**THE INFLUENCE OF LIGNIN ON THE ENZYMATIC HYDROLYSIS OF
PRETREATED BIOMASS SUBSTRATES**

by

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ABSTRACT

During the “enzymatic hydrolysis of the cellulose” component of the overall lignocelluloses-to-bioethanol process, lignin has been shown to be a very influential factor, acting as both a physical barrier and limiting hydrolysis through the adsorption of cellulases. Although hydrophobic, electrostatic and hydrogen bonding interactions between lignin and cellulases have been suggested to influence the hydrolysis efficiency, comparative studies using isolated lignins from different types of biomass which have been pretreatment in different ways have not been done. To gain a better understanding of the effects of lignin on enzymatic hydrolysis, six different substrates: steam and organosolv pretreated softwood (lodgepole pine), hardwood (poplar) and an agricultural residue (corn stover), were prepared. Lignin was isolated from the pretreated substrates by two methods. The lower lignin yields obtained with corn stover when compared to poplar and lodgepole pine suggested that the hydrophobicity of the corn stover derived lignin was lower than the lignin from poplar and lodgepole pine. The characterization of the physical and chemical properties of the isolated lignins showed that the carboxylic acid present in the isolated lignin had a significant influence on the enzymatic hydrolysis yields when lignin was added to pure cellulose. Dehydrogenative polymers (DHP) from ferulic acid adsorbed lower amounts of cellulases and did not decrease hydrolysis yields when compared to the DHP from coniferyl alcohol, showing that the increased carboxylic acid content of the lignin alleviated the non-productive binding of cellulases and increased the enzymatic hydrolysis of the cellulose. Douglas-fir was next steam pretreated at different severities and the lignin was isolated from the water insoluble fraction. The lower hydrolysis yields obtained with the substrates pretreated at 190°C when compared with those treated at 200 and 210°C was attributed to the lower accessible surface area of the substrate pretreated at

190°C rather than lignin-enzyme interactions. Isoelectric focusing analysis after incubation of cellulases with the lignin showed that the positively charged cellulases were preferentially adsorbed, indicating that electrostatic interaction was involved in cellulase adsorption onto the lignin. It was also apparent that the hydrophobicity of the lignin also played a role in the adsorption of cellulases.

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LIST OF ABBREVIATIONS

α	alpha
β	beta
γ	gamma
$^{\circ}\text{C}$	degree Celsius
AIL	acid insoluble lignin
ASL	acid soluble lignin
ATR	attenuated total reflection
AFEX	ammonia fibre explosion pretreatment
AFM	atomic force microscopy
Ara	arabinose
BSA	bovine serum albumin
CA	coniferyl alcohol
CBD	cellulose binding domain
CBH	cellobiohydrolase
CBM	cellulose binding module
CEL	cellulolytic enzyme lignin
CD	catalytic domain
CS	corn stover
cm	centimeter
DF	Douglas-fir
DL	dissolved lignin
DNA	deoxyribonucleic acid

DNS	dinitrosalicylic acid
DHP	dehydrogenative polymers
DP	degree of polymerization
DSC	differential scanning calorimetry
EC	Enzyme Commission
EG	endoglucanase
ERL	enzymatic residual lignin
E _T	empirical parameter
FA	ferulic acid
FPU	filter paper units
FT-IR	Fourier transformed infrared spectroscopy
g	gram
Gal	galactose
Glu	glucose
h (r)	hour(s)
H	histidine
HCl	hydrochloric acid
H ₂ O ₂	hydrogen peroxide
H ₂ SO ₄	sulphuric acid
HMF	5-hydroxymethylfurfural
IU	internal units
kDa	kilodalton
L	liter

LPP	lodgepole pine
m	meter
Man	Mannose
M	molar
mg	milligram
min	minute(s)
mL	milliliter
mm	millimeter
mM	millimolar
MW	molecular weight
MWL	milled wood lignin
nm	nanometer
NMR	nuclear magnetic resonance
OD	oven dry
OS	organosolv pretreatment
PAGE	polyacrylamide gel electrophoresis,
pI	isoelectric point
PCR	polymerase chain reaction
PEG	polyethylene glycol
PS	polystyrene
PTL	protease treated lignin
REL	residual enzyme lignin
rpm	revolutions per minute

