Substrate Factors Limiting the Enzymatic Hydrolysis of Softwood Substrates

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

Department of Wood Science

We accept this thesis as conforming
to the desired standard

THE UNIVERSITY OF BRITISH COLUMBIA

November 1998

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Date Nov 30ᵗʰ 1998

DE-6 (2/88)

Abstract

Past work has shown that softwood substrates are inherently more difficult to pretreat and enzymatically hydrolyse than are hardwoods or agricultural residues. While the recalcitrance of lignocellulosic substrates to enzymatic hydrolysis after pretreatment has been the subject of considerable research, there is a dearth of information regarding the specific factors which influence softwood hydrolysis.

It has been shown that the lignin content and distribution as well as overall accessible surface area, are two of the major factors that influence the hydrolysis rates and yields of many lignocellulosic substrates. The first part of this study investigated the effect of these two factors on the accessibility of cellulases to the cellulose component of a Douglas-fir kraft pulp and three pulps derived from a Douglas-fir refiner mechanical pulp. The refiner mechanical pulp was pretreated to selectively remove the lignin and to also increase the swelling of the pulp without removing the lignin. This resulted in four pulps, a kraft pulp with little lignin, a refiner mechanical pulp with a high amount of lignin, a delignified refiner mechanical pulp, and a refiner mechanical pulp in which the lignin had been sulphonated. The accessibility of cellulose within the four pulps was investigated by measuring the cellulase adsorption capacity, the pore volume and the Simons stain accessibility of each substrate. It was found that, while the adsorption capacity was significantly increased with the more swollen substrate, there was still only approximately 50% hydrolysis of the substrate. It was apparent that lignin removal was necessary in order to facilitate complete hydrolysis. The pore volume of the substrates as well as the Simons stain accessibility was significantly higher with the pulps which contained little lignin reflecting the higher accessibility of cellulose within these substrates.

The second part of this study investigated the effect of fiber size on the adsorption capacity and hydrolysability of three Douglas-fir kraft pulps with different fiber size distributions. It was found that, by decreasing the average fiber size of the kraft pulp, the adsorption capacity and the hydrolysis yields could be greatly increased. The average fiber size was reduced by increasing the fines content from approximately 5% to 20%, which resulted in a significantly increased adsorption capacity with up 96% of added enzyme adsorbed. In contrast, a maximum of approximately 75% of the added protein was adsorbed to the untreated kraft pulp. It was also found that the enzyme loading required to achieve maximum hydrolysis of the kraft pulp could be decreased significantly by decreasing the average fiber size. By observing the changes in fiber size distribution and fiber coarseness, it was determined that the smaller fibers and fines were hydrolysed preferentially resulting in the traditionally observed rapid initial hydrolysis rates. In contrast, if the initial substrate consisted entirely of larger coarser fibers, the rate was greatly influenced by the time taken to disintegrate the fibers during the initial phase of hydrolysis.

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List of Abbreviations

β	beta
μg	microgram
μL	microliter
μm	micrometer
μM	micromolar
CBD	cellulose binding domain
CBH	cellobiohydrolase
CBU	cellobiase unit
CMC	carboxymethylcellulose
CTMP	chemithermomechanical pulp
DP	degree of polymerisation
EG	endoglucanase
FPU	filter paper units
HPLC	high performance liquid chromatography
Ll	length weighted average fiber length
Ln	number average fiber length
Lw	weight weighted average fiber length
m	meter
M	molar
mA	milliamp
mg	milligram
min	minute
mL	milliliter
mm	millimeter
mm	millimeter
mM	millimolar
MW	molecular weight

o.d.	oven dried
PAD	pulsed amperometric detection
PFI	papirindustriens forskningsinstitutt
psi	pounds per square inch
R14	R14 screen of Bauer Mc Nett fiber fractionator
R14-DF	R14 fraction of Douglas-fir kraft pulp
ref-DF	refined Douglas-fir kraft pulp
RMP	refiner mechanical pulp
SEC	size exclusion chromatography
SO_2	sulphur dioxide
TMP	thermomechanical pulp
UV	ultraviolet light
w/v	weight per volume

Acknowledgements

Chapter 1 Introduction

1.1 Background

The potential of enzymes to modify cellulose has been of interest for many years and has resulted in commercial applications in areas such as detergents, food additives, fiber and textile modification, and bioconversion. Research into the use of cellulases grew significantly in the 70's during the fuel crisis when it was realised that fossil fuel supplies were finite and that alternative fuel sources were going to be needed. The goal of much of this early research was to investigate the feasibility of converting biomass to glucose and ultimately to ethanol, so that the ethanol could be used as an alternative to transportation fuels such as gasoline and natural gas/propane. More recently, it is even more apparent that alternative fuels are required as much for environmental reasons as they are for economic reasons. One of the most significant outcomes of the UN Global Climate Conventions in Rio de Janeiro in 1992 and in Kyoto, Japan in 1997 was the acknowledgment that developed countries would have to shoulder the majority of the burden of reducing greenhouse gas to 1990 levels by the year 2000 in order to allow for industrial growth in undeveloped countries. This has been the impetus behind the recent renewed interest in renewable biomass biconversion, since the transportation sector accounts for almost 50% of oil consumption in developed countries (World Energy Council, 1993).

In Western Canada, the most abundant source of lignocellulosic material is softwoods and the various industries involved in its processing, such as lumber and pulp and paper, accumulate large volumes of wood waste with very few environmentally friendly options available for its disposal. Beehive burners have traditionally been used as the primary means of waste disposal in BC sawmills but this is rapidly changing. Since the early 1990's, half of the incinerators operating in BC have been shut down with the remaining incinerators supposedly closed at the end of 1997 (McCloy, 1997). It has been calculated that the amount of waste generated in BC

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annually could fill 3680 barges in a line 185 km long (Hamilton, 1997). Clearly alternative means of disposal are required with the probability that these wastes could provide a cheap feedstock for a potential bioconversion process for ethanol production.

The conversion of wood or agriculture residues to fermentable sugars and then to ethanol has been of interest to numerous researchers (Fan *et al*, 1983; Greithlein *et al*, 1985; Converse *et al*, 1990; Ramos *et al*, 1993). It was apparent early in this research that biomass conversion would not be an efficient means of ethanol production without a pretreatment process to make the substrates more amenable to enzymatic hydrolysis. Pretreatment methods have focused on increasing enzymatic hydrolysis of the cellulose component by making it more accessible to enzymes. One of the most extensively studied procedures has been the acid catalysed, steam pretreatment process (Saddler *et al*, 1982; Vallander *et al*, 1985; Saddler *et al*, 1984; Schwald *et al*, 1984; Puls *et al*, 1985; Eklund *et al*, 1990). These past studies have shown that acid catalysed steam pretreatment, using low levels of SO_2, is one of the most cost effective methods of fractionating the three major wood components while making the cellulose component more amenable to enzymatic hydrolysis. Several studies have indicated that the enhanced accessibility of the cellulose component to enzymes, caused by acid catalysed steam pretreatment, is a result of removal of the hemicellulose component and condensation of the lignin component (Greithlein *et al*, 1984; Schwald *et al*, 1987). This results in the disruption of the lignin-hemicellulose barrier and increases the surface area of cellulose accessible to cellulases. However, it is apparent that the extent to which acid catalysed steam pretreatment improves enzymatic hydrolysis is substrate dependant. The majority of the studies carried out to date to try to determine the efficacy of steam pretreatment methods have used hardwood and agricultural feedstocks. Those few studies which have used softwoods substrates have usually concluded that softwoods are not as easily hydrolysed as hardwoods or agricultural feedstocks (Greithlein, 1984;

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Clark *et al*, 1987; Wong *et al*, 1988; Ramos *et al*, 1992; Maekawa, 1996). The higher recalcitrance of softwood substrates was even more apparent when various post treatments were investigated in order to increase the susceptibility of steam pretreated substrates even further. These included alkali washing with sodium hydroxide to remove lignin followed by peroxide treatment to oxidise the remaining lignin (Ramos *et al*, 1992; Schwald *et al*, 1987). Alkali washing has met with mixed success since it resulted in improved hydrolysis of hardwood substrates but decreased the hydrolysis of softwoods (Ramos *et al*, 1992). These studies proposed various possible explanations for the poor hydrolysis of pretreated softwoods, including the higher extractive content of softwood substrates, recalcitrance of guaiacyl lignin, redeposition of lignin during pretreatment and the lower porosity of softwoods after pretreatment. Whatever the cause, it is apparent that better means of pretreatment are still necessary for softwood feedstocks and that this will require a better understanding of the factors responsible for their low hydrolysis yields.

The question which this thesis attempts to address is "what are the structural features which limit cellulose accessibility in softwoods?" In answering this it should be possible to develop a pretreatment process which will address these features such that softwoods substrates can be made more amenable to enzymatic hydrolysis. In order to better understand the factors responsible for softwood recalcitrance this study has focused on the main factors which differentiate softwood substrates from hardwood substrates, i.e. lignin composition and fiber size/surface area. Softwood fibers generally contains a higher amount of guaiacyl lignin than hardwood fibers and it has been suggested that this lignin component has a greater effect on fiber swelling than hardwood lignin (Ramos et al, 1992). In addition, softwood fibers are generally longer and have greater coarseness values than hardwood fibers thus giving them a lower specific surface area value. Softwood pulps were chosen as the substrates for this study rather than steam

pretreated softwood substrates as they are known to be somewhat more homogenous than steam exploded substrates and there is a substantial body of literature on pulp characteristics which can be sourced to try to explain the observed hydrolysis reactions. It was hoped that by using softwood substrates which have already been well characterised, i.e. pulps, it would be easier to determine the extent to which the structural characteristics of those substrates influence enzymatic hydrolysis.

1.2   The Nature of the Substrate-Lignocellulosic Structure

Wood derived lignocellulose is found in nature as a composite of cellulose microfibrils embedded in a matrix of lignin and hemicellulose. Although the extact nature of the associations between these three components has been a subject of much research and debate, it is known that the proportion of these three components differs in hardwoods and softwoods. Cellulose usually accounts for 40-45% of the wood with hemicellulose and lignin making up 20-30% and 15-25% respectively. In softwoods, the lignin content is higher, in the range of 25-30%.

1.21   Cellulose

Cellulose is a linear homopolymer of ß-1,4 linked D-glucose units. Cellobiose is the repeating unit in the cellulose chain since the successive glucose residues are rotated by 180 degrees relative to each other so that the glucosidic oxygens are pointing alternately in the opposite direction. The resulting straight chain is further stabilised by intramolecular hydrogen bonds and intermolecular hydrogen bonds to other chains. Thus, several chains can form stable sheets held together in a crystalline structure by van der Waals interactions. Cellulose can exist in many crystalline forms, with the main difference between these associations occurring in the relative position of the neighbouring chains and sheets. The structure known as cellulose I, in which the chains are parallel, is the crystalline form most commonly found in nature. Cellulose can also exist in a disordered or 'amorphous' form which is thought to be more accessible to chemical and

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enzymatic attack. The ratio of crystalline to amorphous cellulose is termed the crystallinity index or degree of crystallinity of the substrate. Therefore, cellulose, though homogenous in chemical structure, is not so uniform in physical structure.

Various model cellulose substrates have been used for the purpose of studying the mechanism of action and interaction of individual cellulase enzymes and the effect of substrate characteristics, such as degree of polymerisation and crystallinity, on the rate and efficiency of enzymatic hydrolysis (Nidetzky *et al*, 1994: Tomme *et al*, 1995; Puri, 1984, Peters *et al,* 1991; Lee *et al*, 1983). These model substrates include Avicel, Solka Floc, filter paper, cotton, Valonia cellulose, phosphoric acid swollen cellulose, soluble cellulose derivatives and bacterial cellulose.

1.22   Association with Hemicellulose and Lignin in Fiber Wall

Hemicelluloses primarily function as support material in the plant cell wall and they are usually found in wood as low molecular weight polysaccharides. They are usually composed of heteropolymers consisting of a number of different sugar residues including D-xylose, D-mannose, D-arabinose, D-rhamnose, D-glucose, 4-O-methyl-gluconic acid, D-galacturonic acid and D-glucuronic acid. (Fengel and Wegener, 1983). Though hemicellulose chemical and physical composition varies between species, they are generally composed of short chains with branches and side chains folded back towards the main chain by hydrogen bonding. This structure facilitates their close association with cellulose microfibrils. In softwoods the predominant hemicellulose is glucomannan while hardwoods have higher percentage of xylan based oligomers.

Lignin is said to be instrumental in defining fiber ridgidity and maintaining structural integrity since it forms a matrix with some of the polysaccharides and encrusts the cellulosic material of the plant cell wall. Studies with radioactive carbon (^{14}C) have shown that the p-hydroxy-cinnamyl alcohols, p-coumaryl alcohol (I), coniferyl alcohol (II) and sinapyl alcohol (III) are the

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primary precursors and building blocks of all lignins (Fengel and Wegener, 1983). While softwoods contain mainly guaiacyl lignin which is composed of coniferyl alcohol units, hardwoods contain both guaiacyl and syringyl lignin which is composed of sinapyl alcohol units.

The cell wall of wood fibers can be divided into three main layers, i.e. the primary wall, secondary wall and the warty layer while outside the primary wall is the highly lignified compound middle lamella (Fig. 1). These layers differ from one another in their chemical composition in the orientation of the cellulose microfibrils. The primary cell wall has an irregular microfibril orientation and this thin layer is highly lignified. The secondary cell wall is located inside the primary wall and is composed of three layers, S_1, S_2 and S_3 of which S_2 is the largest. The cellulose fibrils in the S_2 layer form a steep helix around the longitudinal axis, while the S_1 and S_3 from flatter helices. Finally, the warty layer is a thin amorphous layer located adjacent to the fiber lumen in all softwoods and some hardwoods.

As mentioned previously, the chemical association and location of the cellulose, hemicellulose and lignin relative to each other is not clear though the most accepted hypothesis holds that hemicellulose and cellulose are closely associated through hydrogen bonds while the hemicellulose and lignin are covalently linked, forming lignin carbohydrate complexes (Eriksson *et al*, 1980; Iversen, 1985; Karlsson *et al*, 1996; Lundquist, 1983; Newman *et al*, 1985; Yllner *et al*, 1957). The overall distribution of these components varies with species and growing conditions. However, the cellulose content is generally lowest nearer the outside of the fiber, increasing towards the S_2 layer while the cell wall lignin is found in its highest concentration in the secondary cell wall. However, next to the middle lamella, the highest concentration of lignin in wood cell wall is found in the middle lamella. It is generally accepted that within the cell walls, hemicellulose molecules are oriented between the cellulose sheets parallel to the cellulose fibrils while lignin is located randomly in the interfibrillar space (Kerr and Goring, 1975).
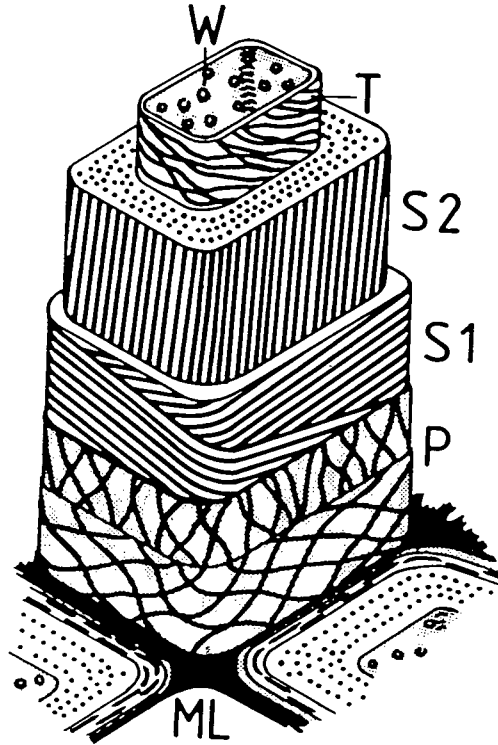
Fig. 1: Model of the cell wall structure wood fibers. Ml =middle lamella, P = primary cell wall,

S1 = secondary cell wall 1, S2 = secondary cell wall 2, T = tertiary cell wall, W = warty layer

(Fengel and Wegener 1983)

While the compositional and structural organisation of wood fibers is relatively well defined, they undergo significant and varied changes when subjected to processes such as pulping and steam explosion. These processes are designed to exploit the inherent characteristics of wood fibers such as their strength and length or their carbohydrate content, as such changing the structural and compositional organisation of the fibers in different ways. Pulping processes are designed to ultimately keep as much of the fibrous structure of the original wood as possible while separating individual fibers and optimizing their inherent characteristics such as strength, flexibility, collapsibility etc. In contrast, steam explosion processes are designed to optimise sugar recovery from the original fibers and retaining the fibrous nature of the wood is not a priority since the objective of the treatment process is to open up the substrate as much as possible. There are some similarities however, between some aspects of pulping processes and steam explosion processes. Thermomechanical pulping involves the separation of individual fibers after a presteaming step by mechanical means, resulting in a pulp with a relatively low average fiber size with little change in chemical composition. While the extent to which the hemicellulose composition of steam exploded wood changes depends on the severity of the treatment, there is generally little change to the lignin and cellulose composition. Various chemical post treatments have been tried in order to increase the enzymatic hydrolysability of the cellulose component of steam exploded wood such as alkali washing the solids left after explosion in order to remove lignin. The resulting delignified substrate is not unlike a chemical pulp such as a kraft pulp, since they too are a result of removal of lignin with alkali. While there is an abundance of literature describing the chemical and morphological changes brought about by different pulping processes, the steam explosion process is not so well defined, particularly with softwood feedstocks. Thus, softwood pulps are ideal for fundamental studies directed at determining which substrate related factors influence cellulose accessibility and hydrolysis.

