, 201**(25):743, 1978.

133. Tsao, G.T., M.R. Ladisch, C.M. Ladisch, and T.-A. Hsu, U.S. Patent, 4,281,063, 1981.

134. Chen, L.-F., and C.-S. Gong, "Enzymatic hydrolysis of cellulose pretreated with zinc chloride and hydrochloric acid," **Biotech. Bioeng. Symp., 12**:57, 1982.

135. Eriksson, K.-E., "Microbial delignification - Basics, potentials and applications," in **Biochemistry and Genetics of Cellulose Degradation**, J.P. Aubert *et al.* (eds.), Academic Press, Limited, pp. 285-302, 1988.

136. Myers, G.C., G.F. Leatham, T.H. Wegner, and R.A. Blanchette, "Fungal pretreatmetn of aspen chips improves strength of refiner mechanical pulp," **Tappi**, pp. 105-108, May, 1988.

137. Daniel, G., T. Nilsson, and B. Pettersson, "Intra- and extracellular localization of lignin peroxidase during the degradation of solid wood and wood fragments by *Phanerochaete chrysosporium* by using transmission electron microscopy and immuno-gold labeling," **Appl. Environ. Microbiol., 55**(4):871, 1989.

138. Grethlein, H.E., U.S. Patent 4,237,226, 1980.

139. Gould, J.M., and S.N. Freer, "High-efficiency ethanol production from lignocellulosic residues pretreated with alkaline H_2O_2," **Biotech. Bioeng., 26**:628, 1984.

140. Gould, J.M., Private Communication.

141. Saemon, J.F., J.L. Bubl, and E.E. Harris, "Quantative saccharification of wood and cellulose," **Ind. Eng. Chem., 17**(1):35, 1945.

142. Stone, J.E., A.M. Scallan, "A structural model for the cell wall of water-swollen wood pulp fibres based on their accessibility to macromolecules," **Cellulose Chem. Technol., 2**:343, 1968.

143. Weimer, P.J., and W.M. Weston, "Relationship between the fine structure of native cellulose and cellulose degradability by the cellulase complexes of *Trichoderma reesei* and *Clostridium thermocellum*," **Biotech. Bioeng.**, 27:1540, 1985.

144. Scallan, A.M., Private Communication.

145. Lin, K.W., M.R. Ladisch, M. Voloch, J.A. Patterson, and C.H. Noller, "Effect of pretreatments and fermentation on pore size in cellulosic materials," **Biotech. Bioeng.**, 27:1427, 1985.

146. Lin, J.K., M.R. Ladisch, J.A. Patterson, and C.H. Noller, "Determining pore size distribution in wet cellulose by measuring solute exclusion using a differential refractometer," **Biotech. Bioeng.**, 29:976, 1987.

147. Grotte, G., "Passage of dextran molecules across the blood-lymph barrier," **Acta Chirugica Scandinavica, Suppl. 211**, 1956.

148. Stephanopoulos, G., **Chemical Process Control**, 1st Ed., Prentice Hall, Englewood Cliffs, NJ, p. 188, 1984.

149. Esterbauer, H., and W. Steiner, Standard Procedure for the Round Robin Test on Enzymatic Hydrolysis, Private Communication.