SDS	sodium dodecyl sulfate
sec	second(s)
SO ₂	sulphur dioxide
SP	steam pretreatment
SPS	steam pretreated spruce
t	time
μL	microliter
μm	micrometer
μM	micromolar
UV	ultraviolet light
v/v	volume per volume
w/w	weight per weight
w/v	weight per volume
Xyl	xylose
Y	tyrosine

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1. INTRODUCTION

1.1 Bioethanol

The development of biorefineries that are capable of producing fuels and commodity chemicals from lignocellulosic biomass is viewed as a potential alternative to the world's current reliance on fossil fuels (Farrell et al. 2006; Himmel et al. 2007; Mabee and Saddler 2010). Our global dependency on fossil fuels has resulted in increased oil prices and emissions of carbon dioxide, which was at less than \$10 (US) a barrel about 10 years ago to a high of almost \$150 (US) a barrel three years ago to the current, approximately \$75-80 (US) per barrel range (August, 2010) (Administration 2010). Biofuels derived from plant sources are not generally regarded as sources of greenhouse-gas emissions under the Kyoto Protocol that went into effect in February 2005, because the amount of carbon dioxide emitted during their use is equivalent to that of absorbed by plants during photosynthesis (Lynd et al. 1991). Therefore, the dual forces of potentially higher fossil fuel prices and their contribution to CO₂ emissions have catalyzed many groups to try to develop cost-effective processes to produce biofuels from lignocellulosics (Chandra et al. 2007; Hoekman 2009). Governments have already recognized the importance of biofuels for transportation fuel. The U.S. Department of Energy has set goals to replace 30% of liquid petroleum-derived transportation fuel with biofuels and to replace 25% of industrial organic chemicals with biomass-derived chemicals by 2025 (Coalition 2005; Ragauskas et al. 2006). The European Union Directive 2003/30/EC ("the Biofuels Directive") adopted in 2003 targeted 2% of all petrol and diesel transport fuels to be biomass-derived by December 2005 and 5.75% by December 2010 (Ragauskas et al. 2006). In Japan, the government launched a national project in 2002 to make full use of biomass mainly for fuel (Kuzuhara 2005; Mabee 2007). To meet these targets, so called the

first generation biofuels (primarily bioethanol, and biodiesel) (Mabee 2007), have been produced from starch and sugar based crops as well as from oil producing plants such as Canola/rapeseed, sunflowers, etc. In 2008, 9 billion gallons, 6.5 billion gallons, 733.6 million gallons of fuel ethanol was produced in the United States, Brazil, and EU, respectively (Association 2010). However, there has been considerable debate about the food-vs-fuels dilemma in using these first generation crops and this has motivated the drive to develop so called second generation biofuels that would be based on lignocellulosic substrates such as agricultural (i.e. straw/stover) and forest (i.e. sawdust, logging waste) residues. However, various technical challenges have stalled the commercialization of biofuels produced from lignocellulosic feedstocks and virtually all of the suggested processes are still in the demonstration phase, requiring further R&D to improve their overall economics and increase the efficiency of the various process steps (Banerjee et al. 2010; IEA 2007). Over the last few years, one effect of utilizing starch and sugars for biofuels has been that the price of these commodity foods has generally increased (Runge and Senauer 2007). This has contributed significantly to the food-vs-fuel debate, which is still not resolved. Thus, the production of biofuels from lignocellulosic substrates such as agricultural and forestry residues is attractive, as this approach is not viewed as a threat to the safety of the food supply. This is one reason why the work described in the thesis is of interest.