1.23 Pulping Processes

1.231 Refiner Mechanical Pulping

Refiner mechanical pulping results in the separation of the individual fibers of the wood with little or no component removal. Thus, the chemical composition of the original substrate is largely unchanged with a high percentage composition of lignin and extractives, as such a mechanical pulp provides the ideal situation in which to examine the effect of lignin composition and fiber size. The fiber separation process involves the use of wood chips and the application of disc refiners of various types for defibration and fibrillation of the raw material. The most common mechanical pulping process involves the use of presteamed chips which are then fed into a disk refiner for defibration at approximately the same temperature and pressure as in the preheated stage. A secondary refining stage is generally carried out at atmospheric pressure. The defibered material is expanded into a cyclone where the steam is removed and the pulp is further refined in one or two stages to get the desired characteristics. The resulting pulp characteristics depend on three principal factors, steam temperature, refining consistency and refining energy. In addition to fiber separation, the grinding process produces much particulate matter from the outer layers of fibers and from completely disintegrated fibers; these are referred to as fines. Thus, a mechanical softwood pulp while containing relatively long individual fibers as well as fiber bundles which were not separated by the grinding process, contains relatively large amounts of small particulate matter which is usually higher in lignin content than the whole fibers and shives. This higher lignin content has been attributed to the fact that the majority of the fine material is derived from the outer layer of the fibers which contains the highest concentration of lignin in the cell wall.

1.232 Kraft Pulping

In contrast to the mechanical pulping process the kraft pulping process involves the selective removal of cell wall components in order to achieve fiber separation. It is an alkaline pulping technique in which sodium hydroxide is the principal cooking chemical with sodium sulphide added as an additional active pulping component. In principle the wood is sent to the digester in the form of chips together with fresh cooking liquor. The normal kraft cook is performed at temperatures between 160 and 180 °C and at pressures between 7 an 11 bar. After cooking the pulp and the spent liquor are discharged at the bottom of the digester at reduced pressure into a blow tank with a capacity of several cooking volumes. The pulp is screened and insufficiently separated fibers and knots are transported back to the digester for repeat cooking. The kraft pulping process results in the delignification of the wood and the dissolution of some of the polysaccharides, the extent of which depends on the alkali concentration. The chemical and physical properties of alkaline pulps depend on the wood species and the pulping conditions. Besides morphological fiber properties, the polysaccharide reactions in alkaline medium and the degree of delignification determine the character of a pulp. Alkaline pulps usually have a dark colour resulting from the redeposition of lignin on the surface of the fibers during the last stages of the cook. This lignin contains chromophoric groups which are responsible for the dark colour. The residual lignin content of hardwood pulps are usually about 2% whereas softwood pulps can contain up to 4% (Fengel and Wegener, 1983). Softwood kraft pulps typically have a predominance of long fibers, the coarseness of which depends on the furnish, eg. Douglas-fir yields a pulp with a high fiber coarseness (weight per length) while a Spruce-Pine-Fir mix yields a lower coarseness pulp. Due to the chemical nature of the fiber separation process, the fiber size distribution of the original furnish is largely unchanged, thus providing a substrate which is ideal for studying the influence of fiber size in enzymatic hydrolysis.

1.3 The Cellulases

Cellulase enzymes are produced almost exclusively by microbes. Although there is some production by plants and protozoa, they have not been studied as extensively as bacterial and fungal cellulases. Cellulases generally consist of at least two separate domains, a catalytic domain and a cellulose binding domain (CBD), which are joined by flexible glycosylated linker region. Based on hydrophobic cluster analysis of the catalytic domains, cellulases have been grouped into 12 families (Tomme *et al*, 1995). Members of the same family should have similar folds and active site topologies and therefore the same reaction mechanisms, retaining or inverting. For those enzymes for which the three dimensional structure is known, the predicted similarities have been confirmed. Most cellulolytic organisms produce multiple cellulases which differ in their ability to hydrolyse different cellulosic substrates. This has resulted in a classification system in which the individual cellulases are characterised according to their ability to hydrolyse specific substrates such as carboxymethyl cellulose and filter paper. The majority of research on cellulases has concentrated on fungal cellulases since they generally are better cellulose degraders than cellulases derived from bacteria. Cellulolytic fungi produce higher amounts of extracellular protein, the cellulases of which are better degraders of crystalline cellulose than bacterial cellulases. Thus, for the purposes of bioconversion the most extensively studied cellulases have been fungal cellulases.

1.31 Fungal Cellulases

The cellulose degrading fungi can be divided into two groups, the first include white-rot fungi such as *Phanerochaete chrysosporium* and *Schizophyllum commune* and soft-rot fungi such as *Trichoderma reesei* and *Penicillium pinophilum* and they produce what are called complete cellulase systems. These complete systems are characterised by their ability to efficiently hydrolyse crystalline cellulose (Wood, 1992; Coughlan, 1990) and are comprised of

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endoglucanases (EC 3.2.1.4), exocellobiohydrolases (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21). Endoglucanases are characterised by their ability to randomly cleave the cellulose chains along their length while exocellobiohydrolases remove cellobiose units from the ends of the cellulose chains. Finally, β-glucosidases catalyse the hydrolysis of cellobiose to glucose. These enzymes have been shown to exhibit a high degree of synergism or cooperativity in the hydrolysis of recalcitrant cellulose, since the random cleavage by endoglucanases creates new chain ends for the action of cellobiohydrolases.

The second group, with some exceptions produce endoglucanases in the absence of cellobiohydrolases and includes brown-rot fungi such as *Postia placenta* (Kleyman-Leyer *et al*, 1992). The mechanism of cellulose degradation of brown-rot fungi differs from that of white rot and soft-rot fungi since it has been postulated that the brown-rot fungi degrade cellulose using an oxidative mechanism in combination with endoglucanases. Many oxidative components are known to be involved in this mechanism including chemical oxidants such as hydrogen peroxide and oxalic acid (Koenigs *et al*, 1974; Green *et al*, 1991) and oxidative enzymes such as cellobiose dehydrogenase (Mansfield *et al*, 1997). One of the most extensively studied cellulase systems due to its high cellulolytic efficiency is that of *Trichoderma reesei*, a mesophillic, soft-rot fungus.

The mesophillic fungus *Trichoderma reesei* produces a complete cellulase system, including four major cellulases, cellobiohydrolase I and II (CBH I, CBH II), and endoglucanase I and II (EG I, EG II). The 3-dimensional structures of both cellobiohydrolases and EG I have been determined (Divne *et al*, 1994; Rouvinen *et al*, 1990; Stahlberg *et al*, 1996). The cellobiohydrolases have been shown to have a tunnel shaped active site which can accommodate many glucose molecules, while EG I has an open groove in which the loops which covered the active site in CBHs are not present. Thus, the 3-dimensional structure of the enzymes matches their proposed

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mechanism of action. Cellobiohydrolases bind the free end of a cellulose chain allowing the glucose molecules to enter the tunnel while endoglucananses are able to adsorb onto the cellulose chain randomly along its length due to the open nature of their active sites. Thus, while these two individual enzyme types are able to affect hydrolysis on cellulosic substrates, the extent of this hydrolysis would depend on the availibility of celllulose chains ends for cellobiohydrolases and the accessibility of the individual cellulose chains for endoglucanases. For lignocellulosic substrates, the situation is even more complex with the cellulose microfibrils enmeshed in a lignin/hemicellulose matrix thus limiting accessibility of cellulose chains and chain ends. Together endoglucanases and cellobiohydrolases can exhibit synergism since one endoglucanolytic action creates two more sites for cellobiohydrolases thus increasing the hydrolysis yield. These issues will be discussed in more detail in the following sections.

1.4 Enzymatic Hydrolysis of Lignocellulosic Substrates

Despite extensive research, our current understanding of the mechanism of cellulose hydrolysis is limited. There has been much speculation and debate regarding the mechanisms involved and despite much research, many substrates particularly softwood derived substrates are not easily or completely hydrolysed even after pretreatment. An important aspect of research to date has therefore been to ascertain the limiting factors involved in the decrease in hydrolysis rate as time progresses. These factors have traditionally been divided into two groups, substrate related factors and enzyme related factors.

1.41 Substrate Factors related to Hydrolysis

In the natural environment, cellulase production by microorganisms are accompanied by a wide range of other enzyme activities produced by the microorganism such as hemicellulases, ligninases and pectinases, which all aid in the hydrolysis of cellulose by degrading the hemicellulose and lignin associated with it. However, for the purposes of an enzymatic

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bioconversion process it is much more efficient to pretreat the substrate to make it more amenable to cellulases. In order to optimize a pretreatment process for lignocellulosic substrates, the factors related to substrate structure which limit cellulose accessibility must be clearly understood. As mentioned previously, the bulk of research to date on the mechanism of hydrolysis of cellulose has been carried out with relatively pure cellulose substrates such as Avicel, Solka floc and or cotton so that we have a relatively clear understanding of how individual cellulases hydrolyse crystalline cellulose. However, much less is known about how cellulases, mixed or individual, hydrolyse heterogenous lignocellulosic substrates such as pulps or steam exploded substrates. While the arrangement of cellulose, hemicellulose and lignin within fiber walls is still not fully understood, the most accepted hypothesis holds that the cellulose chains are associated into elementary fibrils of approxiamtely 3nm diameter and that these fibrils are further associated with lignin and hemicellulose into microfibrils (Fengel and Wegener, 1983). In order for cellulases to hydrolyse these substrates, they have to first be able to access the elementary fibrils within these microfibrils. It can be seen that cellobiohydrolase I from *Trichoderma reesei* is sufficiently small to access the cellulose within microfibrils (Fig.3), however when these microfibrils are associated into macrofibrils and further into the fiber walls, the extent to which CBH I can access the cellulose chains is reduced dramatically. It is apparent that the enzymes would only have immediate access to the surface layers of the microfibrils, and then only to those not encased or surrounded by lignin. Thus, it seems clear that a pretreatment process would have to address cellulose accessibility by opening up the fiber wall and exposing more surface to the enzymes. The acid catalysed steam explosion process addresses these factors by reducing the fiber size and removing hemicellulose and causing the lignin to condense thus increasing specific surface area (Tanahashi *et al*, 1990). It has been shown by many researchers that pretreating substrates in this manner results in greater hydrolysis. However, results vary

widely and softwood substrates in particular do not respond as well as some hardwoods and agricultural substrates to steam pretreatment. Softwoods have a different lignin chemistry than do hardwoods and they are generally composed of longer, coarser fibers, giving them a lower specific surface area for enzyme adsorption since they have less surface area per g of fiber. Thus, it is probable that these and other factors are responsible for the higher recalcitrance of softwood substrates rather than characteristics such as cellulose crystallinity and degree of polymerisation which are oftern studied when using 'model' substrates such as cotton, or bacterial derived cellulose. However, it is worth considering the factors that influence the ease of hydrolysis of crystalline cellulose and defining the characteristics which determine the accessibility of cellulose to the enzyme.

CBH I from Trichoderma reesei
2.09 x 18 nm

20 nm

40 nm

200 nm

Elementary Fibril
3 x 7 x 60 nm

*Cellulose chains consisting
of crystalline and
amorphous regions*

Microfibril
~25nm diameter

*Elementary fibrils associated with
hemicellulose and lignin*

Macrofibril
~500nm diameter

Associated microfibrils

S2 Layer of fiber wall
~1700-3700nm

*Helical arrangement of macrofibrils
Thickness varies within and between species*

Fig 2:   Relative size of wood cell wall components and cellobiohydrolase I from *Trichoderma reesei*

1.411 Cellulose Crystallinity

Cellulose crystallinity was one of the first characteristics of cellulose to be studied in relation to enzymatic hydrolysis. One of the earliest studies looked at the changing degree of crystallinity of pure cellulose substrates by measuring the moisture regain values (Walseth, 1952). This work indicated that these values decreased as hydrolysis proceeded and that this was due to the preferential removal of the amorphous cellulose component. This was the first evidence to suggest that the amorphous component of cellulose is hydrolysed first leaving a more recalcitrant crystalline component unhydrolysed. While several studies have found evidence to support this theory (Fan et al, 1980, 1981; Sasaki et al, 1979; Sinitsyn et al, 1991), others have refuted the existence of any correlation between the degree of crystallinity of a substrate and its susceptibility to hydrolysis, (Puri et al, 1984; Thompson et al, 1992; Ramos et al, 1993). It should be noted that those studies which found a positive correlation between crystallinity and hydrolysis rate used relatively pure cellulosic substrates which had been mechanically treated to change their crystallinity indices. In addition, it has been suggested that these treatments which cause a decrease in crystallinity also result in an increase in specific surface area which may have been the cause of any increase in hydrolysis rate (Converse, 1993). It is difficult to separate these two physical properties of cellulose, some studies have subsequently included both in empirical equations relating the structural features of cellulose to rate of hydrolysis (Gharpuray et al 1983; Fan et al, 1981). While these 'ideal' substrates have been invaluable in clarifying the mechanism of individual enzyme activities, the extent of enzyme synergism and the influence of enzyme activities on substrate characteristics such as crystallinity and DP, they do not provide much insight into the actual action of cellulase enzymes on natural heterogenous, lignocellulose substrates such as steam treated or pulped softwoods. However, studies with these more 'realistic' substrates have failed to demonstrate a positive correlation between crystallinity and

rates of hydrolysis (Tanahasi *et al*, 1983; Ramos *et al*, 1993). In fact, these and other studies have shown that steam pretreatment of lignocellulosic substrates results in an increase in the crystallinity index while at the same time increasing the hydrolysis rate. It is apparent that factors other than crystallinity are influencing the accessibility of cellulose within these substrates. However, it is known that the hydrolysis of crystalline cellulose seems to have more stringent prerequisites than amorphous cellulose as not all cellulase systems are capable of hydrolysing crystalline substrates such as Avicel. Those cellulase complexes which are capable of hydrolysing both forms of cellulose are characterized by the fact that they contain at least one tightly binding cellobiohydrolase (Klysov, 1990). Synergism seems to be particularly important for crystalline cellulose hydrolysis as it has been shown that the presence of a CBH is only of use in the hydrolysis of crystalline cellulose if it is capable of exhibiting synergism with the other cellulase components (Lee *et al*, 1988). Although amorphous cellulose can be hydrolysed by both endocellulases and exocellulases, crystalline cellulose is only minimally hydrolysed when endocellulases alone are added to this substrate. Thus, it is probable that crystallinity influences hydrolysis when synergism is lacking, due to an incomplete cellulase system or an insufficient enzyme loading, both of which are enzyme related factors.

1.412 Accessible Surface Area

There have been various methods used to measure the accessible surface area of cellulosic substrates including the BET (Bennet-Emmit-Teller) method which measures the surface area available to a nitrogen molecule (Fan *et al*, 1980). This technique involves drying the substrate which does not give a measurement which is comparable to the substrate in its swollen state. Another problem with this technique is the difference in size between the nitrogen molecule and a cellulase enzyme. As the nitrogen molecule is smaller, it has access to pores and cavities on the fibre surface that the cellulase enzyme cannot enter. Thus, not surprisingly there has been

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little evidence produced using this technique to support the theory that surface area plays a role in hydrolysis.