1.2 Lignocelluloses for bioethanol

Lignocellulosic biomass for ethanol production includes materials such as agricultural residues (e.g. corn stover and wheat straw), forestry wastes, wastepaper, yard waste, and municipal solid waste (MSW), etc (Galbe and Zacchi 2002; Mabee and Saddler 2010;

Wyman 1996). In the long term, copious amounts of woody and herbaceous crops will be required to support large scale production of second generation biofuels (Galbe and Zacchi 2002; Mabee and Saddler 2010; Wyman 1996). Lignocellulosic substrates generally consist of cellulose, hemicelluloses, and lignin. The chemical composition and morphological characteristics vary depending on kinds of lignocellulosic substrates that are being studied (Baucher et al. 1998) . Cellulose and hemicelluloses, which compose about 65-75% of the overall lignocellulosic biomass composition, can be broken down to form their component sugars for fermentation into bioethanol (Galbe and Zacchi 2002; Himmel et al. 2007; Wyman 1996). Woody plants can be further categorized into hardwoods and softwoods. Softwoods such as lodgepole pine (*Pinus contorta*) and Douglas-fir (*Pseudotsuga menziesii*) are prevalent in North America. Lodgepole pine is one of the most prevalent and commercially valuable tree species in the interior of British Columbia (BC), Canada, covering a quarter of the forested land in BC (McGarritty and Hoberg, 2005). However, the current epidemic of mountain pine beetle (MPB, *Dendroctonus ponderosae*) which has grown over the last 5-10 years has infested more than half of the lodgepole pine forest in BC and it is anticipated that MPB will continue to spread (Woods et al. 2010). . Douglas-fir is a representative softwood species found in the Pacific coast and is valued for its excellent structural properties. Douglas-fir accounted for 60% of BC's lumber production in 1999 (Warren 2001). The Pacific coast region of the United States contains the largest standing net volume of softwood timber and is second in total softwood production when compared to all other regions of the United States (USDA Forest Service 2001). When looking for a representative hardwood species poplar (*Populus*) has been suggested to be of relevance for bioenergy as they are currently used commercially for both pulp and oriented strand board production with

significant residues available in the near term in regions such as Alberta. In the longer term, hybrid poplars are known to be fast growing and more productive, with a larger leaf area index and a longer leaf area duration than parental species under short-rotation forestry regimes (Karp and Shield 2008) showing potential to be grown as bioenergy crops (Sannigrahi et al. 2010).

To assess what would be a likely agricultural sourced feedstock, we looked at corn stover, (from *Zea mays*) which is produced at a rate of 1 dry kg per dry kg of corn grain (Kim and Dale 2004). It has been suggested that this is the likely primary biomass substrate for a potential US second generation biofuels industry as it has been reported that about 38.4 GL per year of bioethanol could be potentially be produced annually from this feedstock (Kim and Dale 2004).

1.2.1 Structure of lignocelluloses

Cellulose, hemicellulose, and lignin are the primary structural components of lignocellulosic materials, although there are considerable differences between grasses, hardwoods and softwoods in terms of their content, composition and structure (Baucher et al. 1998; Jørgensen et al. 2007). For example, maize (corn) stalk accounts for more than half of corn stover biomass, followed by leaves, cobs, and husk (Dhugga 2007). Rind in maize stalk accounts for more than 80% of stalk dry mass, which consist of vascular bundles and sclerenchymatous cells (Dhugga 2007). Wood has a more rigid and recalcitrant structure compared to grass, due to many factors such as the development of secondary xylem (Anderson and Akin 2008; Baucher et al. 1998). Softwood consists of two different cells; tracheids (90-95%) and ray cells (5-10%) (Sjostorm 1981). Hardwoods consist of libriform

cells, vessels, ray parenchyma cell, and fibre tracheids (Sjostrom 1981). The thickness of earlywood and latewood tracheids is generally 2-4 μm /and 4-8 μm . A more detailed description of each of the major lignocellulosic materials is discussed in the following sections.

1.2.2 Cellulose

Cellulose is the main constituent of lignocelluloses. In most wood species, cellulose makes up approximately 40-55% of the total dry substance (Sun and Cheng 2002). In corn stover, cellulose makes up approximately 36-42% of the dry substance (Ohgren et al. 2007; Wyman 1996). Cellulose is a homopolysaccharide composed of β -D-glucopyranose units which are linked together by (1 $\rightarrow$ 4)-glycosidic bonds (Figure 1). Cellulose molecules are typically linear and have a strong tendency to form intra and intermolecular hydrogen bonds. Bundles of cellulose molecules are thus aggregated together in the form of microfibrils, in which highly ordered (crystalline) regions alternate with less ordered (amorphous) regions (Nishiyama 2009).