A more suitable technique for determining the accessibility of cellulases to lignocellulosic substrates is one which measures the area available in the form of pores and cavities in the fiber wall (Stone *et al*, 1969; Greithlein, 1985; Newman and Walker, 1992). This pore volume determination method developed by Stone and Scallan (1969) involves measuring the water stored in pores which is accessible to dextrans of various sizes. This technique measures the cell wall capillaries such as spaces between microfibrils rather than gross capillaries such as the cell lumen, pit apertures, and pit membranes. The pore volume technique is carried out on a non-dried substrate so it is more representative of the actual surface area accessible to the enzyme molecule in solution. However, in order to correlate the pore volume to accessible surface area the pore geometry has to be assumed. Stone and Scallan (1969), the original developers of this technique for lignocellulosic substrates, assumed that the pores consisted of spaces between lamellae or layers of lignocellulosic material. The pore volume technique has been used numerous times to correlate the surface area available in the form of pores to the different accessibilities of various substrates (Greithlein at al, 1985; Wong *et al*, 1988). The first such published correlation found that the relative digestibility of a series of cellulosic substrates was directly proportional to the accessibility of a molecule of 30-40 Å in diameter (Stone *et al*, 1969). It was suggested that this was evidence of the size of the enzyme, a very close estimation in retrospect since CBH I from *Trichoderma reesei* has been shown to be tadpole shaped with a total length of 180 Å while the catalytic core has dimensions of 50 x 60 x 40 Å (Esterbauer *et al*, 1991; Divne *et al*, 1995). Others have found similar correlations between pore volume and hydrolysability of lignocellulosic substrates including a study in which a linear relationship was shown between the initial hydrolysability of a substrate and its accessibility to a molecule of

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nominal diameter 51 Å (Greithlein *et al*, 1984). It has been suggested that removal of hemicellulose and redistribution of lignin during the pretreatment of *Pinus radiata* increases the surface area present in the form of pores thus increasing accessibility to enzymes (Wong *et al*, 1988). These studies also pointed out a significant difference between hardwood substrates and softwood substrates in terms of their pore volume and ease of hydrolysis. It was shown that the pore volume of white pine was only half the value obtained with a mixed hardwood substrate and that it was subsequently hydrolysed less efficiently (Greithlein *et al*, 1984). Thus, it was suggested that hemicellulose and lignin occupy smaller spaces in softwoods such that their removal and/or redistribution results in a smaller pore volume than hardwoods.

1.413 Particle Size/Specific Surface Area

Another parameter which has been shown to influence enzymatic hydrolysis is the average particle size of cellulosic substrates. Since adsorption is a prerequisite step in the hydrolytic process, it seems intuitive that the specific surface area would have an effect on hydrolysis rates since a higher surface area to weight ratio should mean more adsorption sites per mass of substrate. However, with cellulosic substrates such as cotton linters and microcrystalline cellulose there has been very little evidence presented to support this theory, with several studies finding no correlation between average particle size and hydrolysis rates (Sinitsyn *et al*, 1991; Shewale *et al*, 1979; Rivers *et al*, 1988). However, these studies measured *average* particle sizes and the overall differences in specific surface area may not have been of sufficient significance to highlight any influence on rates. An issue which has not been fully resolved by these studies is the extent to which the differences in average particle size influenced enzyme adsorption.

In contrast to the dearth of evidence found to support a correlation between particle size and hydrolysis rates of relatively pure cellulosic substrates, there is some evidence to support such a link with lignocellulosic substrates. It has been shown that the smaller sized fractions within

pulps are hydrolysed preferentially in the initial stages of the hydrolysis reaction, (Mansfield *et al* 1996; Jackson *et al*, 1993). Lignocellulosic substrates are heterogeneous in their particle size composition and it has been shown that within the fibre population of a lignocellulosic substrate, the fines have a significantly higher pore volume than the larger fibers (Laivins and Scallan, 1996). With steam pretreated substrates, it has been shown that increasing the severity of the pretreatment decreases the average particle size and increases the hydrolysis yields (Tanahashi *et al*, 1990; Sawada *et al*, 1987). However, most of this evidence shows only an indirect correlation since it has not been proven that the increased specific surface area causes the higher hydrolysis yields. There is as yet, no evidence to support the theory that increased specific surface area leads to increased adsorption since none of the authors previously mentioned have measured this parameter. It is possible that the higher recalcitrance of softwoods may be due in part to the fact that softwood fibers are generally longer and coarser fibers than hardwood fibers, making them less susceptible to the beneficial particle size reduction caused by steam pretreatment. It has been suggested that the lignin type and distribution within the various lignocellulosic substrates can play a key role in influencing the surface area of the component fibers.

1.414 Lignin Distribution

The removal of lignin and hemicellulose invariably causes extensive changes in the structure and accessibility of cellulose. It is known that their removal leaves the cellulose more accessible and more open to swelling on contact with cellulases (Stone *et al*, 1969; Greithlein *et al*, 1984). Since lignocellulosic materials serve a structural purpose, they are by nature resistant to microbial attack. Thus, the enzymatic hydrolysis of lignocellulosic materials tends to be low when the substrate is relatively intact. Lignin is thought to influence cellulose accessibility to cellulases several ways, for example, it is thought to act as a barrier to the enzymes preventing them from effectively binding to the cellulose (Ucar *et al*, 1988), and it has also been

demonstrated that lignin removal increases the porosity of both kraft and sulphite pulps and that the increase in median pore width corresponded with the average molecular weight of the lignin molecules removed (Tarkow et al, 1968; Stone et al, 1969; Ahlgren et al, 1972). It has also been shown that the pore volume increase observed with such lignin removal corresponds with an increase in a substrates susceptibility to hydrolysis (Wong et al, 1988; Greithlein et al, 1985). An issue which further complicates the picture is the differences observed in the response of hardwood substrates and softwood substrates to chemical treatments designed to redistribute and/or remove lignin. It has been demonstrated by several authors that softwood substrates are inherently more resistant to lignin redistribution or removal and also therefore to enzymatic hydrolysis (Greithlein et al, 1984; Stone et al, 1969; Ramos et al, 1992). In a recent study in which seven substrates of different origin were studied, it was shown conclusively that the softwoods were the most recalcitrant to lignin removal and enzymatic hydrolysis (Maekawa, 1996).

Lignin content is also thought to negatively influence the hydrolysis reaction by irreversibly adsorbing the cellulase enzymes thus preventing their action on cellulose. This has been observed numerous times in the literature especially in relation to steam exploded substrates (Cleresci et al, 1985; Converse et al, 1990; Lee et al, 1995). In a study in which various lignaceous residues were studied, it was apparent that the extent to which lignin adsorbs enzymes depends very much on the nature of the lignin itself (Sutcliffe et al, 1986). It has been shown that lignin adsorption of enzyme is decreased as the severity of pretreatment increases (Ooshima et al, 1990). Thus, it seems that while the predominant effect of lignin removal is the creation of extra surface area, it may also increase the levels of enzyme adsorption since the lignin itself may exert a rate limiting effect on cellulose hydrolysis by non-hydrophobically binding to the cellulase enzymes.

1.42 Enzyme Factors Influencing Cellulose Hydrolysis

Several factors associated with the nature of the cellulase enzyme system have been suggested to be influential in the hydrolysis process. These include end product inhibition of the cellulase complex, thermal inactivation and irreversible adsorption (Saddler at al, 1986; Ladish *et al*, 1983; Cleresci *et al*, 1985; Eklund *et al*, 1990). The problem of end product inhibition has been largely dealt with through the addition of ß-glucosidase which hydrolyses cellobiose to glucose thereby preventing inhibition of cellobiohydrolases by cellobiose (Breuil *et al*, 1990). While thermal inactivation and irreversible adsorption undoubtedly play some role in the hydrolysis reaction, the most significant enzyme related factors which influence hydrolysis are synergism and adsorption.

1.421 Synergism

Synergism which was first described over 30 years ago (Mandels *et al*, 1964), occurs when the combined action of two or more enzymes leads to a higher rate of action than the sum of all their individual actions. It is widely accepted that the cellulases of *Trichoderma reesei* co-operate such that EG I and II act randomly along the cellulose chains and thus produce sites for CBH I and II to act as exo-enzymes releasing cellobiose as the main product. A third enzyme, β-glucosidase, though not strictly a cellulase is necessary to hydrolyse cellobiose, thereby preventing end product inhibition of cellobiohydrolases. The individual components of *Trichoderma reesei* cellulase system have been largely classified according to their activities on substrates such as Avicel and carboxymethyl cellulose (CMC). While the CBH enzymes could hydrolyse the more crystalline Avicel well and CMC only moderately, the endoglucanases could only hydrolyse CMC. Although this classification holds true much of the time, the difference between CBHs and endoglucanases is not always so clearcut. For example the cellobiohydrolases of *Trichoderma reesei* have been shown to have limited endoglucanase

activity and endoglucanases have exhibited relatively high activities on Avicel under certain conditions (Stahlberg *et al*, 1993; Nidetzky *et al*, 1994). Issues like this have created doubts about this simplified picture of synergism. Beyond just *Trichoderma reesei*, not all combinations of cellobiohydrolases and endoglucanases exhibit synergism. What is it that differentiates one CBH from another? An observation which highlights the complexity of synergism is the synergism observed between cellobiohydrolases called exo-exo synergism (Fägerstom *et al*, 1980). There have been many explanations suggested for this phenomenon including one based on the fact that CBH I and II act preferentially from opposite ends of the cellulose chain. CBH I and II act from the reducing and non-reducing ends respectively, making it possible that they are creating new adsorption sites for each other. It has also been suggested that the synergism between CBH I and CBHII of *Trichoderma reesei* is due to the fact that CBH I binds more tightly than CBH II, this allows it to disperse crystalline cellulose through mechanochemical effects, thus creating new sites for CBH II (Klysov *et al*, 1990). More significant in terms of rates of hydrolysis of cellulosic substrates with mixed cellulase preparations is the observation that the degree of synergism observed with many mixtures of cellulases has been shown to be substrate dependant since the enzymes may exhibit synergism on some substrate and not on others. It was shown with the major *Trichoderma reesei* cellulases, that synergism was highest with the more crystalline substrates (Nidetzky *et al*, 1991). However, it has also been observed that synergism between CBH I and EG I was highest with semi crystalline substrates and non existent with a fully crystalline bacterial cellulose such as Valonia cellulose (Henrissat *et al*, 1985). A recent observation with *Clostridium stercocarium* cellulases raises more questions about the link between substrate quality and synergism. This study found that with a range Avicel substrates of different particle sizes synergism was highest with the largest particle size and lowest with the smallest particle size (Riedel *et al*, 1997). With so much evidence pointing towards a link

between cellulose structural characteristics and synergism with ideal substrates, it naturally raises the question as to what role synergism plays in the hydrolysis of more structurally heterogenous lignocellulosic substrates.

It has been suggested that the gradual loss of synergism was one of the key factors which limited hydrolysis efficiency of microcrystalline cellulose (Tan *et al*, 1986). It has also been observed during the hydrolysis of Avicel that there was a gradual decrease in the specific activity of the adsorbed enzyme (Ooshima *et al*, 1991). Another enzyme related factor which must be considered for the significant role it plays in the hydrolysis reaction is the adsorption of cellulases to the substrate.

1.422 Adsorption

The hydrolysis of cellulose is distinct from most enzymatic reactions in that the substrate is insoluble and requires the adsorption of the enzyme prior to hydrolysis. Since the efficient hydrolysis of lignocellulosic substrates requires such interaction between the substrate and enzyme, the adsorption reaction has been extensively studied. Cellulase adsorption is facilitated by a cellulose binding domain (CBD) in addition to a catalytic domain. This modular structure is shared by other polysaccharide degrading enzymes (Wilson *et al*, 1995) and more than one hundred and twenty CBDs have been classified into ten families based on their amino acid sequences (Tomme *et al*, 1995). The catalytic domain and the cellulose binding domain are joined by a linker region which is susceptible to proteolytic cleavage thus facilitating their separation and characterization. The role of CBDs in hydrolysis has not been precisely ascribed due to our current limited understanding of the binding reaction. Van der Waals contacts and hydrogen bonds are dominant forces in carbohydrate binding proteins (Tomme *et al*, 1995). The CBDs generally have a low content of charged amino acids and a high content of hydroxy amino acids. Aromatic amino acid residues, tryptophan and tyrosine, are thought to pack onto the sugar

rings conferring additional specificity and stability to the enzyme substrate complexes (Macarrón *et al*, 1994). The importance of aromatic amino acid residues for adsorption has been demonstrated by site directed mutagenesis and it was shown that the CBD of cellobiohydrolase I interacts with cellulose through three conserved tyrosine residues on the more hydrophillic side of a wedge shaped molecule (Reinikainen *et al*, 1992).

Two explanations have been put forward to explain the interaction of CBDs with cellulose. The most obvious explanation is that the CBD serves to increase the local concentration of enzyme at the cellulose surface. The other theory proposes that the CBD is instrumental in liberating cellulose chains from the surface of crystalline cellulose through a non hydrolytic mechanism (Teeri *et al*, 1992b; Din *et al*, 1991). It has been shown that removal of the CBD reduces the hydrolytic efficiency of the enzymes on crystalline cellulose but not on amorphous cellulose (Tomme *et al*, 1988). It has also been shown that addition of a CBD to an enzyme which does not already have one increases its activity as well as it synergistic interaction with other enzymes (Maglione *et al*, 1992).

It should be noted that most studies have focused on the analysis of binding of purified enzymes to relatively pure 'model' insoluble celluloses. While these studies have cast much light on the mechanism of binding and substrate specificities of the individual cellulases, they do not tell us much about how the enzymes behave in mixtures or how their adsorption profile changes with more lignified substrates. The adsorption of cellulases onto cellulose is a complex reaction which is affected by the physiochemical properties of the substrate in the same way that crystallinity, lignin content and surface area all affect hydrolysis (Lee *et al*, 1982; Converse *et al*, 1993; Ooshima *et al*, 1990). Binding of cellulolytic enzymes is complex due to substrate heterogeneity with areas of differing crystallinity and chemical composition. Even within a perfect crystal, the crystal has inequivalent faces and corners resulting in differing adsorption

specificities of individual cellulase components (Chanzy, 1985). It has been shown with both an exocellulase and an endocellulase from *Irpex lacteus* that adsorption is more thermodynamically favourable with more crystalline substrates suggesting that these cellulases preferentially adsorb to crystalline cellulose (Hoshino *et al*, 1992). It has also been shown that lignin can have a significant effect on cellulase adsorption in that it has been shown to irreversibly adsorb cellulases (Ooshima *et al*, 1990; Sutcliffe *et al*, 1986). The tightness of adsorption has been suggested to be one of the most important factors determining a cellulases effectiveness particularly with respect to synergism (Klysov, 1990). It has been shown that removal of the CBD decreases individual enzyme activity on crystalline substrates but not on amorphous ones. It would seem that adsorption is not as important for amorphous substrates which are more easily hydrolysed while crystalline substrates require the concerted action of more than one enzyme. The adsorption of cellulases is influenced by many factors related to quality including crystallinity, lignin content and surface area. It follows therefore that these factors can influence hydrolysis rates of cellulosic substrates.

In summary, it is apparent that in order to develop a feasible pretreatment process for softwood bioconversion, we need to develop a better understanding of the factors that influence the accessibility and hydrolysis of these substrates. Building on the knowledge gained to date in this field, it is possible to narrow the field of possible influential factors down to gross fiber characteristics such as lignin content and distribution, pore volume, surface accessibility and particle size. Determining how and to what extent, factors such as these influence the ease of hydrolysis of softwood substrates would be of immense benefit in tailoring a pretreatment process to increase accessibility of cellulose.

1.5   Research Approach and Objectives

This project was initiated in order to enhance our understanding of enzyme substrate interaction with regard to lignocellulose hydrolysis. In particular, we wanted to determine the extent to which substrates characteristics such as lignin distribution, surface area and fiber size influence cellulose accessibility. While many research groups have directed their interest at elucidating the mechanism of cellulose hydrolysis, this work has been carried out predominantly with model cellulose substrates. Although past research has yielded an abundance of information with regard to individual enzyme activities, it has predominantly focused on their effect on cellulose crystallinity and any possible reduction in the degree of polymerisation of the cellulose as well as their synergistic interaction. Relatively little information has been revealed about their interaction with heterogenous lignocellulosic substrates. It has been amply demonstrated that hydrolysis of these substrates is not a simple process, with the typically biphasic hydrolysis curve observed proving difficult to adequately explain. Although there have been many suggestions proposed to explain the often observed decrease in hydrolysis rates over time, most of these studies have implied that substrate characteristics such as crystallinity and degree of polymerisation are the most influential. However, there has been little evidence to support such a correlation when using more 'realistic' lignocellulosic substrates.

In an attempt to determine the factors that influence cellulose accessibility, several pulps were chosen as model lignocellulosic substrates. Softwoods have been shown to be more resistant to enzymatic hydrolysis than hardwood substrates. In this work we chose Douglas-fir as a representative softwood substrate. It was hoped that by studying these substrates and their susceptibility to hydrolysis we could determine which factors predominantly influence cellulose accessibility.

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The initial work, which was carried out with Douglas-fir kraft and refiner mechanical pulps (RMP) demonstrated that the cellulose accessibility of the RMP substrate was relatively low in comparison with the kraft pulp. Subsequent work involved various pretreatments of the RMP substrate, in order to modify its lignin chemistry and distribution and to consequently determine if this had any effect on hydrolysis. By studying the effect of lignin sulphonation on fiber swelling and cellulose accessibility, it was hoped that we could get a clearer picture of how the lignin component influenced cellulose swelling and accessibility as well as hydrolysis. We also carried out a mild delignification of the RMP substrate in order to determine how the actual presence of lignin influences the accessible surface area and hydrolysis of lignocellulosic substrates. For example, what role does lignin play when it is present in low concentrations? We investigated this by looking at lignin release from the kraft pulp and the delignified RMP during hydrolysis.

The initial results provided evidence to support the theory that the gross fiber characteristics such as lignin distribution, pore volume and specific surface area predominantly influence the hydrolysis of lignocellulosic substrates rather than cellulose characteristics such as crystallinity. Most of the past studies in this field have had to make the assumption that there is a homogenous distribution of these various characteristics throughout the substrate with only a nominal acknowledgment of the fibrous nature and diversity of the component parts of the substrate. Since lignocellulosic substrates are heterogeneous in their physical and chemical composition, we next looked at the influence of fiber size on enzymatic hydrolysis rates. Softwood substrates in particular can have a very wide distribution of fiber sizes. Do these fibers adsorb equivalent amounts of enzyme per gram of cellulose and are they all hydrolysed at the same rate? We investigated this by preparing substrates derived from the Kraft pulp with different fiber size distributions and compared the adsorption capacities of these substrates as well as their

susceptibility to hydrolysis at varying enzyme loadings. We studied the mechanism of hydrolysis of these substrates by looking at changes in fiber length and coarseness in the initial stages of the hydrolysis reaction. In this way it was hoped to get a better picture of how fiber morphology could influence the mechanism and the rate of hydrolysis of these substrates.

The overall objective of my work was to look at the hydrolysis of softwood substrates with regard to determining the factors that influence hydrolysis rates and yields. The original hypothesis was that the most influential substrate characteristics were; (1) the lignin content, both in terms of its chemistry and its distribution and, (2) the accessible surface area as it relates to cellulose accessibility and specific surface area, i.e. particle size.

CHAPTER 2 Evaluation of the Effect of Cellulose Surface Area and Lignin Distribution on the Enzymatic Hydrolysis of Softwood Substrates

2.1 Background

As mentioned in the introduction, the greater recalcitrance of lignocellulosic substrates has been attributed to many factors such as lignin content and distribution and the pore volume of the substrate (Cowling *et al*, 1975; Greithlein *et al*, 1985; Ramos *et al*, 1992). For example, it was suggested that lignin present in the substrates studied reduced the swelling capacity of the fiber and the amount of cellulose surface area available to the enzyme (Cowling *et al.*, 1976). Several groups have shown a good correlation between the determined pore volume and the enzymatic digestibility of lignocellulosic substrates (Greithlein *et al*, 1985; Stone *et al*, 1969). The extent to which increasing the surface area of a substrate increases its digestibility appears to be influenced by its lignin content and distribution. This was demonstrated in a study in which it was observed that alkali washing of a steam exploded substrate resulted in decreased hydrolysis rates despite an increase in pore volume and the decreased lignin content of the substrate (Wong *et al*, 1988).

Since one of the major roles of lignin is to maintain fiber integrity and structural rigidity, it is not unlikely that its close association with the cellulose microfibrils prevents swelling of fibers, thus limiting enzyme accessibility. It is also probable that the distribution and composition of lignin is as important as the concentration of lignin, in terms of enzyme accessibility and digestibility. For example, these factors have been implicated as being responsible for the higher recalcitrance of softwood derived substrates (Ramos *et al*, 1992) as softwoods have predominantly guaiacyl lignin while hardwoods have a mix of guaiacyl and syringyl lignin. It has also been suggested that guaiacyl lignin restricts fiber swelling and thus enzyme accessibility more so than syringyl lignin (Ramos *et al*, 1992)

In an attempt to correlate substrate accessibility to the efficiency of enzymatic hydrolysis, various groups have measured the initial enzyme adsorption capacity of different substrates and correlated this with the initial rates of hydrolysis (Lee *et al*, 1993; Converse *et al*, 1990). It was found that those substrates containing little or no lignin showed a good correlation between initial hydrolysis rates and adsorption capacity (Lee *et al*, 1993), while substrates with higher lignin contents demonstrated a poor relationship.

In the initial work carried out within my thesis, I first wanted to assess the effect that the lignin content and composition had on substrate characteristics such as pore volume, cellulose accessibility. The enzyme adsorption capacity and hydrolysis of softwood substrates were also investigated. Douglas-fir was chosen as a representative softwood species due to its long, coarse fibers and high lignin content. A comparison of kraft and mechanical Douglas-fir pulps was initially carried out, as the expected differences in fiber size and lignin content of these two substrates was expected to influence both enzyme adsorption and substrate hydrolysis. Thus, it was hoped to ascertain the extent to which lignin affects hydrolysis by comparing the rates of hydrolysis of the untreated RMP with a delignified RMP and a chemically modified RMP.

2.2   *Materials And Methods*

Substrates: Two Douglas-fir pulps were used in this study. The kraft pulp was obtained from Fletcher Challenge, Crofton, BC and a refiner mechanical pulp (RMP) was prepared by MacMillan Blodel from presteamed Douglas-fir chips using a Sprout-Waldron refiner under atmospheric pressure. The kraft pulp contained 76.9% cellulose and 3.4% lignin while the RMP contained 43.6% cellulose and 27.9% lignin. The pulps were analysed in triplicate for lignin and carbohydrate content. Lignin was determined by Klason lignin analysis (acid-insoluble lignin) according to TAPPI standard method T222 os-74. The carbohydrate content of the acid hydrolysate was determined by measuring the monomer sugars by HPLC using a Dionex DX500

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system. Substrates were stored at 4°C without drying and the moisture contents of the substrates were determined in order to calculate the substrate loadings used in experimiments on the basis of dry weights.

Enzymes: A complete cellulase preparation, (Celluclast) (74 IU/ml, 45 mg protein/ml, batch 3035) and a β-glucosidase preparation, (Novozym) (492 CBU/ml, 43 mg protein/ml were obtained from Novo Nordisk A/S, Denmark. They were loaded at a 1:1 ratio of filter paper units to β-glucosidase units in all hydrolysis experiments in order to alleviate any end product inhibition resulting from cellobiose accumulation.

Adsorption Measurements: The Celluclast/Novozym mixture was added to 2% (d.w./v) of each substrate in 50 mM sodium acetate buffer (pH 4.8) at various increasing cellulase loadings from 10 to 240 IU/g of cellulose. All of the reactions were run in triplicate and were placed at 4°C for 90 minutes in order to reach adsorption equilibrium. The adsorption reaction was stopped promptly by centrifugation at 10,000 rpm for 5 minutes, followed by separation of the substrate from the supernatant. The protein in the filtrate was measured using the Bradford protein assay (BioRad, USA). Adsorbed enzyme was determined as the difference between the amount of protein initially added and the amount of unadsorbed protein in the supernatant.

Long-term Hydrolysis: Long term hydrolysis was carried out at 2% substrate concentration in 50 mM sodium acetate buffer (pH 4.8) with agitation at 45°C. The enzyme loadings were as described above, with a 1:1 ratio of CBU:FPU, and samples were run in triplicate. The release of glucose was determined by HPLC using a Dionex DX500 system (Sunnyvale, C.A., USA). The samples were filtered through 0.45μm HV filters (Millipore, Bedford, MA, USA) and a volume of 20 μL was loaded using a Spectra system AS3500 autoinjector (Spectra-Physics, CA, USA).

Elution of the samples was monitored using a Dionex ED40 electrochemical detector with a gold electrode and parameters set for detection of sugars as recommended by the manufacturer.

Fiber Fractionation: Fiber fractionation was carried out using a Bauer McNett Fiber classifier using five consecutive screens of 14, 28, 50, 100 and 200 mesh. For each pulp, 5 samples of 10g dry weight were run through the Bauer McNett in order to get the average fiber size distribution. For the mechanical pulp, one major population was collected between 50 and 200 while another was collected between 14 and 28. The sum of both of these fractions accounted for approximately 65% of the pulp with the remaining 35% not retained by any screen. This remaining 35 % is referred to as fines in this work. The kraft pulp was almost completely retained by the 5 screens with 55% retained by the 14 screen, 22% retained by the 28 screen and approximately 6% fines.

Sulphonation: Sulphonation of the mechanical fibers was carried out according to Beatson et al., (Beatson et al., 1985). Duplicate samples of 10g dry weight of the fibers was suspended in 220 ml of 1M sodium sulphite (pH 7). The fibers were allowed to soak overnight in the sulphite solution in two steel bombs. The bombs were then immersed in water at 140 °C and heated for 1 hour, with 20 minutes allowed for the fibers to come to temperature. Following sulphonation, the fibers were cooled quickly and washed extensively until the filtrate was sulphite free as determined by titration with iodine in potassium iodide. The sulphonated fibers were then analyzed for klason lignin and sugar content as described previously. Sulphonation is thought to occur by nucleophillic attack on quinon methide intermediates formed from phenolic units in lignin. The addition of these sulphonic acid groups increases the hydrophillicity of the lignin thus increasing water uptake and fiber swelling. Changes in fiber swelling were quantified by

determining the fiber saturation point of the substrates using the pore volume technique (Stone *et al.*, 1969), with 56 nm and 5.1 nm dextran probes.

Sodium Chlorite Treatment: The mechanical pulp was subjected to mild delignification in 4.5% sodium chlorite as follows. The sodium chlorite was prepared by adding 90g of sodium chlorite to 2 litres of deionised water and bringing the pH to 4 by addition of acetic acid. Duplicate samples of RMP fibers were suspended in 4.5% sodium chlorite at a 20:1 ratio (v/w) and 2 drops of formic acid were added. The suspension was then kept in a dark room for 10 days and 2 drops of formic acid were added twice a day. After 10 days, the fibers were filtered and washed thoroughly and analyzed for klason lignin and sugar content. Changes in fiber swelling were determined by measuring the pore volume accessible to 56 nm and 5.1 nm dextran probes.

Pore Volume Measurements: The pore volume of control and treated pulps were determined using dextran probes with molecular diameters of 56 nm and 5.1 nm, using a modification of the solute exclusion technique developed by Stone and Scallan (Stone *et al.*, 1969). The dextran probe solution (0.5% w/v) was added to the pulp samples, mixed thoroughly and allowed to equilibrate for 5 hours with frequent, gentle mixing. After equilibration, the pulp samples were allowed to settle and the probe solution withdrawn and filtered through a sintered glass funnel and refiltered through 0.45 um millipore filters. The concentration of the probe solution was determined refractometrically using a Waters 625 liquid chromatography system equipped with a Waters 410 Differential Refractometer (Millipore Corp., Milford, Mass, USA). The volume of inaccessible water was determined as described previously (Mansfield *et al.*, 1996). Since the pore volume technique is very sensitive, at least 5 samples of each pulp were analysed for each pulp and samples of the filtrates from each replicate were analysed in triplicate.

<u>Simons Stain:</u> Staining was carried out using the procedure of Yu *et al*, 1995. The dyes, Pontamine Fast Sky Blue 6BX and Pontamine Fast Orange 6RN were obtained from Pylam Products Co. Inc., Garden City, New York. The orange dye which consists of high and low molecular weight molecules was fractionated in order to isolate the high molecular weight fraction which has a higher affinity for cellulose than the low molecular weight fraction. The fractionation of the orange dye was achieved by passing the dye solution through an Amicon filter which retained molecules above a molecular weight of 50,000 kD.

Triplicate samples of each of the substrate fibers were stained by soaking them at 75°C for 48 hours in a mixture of 1% direct blue dye and 0.2% solution of the high molecular weight fraction of direct orange dye in 2% NaCl. The fibers were then washed with ice water and blotted gently with filter paper to remove unadsorbed dye. They were then stripped with 25% aqueous pyridine at 45 °C. The stripped fibers were filtered and the filtrate diluted to 100 ml with 25% aqueous pyridine solution prior to spectrophotometric analysis of the filtrates.

The absorption was determined at 621.5nm for the blue dye and 450nm for the orange dye. The absorption coefficients were 1.17 $lg^{-1} cm^{-1}$ for the orange dye and 0.133 $lg^{-1} cm^{-1}$ for the blue dye.

<u>Measurement of Lignin Release using Size Exclusion Chromatography</u>: The molecular mass distribution of lignin molecules in the hydrolysate of Douglas-fir kraft pulp and delignified refiner mechanical pulp was determined using Toyopearl HW 55S (TosoHaas, Montgomeryville, PA, USA) packed in a 0.5x20 cm column (Pharmacia, Uppsala, Sweden). Duplicates of each of the substrates was subjected to enzymatic hydrolysis with 60 FPU/g of cellulose and B-glucosidase added as described previously. Control reactions which omitted the addition of cellulases were also prepared. Duplicate samples were taken at intervals from the hydrolysis flasks and the control flasks. The samples were filtered through 0.45um HV filters (Millipore,

Bedford, MA, USA) and a volume of 20 uL was loaded using a Spectra System AS3500 autoinjector (Spectra-Physics, Fremont, CA, USA). The eluent 1M NaOH was run at a flow rate of 0.2ml min^{-1} using a Dionex DX500 HPLC system (Sunnyvale, CA, USA). Elution of the samples was monitored using a Dionex AD20 absorbance detector set at 280nm.

2.3   Results

2.31   Hydrolysis Yields of Untreated Substrates

To try to determine the effect of enzyme loading on hydrolysis of substrates with different lignin contents, we initially assessed the digestibility of a Douglas-fir refiner mechanical pulp (RMP) and a kraft pulp with increasing cellulase loadings (Fig. 3). As expected, the RMP was much more recalcitrant, with a 3 fold increase in enzyme loading (40 to 120 FPU/g) only increasing the degree of hydrolysis from 10 to 20% after 4 days incubation. Substantially higher hydrolysis rates and yields were achieved when the kraft pulp was used as the substrate, with no further benefits obtained by increasing the cellulase loading higher than 60 FPU/g. Although the RMP contained a substantially higher proportion of lignin than did the kraft pulp (Table 1), as the enzyme was added on the basis of cellulose content rather than the amount of whole substrate, it was probable that the inaccessibility of the cellulose to the enzyme was limiting hydrolysis rather than any deficiencies with the enzyme itself.