Figure 1. Structure of cellulose.

1.2.3 Hemicelluloses

In wood, hemicelluloses are typically heterogeneous polysaccharides consisting of D-glucose, D-mannose, D-galactose, D-xylose, L-arabinose, and small amounts of L-rhamnose in addition to D-glucuronic acid, 4-O-methyl-D-glucuronic acid, and D-galacturonic acid. Most hemicelluloses have a degree of polymerization 50-300 (Pu et al. 2008). The amount of hemicellulose in wood and corn stover is usually in the range of between 20 and 30% (Ohgren et al. 2007; Wyman 1996).

1.2.4 Lignin

Lignin is generally composed of polymers of phenylpropane units, guaiacyl (G, coniferyl alcohol), syringyl (S, sinapyl alcohol), and *p*-Hydroxyphenyl units (H, *p*-coumaryl alcohol). The monolignols have been shown to be biosynthesized from glucose via phenylalanine (Humphreys and Chapple 2002). The composition of the lignin is known to vary depending on the source and type of lignocellulosic material that is being studied. For example, for Scots Pine (*Pinus sylvestris*), softwood, the H and G contents of the lignin are 2% and 98%, respectively (Baucher et al. 1998), for Poplar (*Populus trichocarpa*), hardwood, the G and S contents of the lignins are 41% and 59%, respectively (Baucher et al. 1998) and for corn stalks, the H, G, and S contents of the lignin are 4%, 35%, and 61%, respectively. In addition, the cell walls in corn stover contain up to 4% ferulate and up to 3% *p*-coumarate (Saulnier et al. 1999). The lignin content also varies depending on the source of lignocellulosic material. Softwoods generally contain about 25-35% lignin, hardwoods contain 18-25% lignin, while corn stover generally has a lower lignin content (16-22%) when compared with wood (Ohgren et al. 2007; Sun and Cheng 2002; Wyman 1996). The types of

the bonds in inter-unit linkages found in various lignins are shown in Table 1, which vary depending on the source of the lignocellulosic material.

Figure 2. Lignin precursors (Adler 1977).

Table 1. Types of linkages and dimeric structures (Adler 1977)

Linkage type	Dimer structure	Percent of the total linkages	
		Softwood	Hardwood
β -O-4	Arylglycerol- β -aryl ether	50	60
α -O-4	Noncyclic benzyl aryl ether	2-8	7
β -5	Phenylcoumaran	9-12	6
5-5	Biphenyl	10-11	5
4-O-5	Diaryl ether	4	7
β -1	1,2-Diaryl propane	7	7
β - β	Linked through side chain	2	3

Figure 3. Prominent inter-unit linkages found in softwood (SW) and hardwood (HW) (Adler 1977).

Figure 4. The structure of softwood lignin(Adler 1977).

1.3 Pretreatment

As mentioned above, lignocellulosic materials consist mainly of cellulose, hemicellulose, and lignin. The content and structure of each component generally differ between softwoods, hardwoods, and agricultural residues. In the proposed process for bioconversion of lignocellulosics to ethanol, after pretreatment, the cellulose and hemicellulose components are enzymatically hydrolyzed to monomeric sugars and then fermented to ethanol, while the lignin can possibly be burned for energy recovery or used as potential substrate for subsequent chemical production (Chandra et al. 2007; Himmel et al. 2007). In general, the bioconversion of lignocellulosics consists of three process steps: pretreatment, enzymatic saccharification, and fermentation. Techno-economic analysis of the entire bioconversion process from wood estimated that the front-end sub processes (pretreatment, fractionation, and enzymatic hydrolysis) account for a considerable portion (approximately 60%) of the total process cost (Gregg et al. 1998; Nguyen and Saddler 1991). The recalcitrant structure of cellulose and its close association with the lignin and hemicellulose matrix that constitutes a typical lignocellulosic substrate makes it highly resistant to enzymatic hydrolysis (Pan et al. 2005c). Therefore, lignocellulosic substrates must be pretreated to improve enzymatic hydrolysis, while at the same time ensuring maximum recovery of the original material so as to maximize value from the feedstock (Chandra et al. 2007; Kumar et al. 2009a). Pretreatment processes are typically categorized into three types: physical, chemical, or biological pretreatments. One of these pretreatment processes or a combination of the processes is used for the pretreatment step (Chandra et al. 2007; Kumar et al. 2009a). As biological pretreatments have been shown to be time-consuming processes, current pretreatments mainly follow a physical, or chemical