2.32   Substrate Pretreatments

In an attempt to elucidate the role that lignin plays in influencing the enzymatic hydrolysis of lignocellulosic substrates, the RMP was either delignified by a mild sodium chlorite treatment, which reduced the lignin content from 27% to 8%, or swollen without removing the lignin by a sulphonation step. Although there appeared to be a slight increase in the amount of lignin in the sulphonated pulp (Table 1), this was a result of the addition of sulphur groups to the free phenolic groups on the lignin. As expected, there was a proportional increase in the cellulose content of the delignified pulp due to the removal of approximately 70% of the lignin present in the original RMP (Table 1).
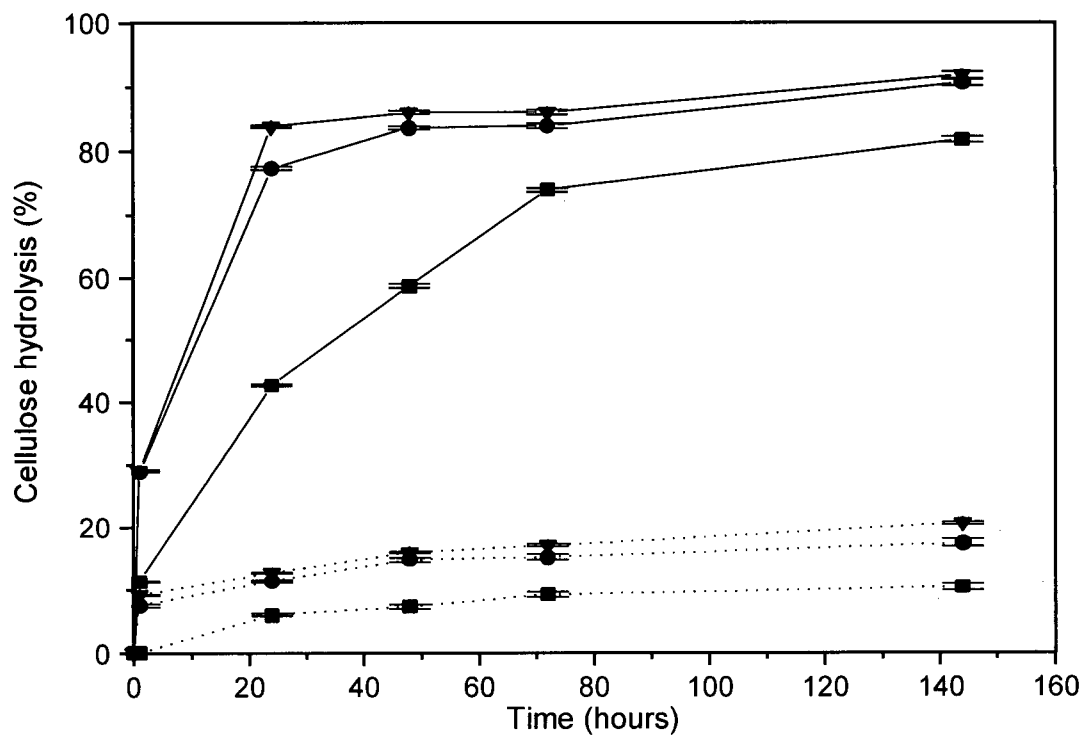
Fig. 3: The effect of increasing cellulase loading on the hydrolysis of a Douglas-fir refiner mechanical pulp(- - -) and a kraft pulp (——) at 40···■···, 60···●···, 120···▼··· FPU/g of cellulose

Table 1: Compositional analysis of a Douglas-fir refiner mechanical pulp (RMP), delignified RMP, sulphonated RMP and a kraft pulp

	Percentage composition of pulps			
Component	Kraft Pulp	Untreated RMP	Delignified RMP*	Sulphonated RMP
Arabinose	0.5	1.1	1.1	1.0
Galactose	0.6	2.4	2.5	1.9
Glucose	77.0	42.5	54.0	43.8
Xylose	5.9	3.2	5.5	3.5
Mannose	6.5	8.3	10.4	8.3
Lignin	4.4	27.3	8.2	30.9
Total	95.6	84.8	81.7	89.4

* Yield after sodium chlorite delignification was ~76%

2.33   Enzyme Adsorption Profiles

As the initial low hydrolysis yield of the unmodified RMP was attributed to the lack of accessibility of the cellulose to the enzymes, the capacity of the four pulps to adsorb increasing concentrations of enzyme was investigated (Fig. 4).   In order to minimize hydrolysis, the adsorption was carried out at 4 $^{\circ}$C to ensure that the initial adsorption values were not affected by any substrate changes resulting from hydrolysis. The untreated RMP adsorbed the least amount of protein per g of fiber, with a maximum of approximately 25% of the added protein being adsorbed. When compared to the kraft pulp, it can be seen that the RMP adsorbed less enzyme on the basis of protein adsorbed per mg of cellulose (Fig.4). Both of the treated RMP's showed a greater adsorption capacity than did the kraft pulp despite the latter pulp having the lowest lignin content (Table 1). Although the sulphonated RMP contained a significantly higher lignin content than did the delignified pulp or the kraft pulp, it adsorbed the highest amount of enzyme. Thus, on the basis of initial enzyme adsorption, it would seem that the sulphonated RMP adsorbed significantly more protein per mg of cellulose than the delignified RMP, the kraft pulp and the untreated RMP

2.34   Fiber Length Distributions

Previous work had indicated large differences in the particle size of Douglas-fir kraft and mechanical pulps, with a much higher content of smaller "fines" resulting from the mechanical action of producing a refiner mechanical pulp (Mansfield *et al.*, 1996). To determine if the swelling or delignification of the RMP had altered the gross physical characteristics of the pulps, they were each passed through a Bauer-McNett fiber classifier (Table 2). The pulps were washed through 5 consecutive screens, the first screen retaining the longest fibres and the last the shortest. Any fiber particles which passed through the last screen were termed as "fines". More than 76% of the kraft pulp was associated with the longer fibers retained on the R14 and R28

screens while more than 32% of each of the mechanical pulps was found to be in the fines fraction. The delignification and sulphonation steps did not affect the fiber distribution to a significant extent (Table 2). Thus, it was apparent that the gross fiber characteristics of the RMP could not explain the significant differences in the adsorption capacity of the mechanical pulps as the fiber size distribution was essentially unchanged, with the fines still accounting for a significant proportion of the pulps.
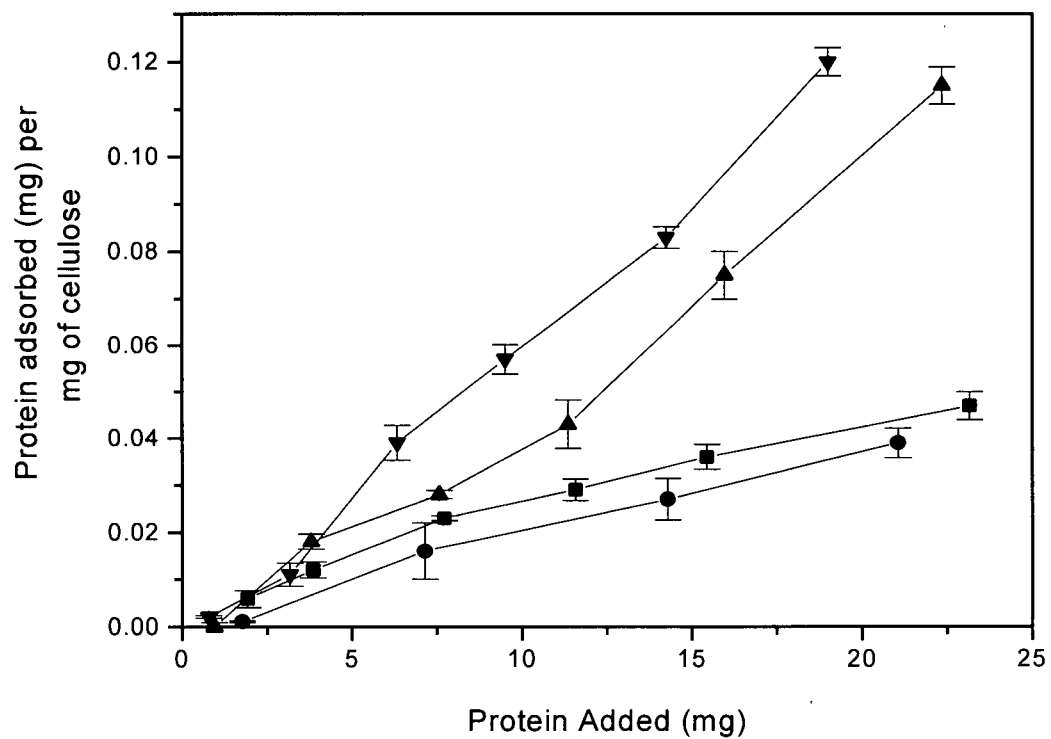
Fig.4: Adsorption capacities of a Douglas-fir kraft pulp—■—,
refiner mechanical pulp (RMP)—●—, delignified RMP—▲—
and sulphonated RMP—▼—

Table 2: Fiber length profile of a Douglas-fir refiner mechanical pulp (RMP), delignified RMP, sulphonated RMP and a kraft pulp

Percent of original pulp in each fraction					
Pulp	R14	R28	R50	R150	R200	Fines
Kraft	55.2	21.3	10.8	4.4	1.9	6.4
Untreated RMP	0.8	11.8	22.9	17.3	12.6	34.6
Delignified RMP	0.8	14.1	27.8	13.9	9.5	34.7
Sulphonated RMP	0.8	12.4	26.0	16.5	11.9	32.1

2.35 Hydrolysis Yields of Pretreated Substrates

In order to determine if there was a correlation between the adsorption capacity of the pulps and their ease of hydrolysis, an enzyme loading of 60 FPU/g of cellulose, which had previously been shown to be adequate for hydrolysis of the kraft pulp, was added to each of the pulps (Fig. 5). Although, the kraft pulp and the RMP showed similar hydrolysis profiles to those described previously, there was a dramatic increase in both the rate and completeness of hydrolysis of the delignified pulp compared to the untreated RMP. In contrast, although the sulphonated pulp showed an initial increase in rate, its final hydrolysis yield was still only 50% of the available cellulose which was double that of the untreated RMP.

It was apparent that there was a good relationship between the adsorption capacity of the delignified RMP and kraft pulp (Fig. 4) and the rate of hydrolysis (Fig. 5), with the substrate which adsorbed the most enzyme being the most efficiently hydrolyzed. However, in contrast to the delignified RMP, the high adsorption capacity of the sulphonated RMP did not translate into the same ease of hydrolysis suggesting that the adsorbed enzyme was incapable of hydrolysing cellulose. In addition, the untreated RMP, despite adsorbing similar amounts of protein per mg of cellulose as the kraft pulp, was only minimally hydrolysed. It is apparent that the adsorption capacity of a substrate can be correlated to its hydrolysis yield only with substrates which contain little or no lignin.

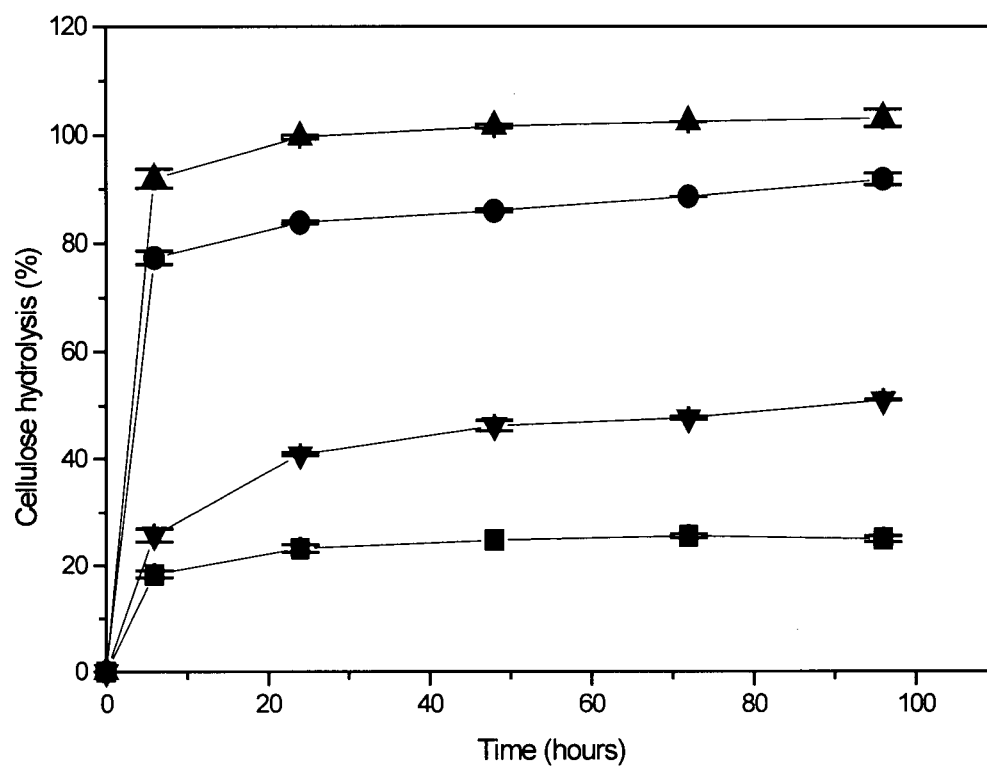Fig. 5 Time course hydrolysis of Douglas-fir kraft pulp—●—, refiner
mechanical pulp (RMP) —■—, sulphonated RMP—▼— and
delignified RMP—▲— at 60 FPU per g of cellulose

2.36 Measurement of Substrate using Pore Volume Technique

To see if these treatments, had in fact enhanced cellulose accessibility, the pore volume of each of the pulps was assessed by determining the pore volume accessible to both a 56 nm and a 5.1 nm probe (Table 3). A solute of 56 nm is considered to be too large to penetrate beyond the fiber surface (Stone *et al.*, 1969), thus the water inaccessible to this probe is the total water in the fiber and is referred to as the fiber saturation point. The 5.1 nm probe was chosen as the most indicative of a cellulase enzyme in terms of size and has been shown to be within the range most affected by pretreatments (Greithlein *et al.*, 1984). Though the median pore width (i.e. the pore size above and below which 50 % of the total pore volume exists) is not measured in this work, the pore volume inacccessible to a 5.1 nm probe is used as an indicator of this parameter.

As expected, the lowest pore volume was found with the untreated RMP while the delignified and sulphonated RMP substrates showed substantially enhanced access to the 56 nm probe. Although the kraft pulp showed a lower pore volume when using the 56 nm probe, it was significantly higher at the 5.1 nm level than either the delignified or sulphonated RMP. The sulphonated pulp had a considerably higher fiber saturation point than did the kraft pulp and to a lesser extent, the delignified RMP. However, since this did not translate into a substantially enhanced hydrolysis rate, it is possible that the significantly lower pore volume accessible to the 5.1 nm probe reflected the higher median pore width of the pulp. Thus, because the increased swelling is not caused by any significant increase in porosity, the accessibility and ability of the enzyme to hydrolyze the sulphonated RMP was still restricted.

Table 3: Accessibility of a Douglas-fir refiner mechanical pulp (RMP), delignified RMP, sulphonated RMP and a kraft pulp

Pulp	Pore Volume (ml/g) inaccessible to 56 nm probe		Pore Volume (ml/g) inaccessible to a 5.1 nm probe	
Kraft	1.2	+/- 0.078	0.9	+/- 0.017
RMP	0.14	+/- 0.021	0.1	+/- 0.015
Delignified RMP	2.1	+/- 0.014	0.6	+/- 0.032
Sulphonated RMP	2.5	+/- 0.063	0.3	+/- 0.043

2.37   Measurement of Substrate Accessibility using Simons Stain Method

Although, the pore volume method has many advantages, such as allowing the measurement of surface area without previous drying of the substrate, it only measures the surface area available in the form of pores and does not measure the external surface area of the substrate. In order to measure the complete surface area of the substrates a modified Simons stain technique was used (Yu *et al*, 1995). This method allows measurement of surface area both in the form of pores and total external surface area and it is also sensitive to variations in the accessibility of the interior structure of fibers (Yu *et al*, 1995). The technique involves the use of two cotton dyes which compete for the free hydroxyl groups on cellulose. The dyes are of different size and colour, an orange dye of large molecular weight (>50,000) and a low molecular weight blue dye (<10,000). The orange dye has a stronger affinity for cellulose than does the blue dye so that cellulose will preferentially bind this dye when both are present. However, due to the larger size of the orange dye, it can only bind to the cellulose surface where it has sufficient space. Thus, the blue dye adsorbs to the smaller spaces and pores into which the orange dye cannot reach. In this way it is possible, by measuring the amounts of each dye adsorbed, to determine the quality of the accessible surface area of a substrate in terms of the actual size of the spaces available to an enzyme on a fibers surface and in the form of pores.

When the accessible surface area of all Douglas-fir pulp derived substrates was measured, it was apparent from the amounts of each dye adsorbed to the different substrates, that there was a significant difference in the available cellulose surface area of each substrate. The delignified RMP and the kraft pulp adsorbed significantly more orange dye per mg of cellulose than did the untreated RMP and the sulphonated RMP. This suggested that the majority of the cellulose available within these two substrates was accessible to large molecules (Fig.6). In contrast, the sulphonated RMP and the untreated RMP adsorbed more of the blue dye indicating that these

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substrates had a significant proportion of their exposed cellulose surface in the form of small spaces and pores, which the larger orange dye could not penetrate. It is probably not a coincidence that these substrates still contain high concentrations of lignin as it seems that lignin removal opens up the substrate, enhancing the accessibility of the cellulose. It is apparent that there is a correlation between the cellulose surface area measured by the Simons stain technique and the ease of hydrolysis of each substrate. The sulphonated RMP adsorbed more dye than did the untreated RMP as indicated by the 54% increase in adsorption of the orange dye, which correlated with the hydrolysis yields resulting in twice as much hydrolysis (Fig.5). The cellulose within the sulphonated RMP was therefore made significantly more accessible to cellulases by the sulphonation process, although it was not sufficient to allow complete hydrolysis of the substrate. The delignified RMP adsorbed the highest amount of orange dye per mg of cellulose and was hydrolysed most completely and at the fastest rate. The kraft pulp, despite having less lignin than the delignified RMP, adsorbed less orange dye and less blue dye suggesting that the cellulose within this substrate was not as accessible.

The exact structure or size of the high molecular weight orange dye is not known but it has been shown that this fraction consists of two populations, one 5-7 nm in diameter and the other 12–36 nm in diameter (Yu *et al*, 1995). These dimensions are close to the known dimensions of some of the cellulase components such as the cellobiohydrolase I of *Trichoderma reesei* which is 18 nm in length with the catalytic core having dimensions of 5x6x4 nm (Divne *et al*, 1994). It is therefore not surprising that there is such a good correlation between the accessibility of the fibers to the orange dye and to the adsorption of the cellulases.
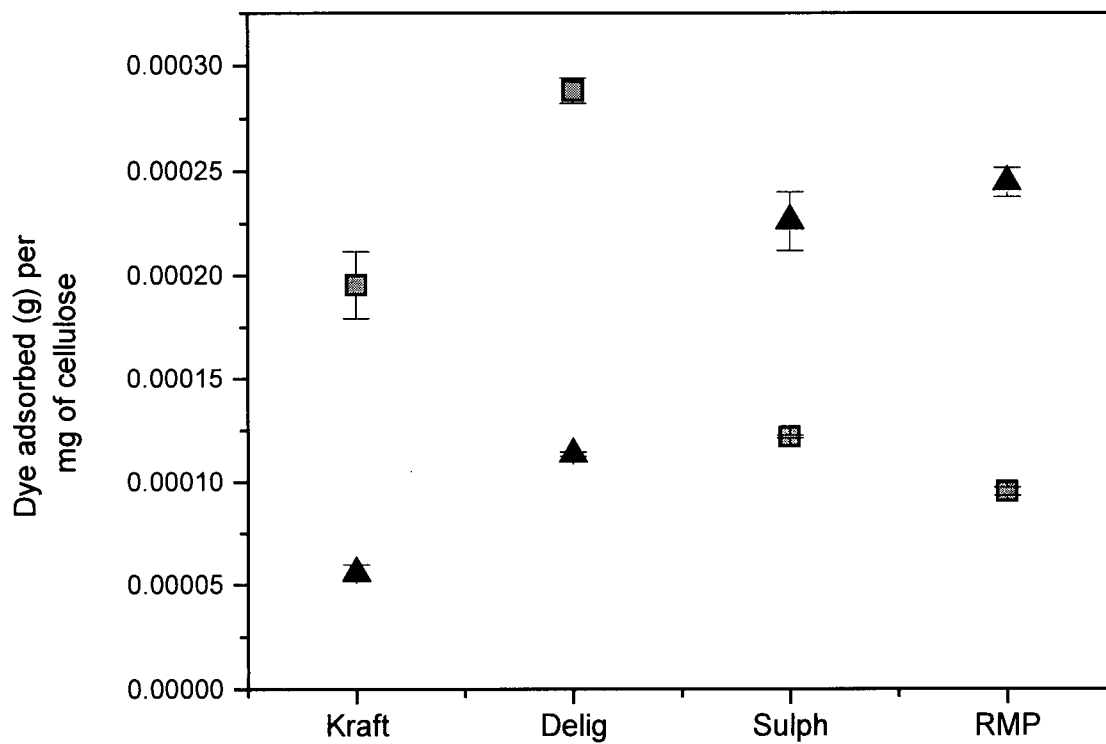
Fig.6: Amount (g) of Simons stain dye orange ▨ and blue ▲ adsorbed to a Douglas-fir kraft pulp, refiner mechanical pulp (RMP), delignified RMP and sulphonated RMP per mg of cellulose

2.38 Measurement of Lignin Release during Hydrolysis

While much of the data presented to date has provided some insights into the effect of accessible surface area and lignin content on the enzymatic hydrolysis of softwood substrates, there is little evidence to indicate that these factors played a role in the differences observed between the hydrolysis rates and yields of the delignified RMP and the kraft pulp. The kraft pulp was hydrolysed incompletely and at a slower rate than was the delignified RMP despite having a lower lignin content and similar pore volume and stain accessibility. The lignin released during hydrolysis of the kraft pulp and the delignified RMP was measured in order to try to determine if the residual lignin played a role in the observed differences in hydrolysis yields.. The residual lignin left after the kraft pulping process is chemically resistant and is more concentrated near the fiber surface where it is closely associated with both cellulose and hemicellulose (Karlsson *et al.*, 1996; Yllner *et al.*, 1957; Heijnesson *et al.*, 1995). In order to determine the extent to which this lignin affects hydrolysis, we measured the lignin released during hydrolysis of the kraft pulp and the delignified refiner mechanical pulp with 60 FPU per g of cellulose over a period of 72 hours (Fig. 7). It was apparent that the majority of the kraft lignin was not released during hydrolysis but remained closely associated with the residual unhydrolysed material. In contrast, lignin was slowly released during hydrolysis of the delignified pulp such that the percent lignin content increased from approximately 8% of the original substrate to 14 % of the residue left after hydrolysis while that of the kraft pulp increased from approx. 4% to 36% respectively. This would suggest that the lignin in the delignified RMP was only loosely associated with the cellulose such that it did not interfere with the cellulases during hydrolysis. In contrast, the kraft lignin was very resistant during hydrolysis and remained associated with the residue. This may provide a partial explanation for the incomplete hydrolysis of the kraft pulp since the residual cellulose may be closely associated with the lignin.
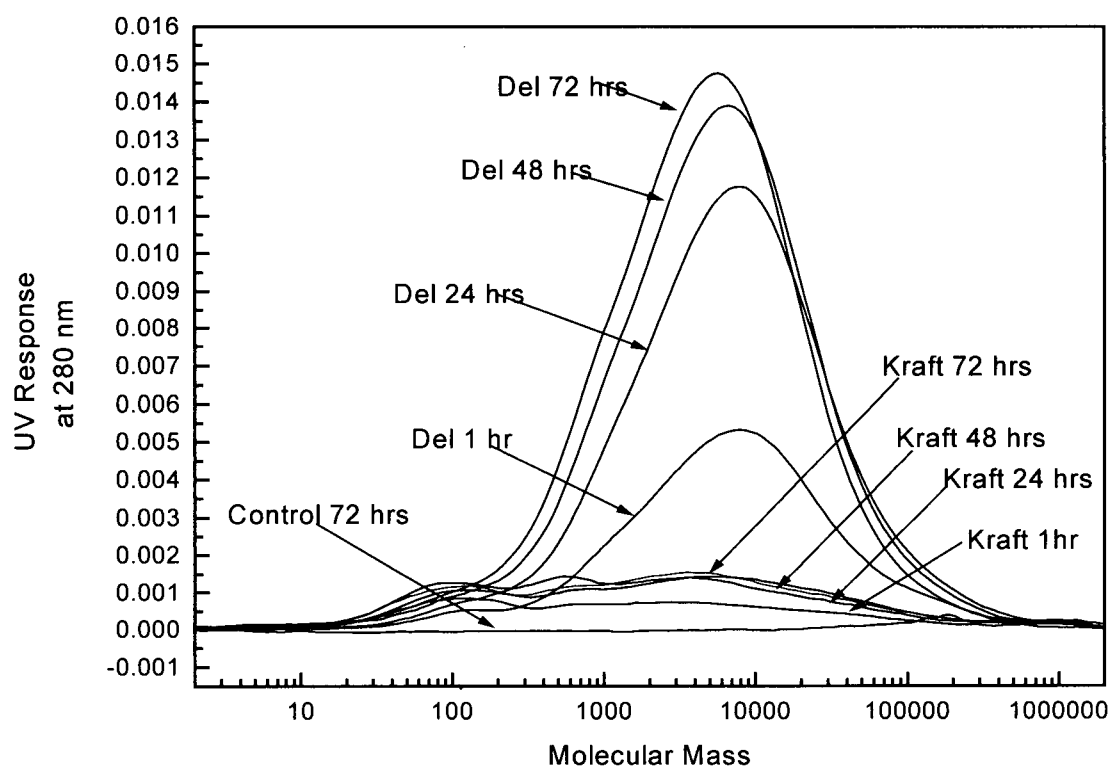
Fig. 7: Relative amounts of lignin released by a Douglas-fir kraft pulp (kraft) and delignified refiner mechanical pulp (Del) during hydrolysis with 60 FPU per g of cellulose for 72 hours (control is delignified RMP without enzyme after 72 hours)

2.4 Discussion

The accessible surface area has been cited as one of the most important factors determining the ease of hydrolysis of a lignocellulosic substrate (Stone *et al.*, 1969; Wong *et al.*, 1988; Burns *et al.*, 1989). However, the extent to which lignin removal and the consequent increase in cellulose accessibility may contribute to surface area and enzymatic digestibility has not been determined. While the major inhibitory role of lignin has been attributed to its effect on fiber swelling, our results indicated that the lignin still had a significant effect on cellulose digestibility, even when it no longer prevented fiber swelling.

The key role that lignin plays in restricting enzyme accessibility to the mechanical pulp was clearly indicated by the dramatic increase that delignification and sulphonation played in enhancing enzyme adsorption. Both of the RMP derived substrates had a higher enzyme adsorption than did the kraft pulp perhaps reflecting the smaller average fiber size which resulted in a higher specific surface area. However, the similarity between the two pretreated RMP substrates ends there. While the sulphonated RMP initially adsorbed as much cellulase as did the delignified pulp, the hydrolysis yields were considerably lower. Pore volume determination indicated that, although sulphonation increased the fiber saturation point dramatically, the substrate was not made more porous as the pore volume inaccessible to the 5.1nm probe was low. Previous work (Scallan *et al.*, 1977) has shown that sulphonation introduces hydrophilic sulphonic acid groups into the lignin resulting in a more swollen substrate. Softwoods are particularly susceptible to sulphonation as guaiacyl lignin contains more free phenolic groups than syringyl. Thus, the increased hydrophillicity brought about by sulphonation of the RMP resulted in a substantially more swollen pulp. Despite a substantial increase in fiber swelling and enzyme adsorption, the sulphonated RMP was still not hydrolysed completely. This suggests that although the enzyme is able to adsorb to the substrate, it is still not capable of accessing all of the

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cellulose. The increased adsorption of enzyme may be a surface phenomenom caused by adsorption on the more hydrophillic lignin component as well as to cellulose since increased cellulose accessibility alone could not explain the dramatic increase in adsorption without a concurrent increase in hydrolysis yields. The Simons stain data supports this hypothesis since the sulphonated RMP adsorbed significantly less of the high molecular weight orange dye than did the delignified RMP and the kraft pulp which had much lower lignin contents. Since the orange dye adsorbs to the free hydroxyl groups of cellulose where it has sufficient access, it seems that the lignin is still preventing such access in the sulphonated RMP. Simons stain has been used previously to determine the effect of biomechanical pulping on the accessibility of the interior structure of the fiber (Akhtar *et al*, 1995). However, it has not been used for this application previously, though it does seem to be a very good indicator of the accessibility of cellulose within different lignocellulosic substrates due to the preferential adsorption of the dyes to cellulose and the comparability of the high molecular weight orange dye and cellulase enzymes.

Delignification of the RMP resulted in a similar increase in enzyme adsorption which, in contrast to the sulphonated RMP resulted in dramatically increased hydrolysis. This provided further evidence of the effect of lignin on the accessibility of cellulose within lignocellulosic substrates. The delignified RMP was not as swollen as the sulphonated RMP since its fiber saturation point was slightly lower. However, the pore volume inaccessible to a 5.1 nm probe was higher, indicating that lignin removal has a significant effect on the creation of smaller pores within a fiber. This was further indicated with the Simons stain data since the delignified RMP adsorbed much more of the high molecular weight orange dye than did any of the other substrates. Thus, it seems that the removal of lignin resulted in a dramatic increase in the accessibility of cellulose to this dye, at the expense of the blue dye, for both the kraft pulp and the delignified RMP. The Simons stain data also suggests that the removal of lignin increases the size of the existing pores

and spaces rather than creating new ones since any increase in the adsorption of the orange dye was always accompanied by a decrease in the adsorption of the blue dye.

Since the pore volume method and the Simons stain method both measure relative accessibility of substrates it is difficult to compare them. Although the pore volume data indicated that the delignified RMP has a lower proportion of small pores than does the kraft pulp, the Simons stain data suggested the opposite since the delignified RMP adsorbed more of the smaller blue dye. This apparent discrepancy could probably be explained by the fact that the delignifed RMP had a significantly smaller average particle size than did the kraft pulp such that it had a higher specific surface area. It is possible that the higher adsorption of both dyes by the delignified RMP was due to this substrates higher surface area to weight ratio. As the pore volume method does not measure the outside surface area of fibers it should not be influenced by fiber size. The higher pore volume inaccessible to the large probe can be explained by the high fines content of the delignified RMP. It has been shown that fines are significantly more swollen than are the other fiber fractions (Laivins et al, 1996), with a linear correlation shown between fines content and the level of swelling of mechanical pulps. The higher fines content and lower average fiber size of the delignified RMP could also explain the higher adsorption capacity of this substrate compared to the kraft pulp. It was apparent, from the low accessibility of the untreated RMP prior to pretreatment, that the effect of fiber size and fines content on fiber swelling was overshadowed by the constricting influence of lignin prior to pretreatment. Previously, it was shown that hemicellulose removal substantially increased the pore volume of acid treated softwoods (Greithlein et al., 1984). This provided another possible explanation for the higher porosity of the kraft pulp since this substrate had undergone more extensive lignin and hemicellulose removal than had the other substrates.

It was apparent that the kraft pulp was more slowly hydrolyzed than the delignified RMP, with incomplete hydrolysis obtained even at the very high enzyme loadings, indicating that the residual cellulose may be closely associated with lignin. The lignin release data suggested that the residual kraft lignin was significantly more recalcitrant than that of the delignified RMP. The delignified RMP released lignin slowly but consistently over the period of hydrolysis while little lignin was released from the kraft pulp. It has been shown that kraft pulp contains lignin-carbohydrate complexes, which are relatively stable and resistant to chemical attack and that a considerable amount of the residual lignin in kraft pulps is associated with cellulose, particularly in softwood pulps (Karlsson *et al.*, 1996). It has also been shown that degraded xylans are precipitated on the surface of fibers during pulping (Yllner *et al.*, 1957) and that kraft fibers have a relatively high concentration of lignin on their surface (Heijnesson *et al.*, 1995). Although the low lignin content of a kraft pulp would suggest that this substrate would be readily hydrolyzed, it was obvious that other factors such as the presence of longer fibers, redeposition of lignin on the fiber surfaces and limited pore volume, all influence the ease of enzymatic hydrolysis.

CHAPTER 3   The Effect of Gross Fiber Characteristics on the Accessibility and Hydrolysis of Softwood Substrates

3.1 Background

Much of the past work in this area has tried to explain the typically observed biphasic rate of hydrolysis by substrate characteristics such as crystallinity, degree of polymerization, lignin content, surface area etc. In this way, some authors have suggested that the initial rapid phase of hydrolysis is due to, for example, the more rapid attack of amorphous cellulose regions with the secondary, slower hydrolysis due to increasing crystallinity or recalcitrance of the residual substrate (Fan et al, 1980; Walseth et at, 1952; Puri et al, 1984). However, most of these past studies have had to make the assumption that there is a homogenous distribution of these various characteristics throughout the substrate with only a nominal acknowledgment of the fibrous nature and diversity of the component parts of the substrate.