approach, or a combination of the two. Usually, there has to be some form of physical size reduction, such as occurs in making chips prior to pulping. The lignocellulosic substrate is then processed to try to separate the cellulose, hemicellulose and lignin components and to increase the cellulosic components susceptibility to enzymatic hydrolysis (Chandra et al. 2007; Kumar et al. 2009a). Although the ideal pretreatment method would involve complete separation and isolation of each component from the lignocellulosic substrate, this type of separation has proven to be impractical for both technical and economic reasons. Therefore, pretreated lignocellulosic substrates usually contain some amount of hemicellulose and, particularly, lignin associated with the cellulosic rich, water- or solvent-insoluble stream obtained after pretreatment. The amount of each component remaining associated with the cellulosic fraction varies, depending on the kind of pretreatment and the severity of the pretreatment (Ohgren et al. 2007; Shevchenko et al. 2001).

Typically, the effectiveness of enzymatic hydrolysis of cellulose is evaluated by both the hydrolysis rate and glucose yield based on the initial cellulose content prior to pretreatment of the lignocellulosic substrate. Hydrolysis of cellulose is generally characterized by an initial logarithmic phase, associated with the rapid release of soluble sugars, followed by a declining rate of sugar production as the reaction proceeds (Mansfield et al. 1999). In order to increase the efficiency of enzymatic hydrolysis, several pretreatment methods have been developed such as steam pretreatment (Boussaid et al. 2000a), organosolv pretreatment (Neilson et al. 1983; Pan et al. 2005c), ammonia fibre expansion/explosion (AFEX) (Wyman et al. 2005b), lime pretreatment (Chang et al. 2001; Kaar and Holtzapple 2000), and dilute acid pretreatment (Saha et al. 2005). Each of the pretreatment processes has drawbacks and advantages (Chandra et al. 2007). The content of lignin in the resulting,

cellulosic rich pulps varies depending on the pretreatment method used (Sanchez and Cardona 2008). For example, earlier comparative pretreatment studies showed that only small amounts of lignin in corn stover could be removed by dilute acid, whereas substantial amounts of lignin were removed by lime pretreatment (Wyman et al. 2005a). In other cases, the composition of the materials after AFEX pretreatment was essentially the same as that of the original biomass (Wyman et al. 2005a), while the lignin content of steam pretreated substrates increased when compared to the initial substrates, mainly because of the solubilization and removal of the hemicellulose during pretreatment (Shevchenko et al. 2001). Earlier work showed that organosolv pretreatment decreased the lignin content in pretreated pulps due to the solubilization of the lignin into the organic solvent (Pan et al. 2006; Sarkanen et al. 1981). In our group, steam and organosolv pretreatments have been the most frequently studied processes and were the pretreatments used for the work reported in this thesis.

1.3.1 Steam pretreatment

The advantages of steam pretreatment include favorable economics, low water usage, and energy savings (Duff and Murray 1996). The steam pretreatment process is an efficient process for the treatment of hardwoods and agricultural residues. However, due to their recalcitrant nature, softwoods generally require the addition of a catalyst such as sulfur dioxide (SO_2) or sulfuric acid (H_2SO_4) to be fully effective (Boussaid et al. 2000b; Mais et al. 2002; Robinson et al. 2002; Shevchenko et al. 2001). After steam pretreatment, the lignin content of the pretreated substrate increases, when compared with the initial biomass, mainly because of the solubilization of the hemicellulose into the water soluble stream (Shevchenko et al. 2001). During the process, the substrate is treated with high temperature and pressure