While there is some evidence that factors such as crystallinity can have a negative influence on the hydrolysis rates of pure cellulosic substrates, the effects of fiber related characteristics such as the influence of the lignin-hemicellulose barrier and the cell wall thickness of the component fibers probably outweigh the effect of microscopic characteristics such as DP or crystallinity (Greithlein et al, 1985; Thompson et al, 1992; Wong et al, 1988). Unlike pure cellulosic substrates, lignocellulosic substrates are heterogeneous and are generally composed of a wide range of particle sizes. Previous studies with various pretreated lignocellulosic substrates have found positive correlations with factors such as pore volume and surface area (Gharpuray et al, 1983, Greithlein et al, 1984). Thus, it is probable that the gross structural characteristics of lignocellulosic substrates are at least as influential in determining the degree and efficiency of hydrolysis as are fine structural characteristics such as crystallinity and DP.

In the previous chapter of this thesis it was shown that the lignin content of Douglas-fir pulps had a significant effect on hydrolysis rates and that the accessible surface area could be correlated with hydrolysis rates and yields. However, it was also found that there were differences in the rates of hydrolysis of a delignified refiner mechanical pulp and a kraft pulp which could not be explained by differences in lignin content and surface area alone. This was attributed in part to the recalcitrance of the residual lignin as well as the large average particle size of the kraft pulp. In the work described here, the effect of gross fiber characteristics such as fiber size and coarseness on accessibility to enzymes and hydrolysis rates was investigated. Coarseness is a parameter more commonly measured in papermaking as it gives an estimation of the weight to length ratio of a population of fibers. Thus, the fiber coarseness values could be used in order to obtain an estimation of the changes in mass per length of fiber as hydrolysis progresses. In this way, it was possible to determine how factors such as fiber coarseness and size influence enzyme adsorption and subsequent hydrolysis rate and yields.

3.2 *Materials and Methods*

Substrate Preparation: The Douglas-fir kraft pulp was obtained from Fletcher Challenge, Crofton, BC. Its chemical composition was described in the previous section. The pulp was subjected to refining in a PFI laboratory refiner at 5000 revolutions resulting in a substrate in which the fines content was increased from approximately 5% to 20% (ref-DF). Refining in a PFI mill typically increases the fines content by a delamination process in which the surface layers of the larger fibers are removed and broken down into fines. The pulp was fractionated using a Bauer McNett fiber classifier using 5 consecutive screens of 14, 28, 50, 100 and 200 mesh. The largest fibers, which were retained on the 14 mesh screen (R14), were collected and used for this study (R14-DF).

Enzymes: As described in previous chapter.

Adsorption Measurement: As described in the previous chapter.

Long Term Hydrolysis: As described in the previous chapter.

Fiber Length Analysis: The fiber length distribution and fiber coarseness of the substrates was measured before and during hydrolysis using a Kajaani FS200 fiber analyser. Samples were prepared in duplicate, by boiling them after the required hydrolysis period for 10 minutes to stop hydrolysis. They were then filtered and the residual substrate was analysed in triplicate for its fiber length distribution and fiber coarseness. Using this procedure all of the fibers between the length of 0.05mm to 7mm could be counted and a profile of the overall fiber length of the substrates was determined. This method is based on the characteristic scattering of a laser beam by the fiber cell wall due to its property of birefringence. It also measures the fiber coarseness of each substrate i.e. weight per length of fiber.

3.3   *Results*

3.31   Substrate Preparation

As mentioned previously, past work has shown that softwood substrates are inherently more recalcitrant to hydrolysis than hardwood substrates (Ramos *et al*, 1992; Greithlein *et al*, 1985), and this was attributed to factors such as lignin content and pore volume. In the previous chapter of this thesis it was shown that Douglas-fir kraft pulp could be hydrolyzed relatively efficiently, although this required high enzyme loadings. This was attributed in part to the large fiber size and coarseness of Douglas-fir pulp. In order to study the effect of fiber size on hydrolysis of this softwood substrate, it was subjected to various pretreatments, in order to change its average fiber size. The effect of these changes on enzyme adsorption and hydrolysis rates was also studied in order to determine the extent to which these factors influence the hydrolysis reaction.

The average fiber size of the Douglas-fir kraft pulp was reduced by subjecting it to a refining process using a PFI laboratory refiner. The substrate created by the refining process (ref-DF) had a percent composition of fines of approximately 20%, which was about a four-fold increase over the original pulp. A pulp with increased fiber average fiber size was also produced by fractionating the original kraft pulp, using a Bauer McNett fiber analyser to isolate the largest fibers on a 14-mesh screen. The resulting substrate (R14-DF) consisted predominantly of fibers between 2 and 5 mm in length. In this way it was possible to obtain two substrates which differed significantly in their fiber size distribution but were similar in their chemical composition, enabling comparison of rates and yields of hydrolysis on the basis of fiber size alone. The resulting substrates were very similar in terms of chemical composition with the exception of the R14-DF substrate which had a marginally lower lignin content than either the whole pulp or the ref-DF substrates (Table 4).

3.32 Adsorption Capacity Measurements

In order to determine the effect of changes in fiber size distribution on enzyme accessibility the adsorption capacity of the refined pulp (ref-DF) was measured and compared to that of the original kraft pulp and the R14 fraction (R14-DF) (Fig 8). The adsorption capacity of the ref-DF substrate from which the fines had been removed was also measured to determine the influence that an increased fines content might have had on adsorption capacity. As expected, the ref-DF substrate had a significantly greater adsorption capacity than did the original pulp or the R14-DF substrate. It was found that the adsorption capacity was greatly increased by the refining process with up to 96% of added enzyme adsorbed, regardless of the amount of enzyme added. In contrast to the linear correlation that was obtained between the added and adsorbed enzyme with the ref-DF, the original pulp reached its maximum adsorption once an addition of approx. 10mg of enzyme was reached, after which it began to taper off with more enzyme left unadsorbed by

the substrate. Since refining significantly increased the fines content of the pulp, it consequently also increased the specific surface area available for enzyme adsorption. Removal of the fines from the ref-DF substrate also reduced the adsorption capacity, thus it was apparent that the amount of enzyme adsorbed per gram of cellulose varies with fiber size and that small fibers and fines adsorbed significantly more enzyme per g than did the larger fibers.
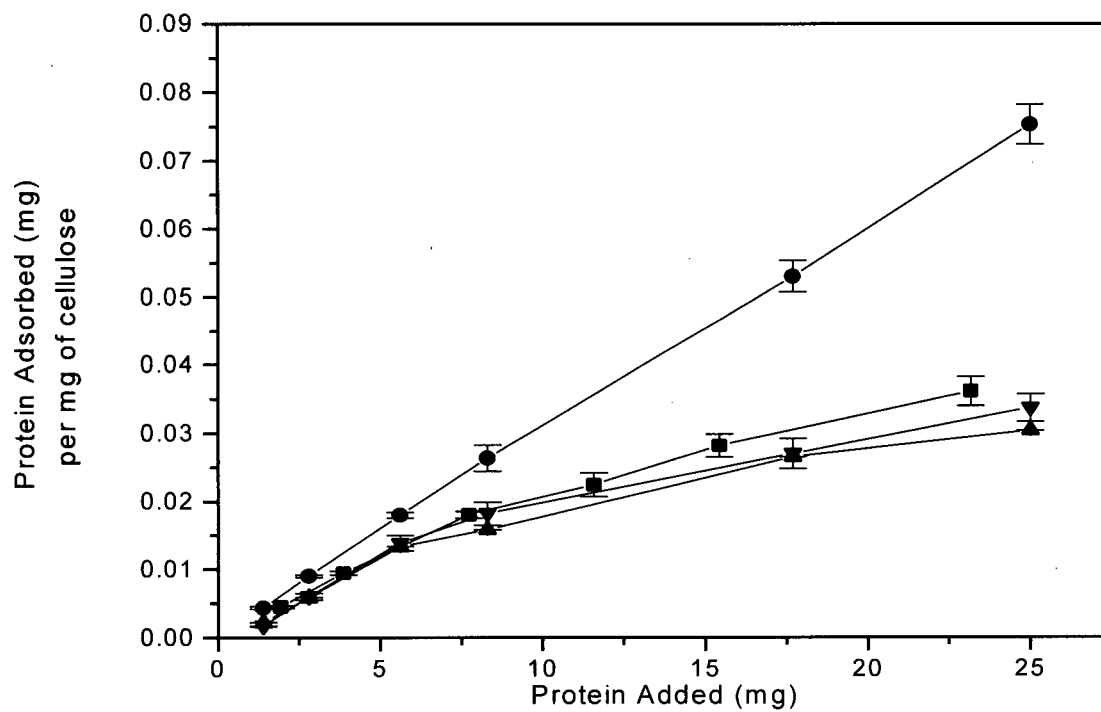
Fig 8: Adsorption profile of Douglas-fir kraft pulp—■—, refined kraft pulp (ref-DF)—●—, refined kraft pulp without fines —▲— and R14 fraction of kraft pulp (R14-DF) —▼—

3.33   Substrate Hydrolysis Yields

In order to determine how these differences in adsorption capacity relate to hydrolysis rates, the susceptibility of the ref-DF substrate and the R14-DF substrate to hydrolysis was measured at increasing cellulase loadings of 5, 10 and 20 FPU/g of cellulose over a period of 72 hours (Fig. 9 inset).

It was apparent that the ref-DF was hydrolyzed at a faster rate (Fig.9) and more completely than either the R14-DF or the original pulp. It was also apparent that less enzyme was required to obtain maximum hydrolysis of the ref-DF substrate. For example, in the initial 1.5 hours of hydrolysis of the whole pulp, an enzyme loading of 20 FPU/g of cellulose was required in order to reach a hydrolysis yield of 10% while only 5 FPU/g was required with the ref-DF. The observed correlation between adsorption capacity and hydrolysis yields of these substrates suggested that the fiber size influenced the susceptibility to hydrolysis by controlling the initial enzyme adsorption. The difference in hydrolysis rates and yields of the three substrates was apparent after 1.5 hours, suggesting that the initial stage of the hydrolysis reaction was critical in determining the final yield.
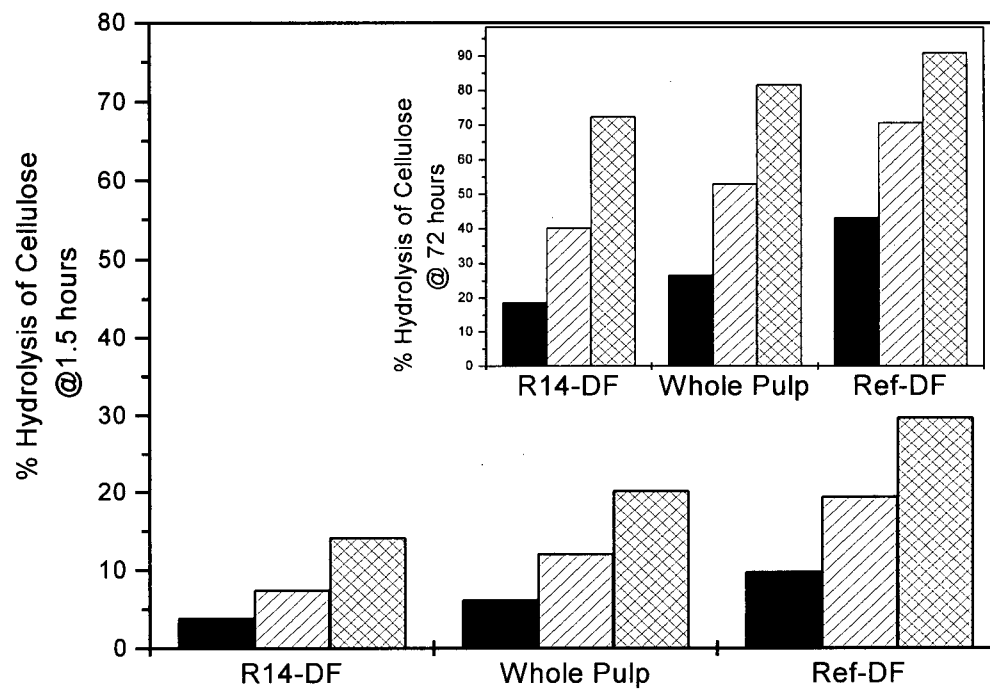
Fig. 9: Hydrolysis after 1.5 hours and 72 hours(inset) of Douglas-fir kraft pulp (Whole Pulp), refined kraft pulp (ref-DF) and R14 fraction of kraft pulp (R14-DF) at 5 FPU ▉ ,10 FPU ▨ and 20 FPU ▩ per g of cellulose

3.34 Measurement of Fiber Size Distribution during Hydrolysis

What is happening to the fibers in this initial stage of hydrolysis that so dramatically affects the ultimate yield? This was investigated by comparing the changes in fiber size distribution and coarseness of the ref-DF and R14-DF during hydrolysis with 10 FPU/g of cellulose. These substrates had significantly different initial fiber size distribution profiles, with the R14-DF consisting predominantly of long fibers, with approximately 70% of the fiber population having a length of between 2 and 4 mm (Fig. 10). The ref-DF substrate had a wider fiber length distribution with approximately 80% of the component fibers between 0.2 and 2 mm in length (Fig. 11). After 20 minutes of hydrolysis, the population distribution of the R14-DF had already undergone significant changes with the creation of smaller fibers while the ref-DF was not significantly changed. After 45 minutes, the population distribution of the ref-DF had still not changed despite the fact that approximately 8% of the substrate had been hydrolyzed. In contrast, although only 5% of the R14-DF substrate was hydrolyzed, the fiber length profile had changed dramatically from initially having very few small fibers (0.2-1.2 mm), to having approximately 24% of its population in this range after 45 minutes of hydrolysis.

It seems that the fines (< 0.05mm) which were not detected by the Kajaani Fiber Analyzer, were quickly hydrolyzed in the initial stages of ref-DF hydrolysis, resulting in no observable difference in the fiber population distribution. It was possible that a certain percentage of the fines of this pulp were not detected by the Kajaani Fiber Analyser since the fines content of the refined pulp was originally determined by passing the fibers through the Bauer McNett. The fines were defined as those fibers which passed through the smallest mesh screen which normally retained fibers greater than 0.15mm in length. Since approximately 20% of the pulp by weight was in the form of particles smaller than this it is probable that a significant proportion of these particles are smaller than 0.05mm and are thus not detected by the Kajaani fiber analyser.

The observed changes in fiber length distribution of the R14-DF and the ref-DF substrates indicated that the mechanism of hydrolysis of these substrates was different as the major shifts in distribution were observed with the long fibers of the R14-DF substrate and with the fines fraction of the ref-DF.

Fig.10: Changes in population and weighted (inset) fiber length distribution of R14 fraction of Douglas-fir kraft pulp resulting from hydrolysis at 0 hours————, 20 min-----, 45 min········ and 2 hours---·---·-- with 10 FPU per gram of cellulose
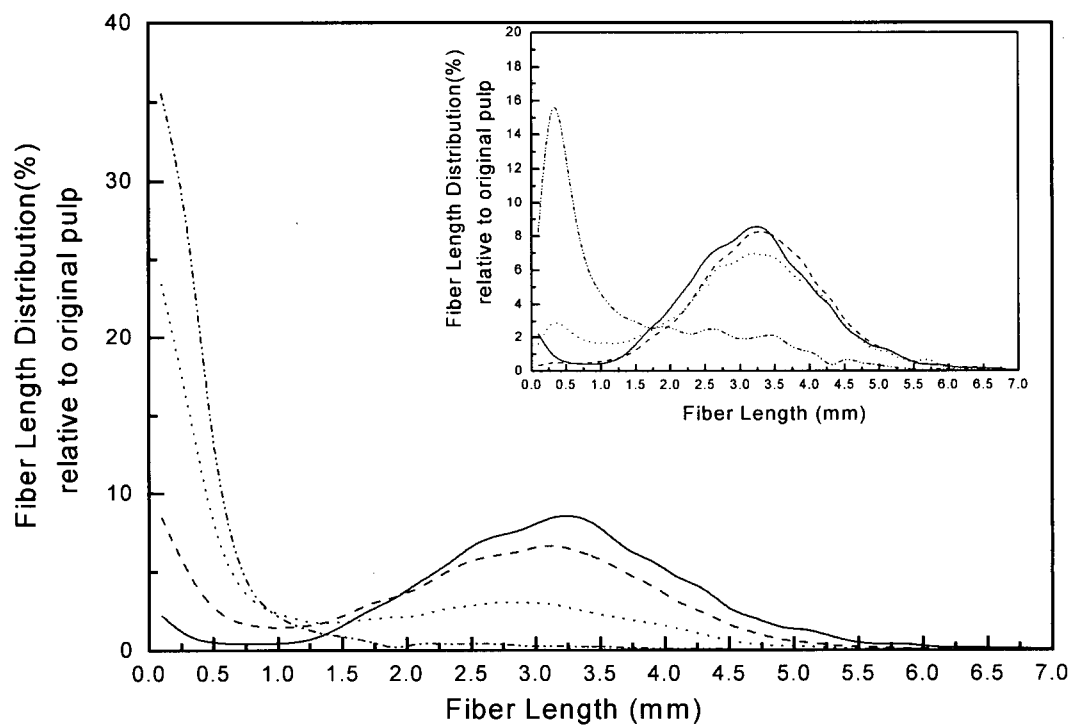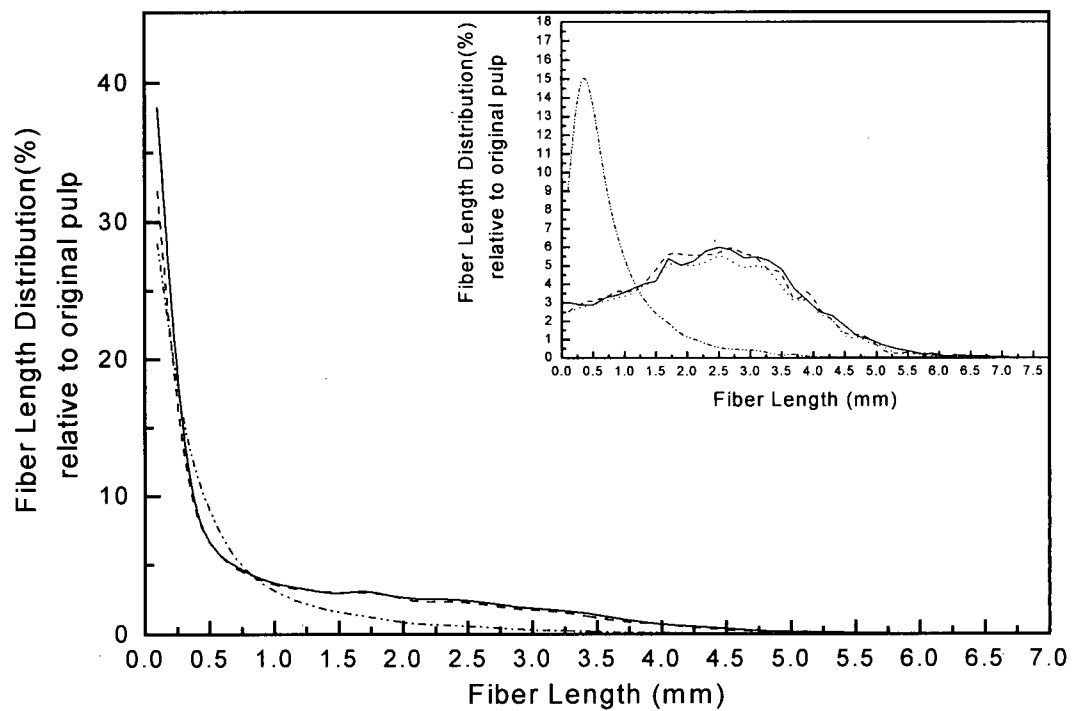
Fig.11: Changes in population and weighted (inset) fiber length distribution of refined Douglas-fir kraft pulp resulting from hydrolysis at 0 hours————, 20 min-----, 45 min········ and 2 hours---------with 10 FPU per gram of cellulose.

3.35 Coarseness Measurements during Hydrolysis

To try to better relate the gross fiber characteristics to the overall rate of hydrolysis, the coarseness of the residual pulp was measured during hydrolysis. Fiber coarseness is a measure of the amount of fiber material per length of fiber and is generally used to give an indication of a fibers cell wall thickness. Therefore, measuring the changes in fiber coarseness as well as fiber length during hydrolysis should give an indication of how the fiber is being hydrolyzed, i.e., whether it is being broken down into shorter fibers or peeled along it's length. The observed changes in fiber coarseness agree with the fiber length changes in that the two substrates undergo different changes suggesting different mechanisms of hydrolysis (Tables 5 & 6).

After 45 minutes of hydrolysis, the coarseness of the R14-DF substrate was decreased by approximately 8% (Table 5) while the ref-DF was unchanged (Table 6). As the coarseness of a population of fibers is largely determined by the longer fibers, it is most sensitive to changes in the weight of this fraction. Thus, the observed changes suggested that the R14-DF fibers were bing made less coarse by hydrolysis as well as being disintegrated into shorter fibers. This yielded a lower degree of hydrolysis (approx. 5%) than did the rapid erosion of fines and smaller fibers observed with the ref-DF substrate (approx. 8%).

After 2 hours of hydrolysis, when the population distribution of the ref-DF substrate began to change, the coarseness was decreased by 16 % while the R14-DF substrate had decreased by a further 18%. At this point, it appeared that the proportion of small fibers in the ref-DF substrate had increased at the expense of the larger fibers causing the observed decrease in coarseness. The differences in the mechanism of hydrolysis of the two substrates can also be seen with the average fiber length data (Tables 5 & 6). The number average (Ln) is the average fiber length based on the actual numbers of fibers of each length. The length weighted average (Ll) is

weighted such that the total length of the fibers at each length is taken into account and weight weighted average (Lw) fiber lengths are weighted such that the total weight of the fibers at each length is accounted for. The average fiber length values of the substrates were different prior to hydrolysis, with the R14-DF substrate having predominantly long fibers which constituted the majority of its total length and weight. In contrast, the ref-DF had lower values for the three fiber length averages due to the presence of a high percentage of small fibers and fines which particularly affected the Ll and Ln values. The length averages of the R14-DF substrate were reduced after 20 min, 45 min and 2 hours of hydrolysis. As expected, the fibers length averages of the ref-DF substrate did not begin to change until after 2 hours of hydrolysis when the initial fines have been hydrolyzed and the distribution of the fibers detected by the Kajaani Fiber Analyzer had begun to change.

After 2 hours the population distribution of both substrates was similar with the majority of the longer fibers reduced to small fibers. However, the weighted distributions (Fig 10 & 11 insets) show that, although the majority of the residual fibers in the R14-DF substrate were in the shorter fiber length region, the long fibers which were left still constituted a significant proportion of the substrates total mass. Similarly, a significant proportion of the R14-DF substrate was still present in the range of 2 to 5mm in length. Therefore, after 2 hours of hydrolysis, the ref-DF had undergone approximately 20% hydrolysis and the remaining fibers were in the form of small fibers and fines. In contrast, R14-DF had only undergone approximately 8% hydrolysis and still contained a significant proportion of its mass in the form of long coarse fibers.

Table 4: Compositional analysis of Douglas-fir kraft pulp R14 fraction (R14-DF), refined pulp (ref-DF) and whole pulp

Pulp Fraction	Arabinose	Galactose	Glucose	Xylose	Mannose	Klason Lignin	Acid-Insoluble Lignin
14 R	0.41	0.54	74.94	5.47	6.26	3.88	0.36
Whole Pulp	0.41	0.53	74.27	5.75	6.39	4.40	0.36
Refined Pulp	0.40	0.55	74.51	5.72	6.30	4.65	0.36

Average of 3 replicates (+/- 0.06)

Table 5: Changes in fiber length averages of the R14 fraction of Douglas-fir Kraft pulp resulting from hydrolysis over time at 10 FPU per g of cellulose

Time	Ln (mm)	Lw (mm)	Ll (mm)	Coarseness (mg/mm)
0 min	3.09 ±0.02	3.78 ±0.01	3.49 ±0.03	0.35 ±0.01
20 min	2.46 ±0.04	3.59 ±0.04	3.26 ±0.04	0.35 ±0.01
45 min	1.4 ±0.04	3.45 ±0.08	2.88 ±0.06	0.32 ±0.01
2 hr	0.47 ±0.02	2.57 ±0.06	1.37 ±0.01	0.26 ±0.01

Table 6: Changes in fiber length averages of refined Douglas-fir Kraft pulp resulting from hydrolysis over time at 10 FPU per g of cellulose

Time	Ln (mm)	Lw (mm)	Ll (mm)	Coarseness (mg/mm)
0 min	1.0 ± 0.03	3.11 ±0.02	2.42 ±0.02	0.25 ±0.001
20 min	1.03 ±0.01	3.03 ±0.04	2.38 ±0.04	0.25 ±0.008
45 min	1.03 ±0.03	3.02 ±0.02	2.36 ±0.03	0.25 ±0.007
2 hr	0.52 ±0.04	2.35 ±0.02	1.36 ±0.06	0.20 ±0.01

3.4 Discussion

This work has shown that the gross fiber characteristics of lignocellulosic substrates have a significant effect on the mechanism as well as the rates and yields of hydrolysis. It is apparent that, as well as microscopic characteristics such as crystallinity and degree of polymerization, gross factors such as fiber size and cell wall thickness can influence the hydrolysis of lignocellulosic substrates. The heterogeneous nature of these substrates is reflected in the wide range of fiber characteristics, such as fiber length and coarseness that were observed in the various substrates. Most methods of analysis measure averages and include assumptions that the characteristics measured are homogenous throughout the substrates. In the previous chapter of this thesis it was shown that the lignin content of Douglas-fir pulps had a significant effect on hydrolysis by limiting the enzymes accessibility to the cellulose. However, it was also apparent that other factors were influencing the hydrolysis rates of the low lignin substrates with the adsorption values strongly indicating that the significant differences in the fiber size distribution of the substrates were a major contributing factor to the observed differences in hydrolysis rates. The data presented here suggests that the heterogeneous distribution of fiber sizes within a lignocellulosic substrate can have an influence on its accessibility to enzymes and its hydrolysis. The adsorption data showed a marked correlation between particle size and adsorption capacity with the adsorption capacity decreasing with increasing average particle size. The ref-DF substrate which contained the highest amount of small fibers and fines had the highest adsorption capacity while the R14-DF substrate had a significantly lower capacity due to its large particle size and consequent low specific surface area. The adsorption data correlated positively with hydrolysis rates and yields since the ref-DF was hydrolysed at a significantly faster rate than was the original pulp and the R14-DF substrates.

Previous work has shown that the mechanism of hydrolysis of small particles with higher specific surface areas differs substantially from that of larger particles, with the smaller particles preferentially degraded by an 'erosion' type of mechanism while the larger particles appeared to undergo a 'peeling' process (Peters *et al*, 1993). In related work it was found that treating pulps with low enzyme doses resulted in increased pulp freeness (Jackson *et al*, 1993; Mansfield *et al*, 1996). This indicated the preferential hydrolysis of fines, since freeness is a measure of a pulps ability to retain water and is greatly influenced by the fines content.

There have been some previous studies which uncovered morphological differences between the fines of various substrates and the other component fractions of these substrates which may go further to explain their hydrolysability. For example, it has been shown that fines from spruce wood have a lower ratio of interior cellulose to surface cellulose than the whole wood, probably a result of their greater surface area to weight ratio (Leary *et al*, 1986). Another study found that increasing the amount of fines in a refiner mechanical pulp resulted in equivalent increases in pore volume of the pulp, again this could be due to the greater specific surface area of fines (Laivins *et al*, 1996). In this work, the increased specific surface area of the fines allowed them to adsorb more enzyme per g than the larger, coarser fibers. This increased adsorption capacity showed good correlation with the hydrolysis yields, in that the substrate with the highest fines content and highest adsorption capacity (ref-DF) was hydrolysed at a faster rate. Thus, at least in the initial stages of the reaction, it seems that the fiber size influences hydrolysis rates since those substrates with a smaller average fiber length and size were hydrolysed more efficiently. It is clear that the heterogeneous distribution of fiber sizes within a lignocellulosic substrate influences enzyme accessibility to enzymes and thus also substrate hydrolysis.

Chapter 4 Conclusions

The objective of this thesis was to investigate the factors influencing the accessibility and hydrolysis of softwood substrates. In doing so, it was hoped that we could gain a more indepth understanding of how the hydrolysis reaction is influenced by the particular structural characteristics of softwood substrates. The ultimate focus of this fundamental research is to facilitate the development of a pretreatment process suitable for optimising the use of softwood feedstocks for a bioconversion process. In determining how factors such as lignin content and distribution, and fiber morphology influence the accessibility of the cellulase enzymes to the cellulose, it should be easier to develop specific pretreatment steps in order to optimise cellulose accessibility.

It was apparent that lignin is the single most important factor influencing the hydrolysis of Douglas-fir substrates, both through its effect in fiber swelling and substrate porosity. The sulphonation work clearly demonstrated, that while the lignin could be modified such that it no longer prevented substrate swelling and facilitated increased enzyme adsorption, hydrolysis was still incomplete. In contrast, removal of the lignin resulted in complete hydrolysis of the cellulose after only 24 hours. It was apparent from the pore volume data, that lignin removal resulted in a more swollen substrate with an increased porosity. The Simons stain results showed that this increased porosity resulted in the increased accessibility of the cellulose component. In particular, the removal of lignin increased the number of pores below 5.1 nm, suggesting that the lignin had occupied spaces within the substrate and had previously hindered access of the enzyme to cellulose. Thus, it is clear that partial if not complete lignin removal is essential in order to achieve rapid and complete hydrolysis.

Subsequent to lignin removal, it is clear that the average particle size of the Douglas-fir substrates plays a significant role in determining their adsorption capacity and hence rate of hydrolysis. Prior to lignin removal, particle size did not influence the adsorption of enzyme a point which can be inferred from the low adsorption capacity of the untreated RMP. However, once the lignin composition had been sufficiently lowered, it was apparent that the delignified RMP was hydrolysed at a faster rate and more completely than the kraft pulp despite its higher lignin content. The incomplete hydrolysis of the kraft pulp may be explained by the slow and incomplete release of lignin as hydrolysis progressed. Further investigation of the effect of fiber size indicated clearly that the fiber size distribution of the kraft pulp played a major role in determining the rate of hydrolysis as well as the amount of enzyme necessary to achieve it. It was found that by specific surface area of the kraft pulp, the adsorption capacity of the substrate could be increased significantly and the resulting hydrolysis rate was similarly increased. Investigation of the particle size distribution as hydrolysis progressed demonstrated that the smaller particles within a heterogenous substrate are hydrolysed rapidly in the initial stages of hydrolysis leaving a higher percentage of larger particles which are broken down more slowly by the enzymes. It was also apparent that the enzyme loading required in order to achieve optimum hydrolysis could be reduced by decreasing the average particle size of the substrate.

Future Work

While this thesis has answered some fundamental questions regarding the recalcitrance of softwood substrates to enzymatic hydrolysis, there is still much to be learned about how the information obtained relates to potential pretreatment protocols and to other softwood feedstocks.

Future fundamental work should involve a more indepth study of the influence of lignin content and fiber size by looking at steam exploded substrates since these will be the ultimate feedstock for a bioconversion process. In addition, the influence of fiber size could be further investigated by using a wider range of substrates and fiber sizes. A study of the pore volume distribution of the individual fiber sizes may shed some light on the influence of fiber morphology on ease of hydrolysis. For example, it may be that coarser fibers are less porous and thus less accessible to cellulases than less coarse fibers even after lignin removal. The cell walls of coarse fibers such as Douglas-fir may be more resistant to the beneficial effects of lignin removal simply due to their denser cell walls. Comparison of the pore volume of these fibers and their Simons stain accessibility to less coarse softwood fibers would certainly help to determine if this is so.

More applied work could involve the investigation of the how the results of this work could be exploited in order to improve the pretreatment methods for softwood substrates. This should involve optimisation of the pretreatment process such that there is sufficient removal of lignin and reduction of fiber size to increase hydrolysis yields. There may be a compromise between these two factors such that complete lignin removal is not necessary if the fiber size has been sufficiently reduced. The influence of fiber size on the enzyme loading should also be further investigated since this is a particularly costly part of the bioconversion process. The potential to minimise this cost is thus promising and warrants further investigation.

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