followed by a sudden release to separate the individual fibres within the woody substrate, although the rapid release/explosion has been shown to not be necessary for effective steam pretreatment (Brownell and Saddler 1987). At high pretreatment severities, hemicellulose can be degraded to furfural and 5-hydroxymethyl furfural (HMF) that inhibit subsequent fermentation, whereas lignin tends to remain in the substrate (Ewanick et al. 2007; Kabel et al. 2007; Shevchenko et al. 2001). Thus, as the severity of the pretreatment is elevated, the resulting proportion of lignin in the substrate will increase. At low severity pretreatment conditions, the residual hemicellulose and lignin tend to hinder enzyme access to cellulose when compared to substrates treated at high severities (Ewanick et al. 2007). After SO₂-catalyzed steam pretreatment, the lignin tends to be lower in β -5 and β -O-4 aryl ethers linkages (Hemmingson 1986; Hemmingson and Newman 1985) and it has been shown that the addition of SO₂ causes acid-catalyzed condensation reactions (Robert et al. 1988; Shevchenko et al. 2001). Pretreatment using SO₂-catalyzed steam explosion has been applied to a number of softwood species with varying degrees of success. In particular, Douglas-fir has been the subject of a great deal of past steam pretreatment work (Boussaid et al. 2000a; Saddler et al. 1982; Saddler et al. 1983) in our research group. However, even with optimized steam pretreatment conditions, delignification after pretreatment (post-treatment) was necessary in order to obtain sufficient hydrolytic conversion and subsequent fermentation of the glucose to ethanol. Oxygen-alkali and hydrogen peroxide post-treatments that removed relatively small amounts of lignin were found to dramatically improve hydrolysis yields (Pan et al. 2004a; Yang et al. 2002). Steam pretreated Douglas-fir substrates followed by a cold NaOH treatment resulted in a 30% improvement in hydrolysis yields (Pan et al. 2005b). Thus, steam pretreatment, without a subsequent post-treatment step, has generally been shown to be

more successful when applied to corn stover and poplar rather than softwoods.

1.3.2 Organosolv pretreatment

Organosolv pretreatment has been shown to be a promising pretreatment method in which lignin is removed with an organic solvent such as methanol, ethanol, acetone, or ethylene glycol with or without acidic catalysts or alkaline chemicals (Neilson et al. 1983; Pan et al. 2005c). The advantages of organosolv pretreatment include the potential for obtaining valuable lignin-based co-products and more importantly, the production of substrates readily hydrolyzed by cellulases (Neilson et al. 1983; Pan et al. 2005c). The organosolv pretreatment decreases the lignin content in the substrate by breaking bonds such as α -aryl ether and arylglycerol- β -aryl ether (β -O-4) in the lignin macromolecule (Sarkanen et al. 1981), imparting significant changes in the lignin structure, including increases in phenolic and methoxyl groups, and decreasing the average molecular weight of the lignin (Gilarranz et al. 2000). Compared with steam pretreatment, organosolv lignin from a mixed hardwood exhibited significantly lower amounts of β -O-4 structures (Argyropoulos 1994; Soderstrom et al. 2002) which indicated that a greater degree of delignification occurred during the organosolv pretreatment when compared to steam pretreatment. As the hemicellulose is extensively removed by the organosolv pretreatment (Pan et al. 2005a), it produces a substrate that is highly susceptible to subsequent enzymatic hydrolysis (Neilson et al. 1983; Pan et al. 2005a). However, even after the organosolv pretreatment, the residual lignin associated with the cellulose has been shown to adsorb cellulases, reducing our ability to recover cellulases in the supernatant in enzyme recycle strategies (Tu et al. 2007a).

1.4 Enzymatic hydrolysis of lignocelluloses

The enzymatic hydrolysis of cellulose is known to be affected by both enzyme- and substrate-related factors (Chandra et al. 2007; Mansfield et al. 1999). Therefore, efforts to reduce the cost of enzymatic hydrolysis have focused on both enzyme- and substrate-related factors (Mansfield et al. 1999; Sheehan and Himmel 1999; Zhang et al. 2006). Great progress has been made in projects carried out by enzyme companies such as Novozymes and Genencor and groups such as US's National Renewable Energy Laboratory (NREL) in reducing the cost of enzymatic hydrolysis (Aden and Foust 2009; Merino and Cherry 2007; Novozymes 2005). However, greater improvements are still required and more recent research has been funded by the U.S. Dept. of Energy to further decrease the cost of the enzymes, increase their specific activities and broaden the substrate range on which the enzymes are being evaluated. It has been reported that an approximate further 3-fold enzyme cost reduction (from 0.32 to 0.10 \$/gal Ethanol) is necessary to reach cost targets for the eventual commercialization of the bioconversion of pretreated corn stover to ethanol (Aden 2008). More recent cost estimates have been difficult to obtain because the cost is determined between suppliers and purchasers and no commercial scale plants were operated (Aden and Foust 2009).

In addition to enzyme-related factors, substrate-related factors also affect enzymatic hydrolysis. It has been reported that the efficient enzymatic hydrolysis of cellulose is determined by several factors such as the specific surface area, pore size, crystallinity, and degree of polymerization of the cellulose (Chandra et al. 2007; Grethlein 1985; Grous et al. 1986; Mooney et al. 1999; Shevchenko et al. 2000). Although early work using relatively pure cellulosic substrates showed that there was some correlation between crystallinity and

the rate of hydrolysis (Fan et al. 1980), subsequent work with lignocellulosic substrates showed that this was a simplified interpretation, when using more heterogeneous substrates, such as pretreated biomass, the contribution of other substrate characteristics and components, such as lignin, were found to be just as important (Chandra et al. 2007). Several researchers have found that when all other substrate factors are maintained at a similar level, changes in the crystallinity of lignocellulosic substrates do not have a significant effect on the rate or extent of hydrolysis (Puri 1984; Ramos et al. 1993).

Earlier work has shown that the rate and extent of enzymatic hydrolysis is directly related to the surface area of the substrates (Mooney et al. 1999). When pulp substrates with equal amounts of lignin (present in Douglas-fir kraft and mechanical pulps) were hydrolyzed, it was found that the fines (smaller particles) of the delignified mechanical pulps were hydrolyzed considerably faster than were the longer fibres of the kraft pulps (Mooney et al. 1999). Direct correlations have been found between the initial pore volume or interior surface area of lignocellulosic substrates and the extent of their hydrolysis (Grethlein 1985; Grous et al. 1986; Shevchenko et al. 2000). It has been proposed that the efficacy of cellulose hydrolysis is enhanced when pores of the substrates are large enough to accommodate both large and small enzyme components to maintain the synergistic action of the cellulase enzyme system (Tanaka et al. 1988). The rate-limiting pore size for the hydrolysis of lignocellulosic substrates was reported to be 5.1 nm (Grethlein 1985).

In addition to the physical properties of substrates mentioned above, it is known that the structure and location of hemicellulose and lignin can affect the hydrolysis efficiency (Chandra et al. 2007). As hemicellulose can cover the surface of cellulose and prevent the access of cellulases to the cellulose, the removal of hemicellulose may be essential to

facilitate complete cellulose hydrolysis. However, of the three main components of lignocellulose, hemicelluloses have been shown to be the most sensitive to changes in pretreatment conditions (Bura et al. 2002). Therefore, compared to the removal of lignin, the removal of hemicelluloses during pretreatment has not been a major issue. Instead, pretreatment studies have focused on minimizing the degradation of hemicellulose to products such as furfural and hydroxymethyl furfural so that the sugars can be obtained in a “useable” form. This can be achieved by using less severe steam pretreatment conditions which, through mild acid hydrolysis (sometimes termed autohydrolysis in uncatalysed conditions) solublizes the hemicellulose resulting in about 80-85% recovery of the original hemicellulose sugars. However, these less severe pretreatment conditions mean that the lignin associated with the cellulose in the water insoluble fraction is more problematic.

Lignin has been implicated in decreasing the efficiency of enzymatic hydrolysis either by physically decreasing accessibility to or by the adsorption of cellulases (Chandra et al. 2007). As mentioned earlier, the content and structure of lignin differs according to the type of lignocellulose. Although the removal of most of the lignin component in lignocellulose is technically possible by current pulping processes, these processes are currently not economically feasible for application in bioconversion of lignocellulose. For example, as the current price of kraft pulp is approximately US \$990 per ton (northern bleached softwood kraft pulp according to the PIX Pulp Benchmark Index, Sep. 2010), and the theoretical ethanol yield from glucose is 51.14%, it is not economically feasible to use kraft pulp as the substrate for the ethanol production. The benchmark for achieving production of ethanol from cellulosic feedstocks that would be “cost competitive with corn-ethanol” has been quantified as \$1.33 per gallon (Aden 2008).

Thus, it is highly likely that no matter which type lignocellulosic substrate, pretreatment process, or fractionation methods are used in the bioconversion of biomass, the resulting cellulosic substrate will always contain some amount of lignin with varying structures.

1.5 Effect of lignin on enzymatic hydrolysis and cellulases recovery

In the saccharification process, pretreated substrates are hydrolyzed to glucose by cellulases and β -glucosidase. Although current pretreatment methods such as steam pretreatment and organosolv pretreatment are promising, further studies are required because cellulases are unable to hydrolyze pretreated lignocellulose efficiently (Chandra et al. 2007; Himmel et al. 2007). One of the major obstacles to efficient hydrolysis is the fact that cellulases work in a heterogeneous system where the enzymes are soluble and must hydrolyze an insoluble substrate. Thus, additional difficulties are presented to the enzymes when they encounter ancillary components of the substrate such as lignin which may restrict their access to cellulose. Two possible mechanisms by which the presence of lignin decreases the yield of cellulose hydrolysis are a “physical”, i.e. steric hindrance of the cellulose (Mooney et al. 1998) and the adsorption (either reversible or irreversible) of cellulases on lignin rather than cellulose (Berlin et al. 2006; Palonen et al. 2004; Sewalt et al. 1997).

Cellulases have been proposed to adsorb to lignin via hydrophobic interactions (Eriksson et al. 2002; Ooshima et al. 1986), ionic interactions (Berlin et al. 2006), and hydrogen bonding interactions (Berlin et al. 2006; Pan 2008; Sewalt et al. 1997). However, the exact mechanism by which cellulases interact with lignin and result in the reduction in hydrolysis effectiveness have yet to be fully resolved. An elucidation of the effects that lignin

has on enzymatic hydrolysis will not only help in our understanding of the more fundamental, mechanistic enzyme-substrate interactions, it should also contribute to the development of efficient pretreatment methods and improve the action of the cellulase enzymes. A main goal of the work described in this thesis is to further elucidate the factors that influence the interaction between cellulases and lignin during hydrolysis of a range of representative pretreated lignocellulosic substrates.

1.6 Interaction between proteins and solid surfaces

In order to gain a general sense of how cellulases may potentially interact with lignin, it would be useful to examine some of the general theories presented to explain protein interactions with solid surfaces. The adsorption of protein is reported to be affected by the properties of both the protein and the solid surface (Norde 1986). Protein adsorption is a complex process that depends on a number of parameters, such as electrostatic attraction, hydrogen bonding and the hydrophobic/hydrophilic characteristics of both the protein and the surface on which it is adsorbed (Haynes and Norde 1994). Although the primary force of protein adsorption seems to be hydrophobic interactions (Schmaier et al. 1984), electrostatic contributions also play an important role, particularly for more hydrophilic surfaces (Norde 1986).

Of the protein properties that can potentially affect adsorption, molecular weight, hydrophobicity, electric charge, and stability are reported to be the most important (Norde 1986). Like all proteins, the components of the cellulase enzyme system each vary in characteristics such as their molecular weight, hydrophobicity, electric charge and stability, all of which will consequently affect their adsorption to the lignin component of pretreated

substrates.

1.7 Properties of cellulases

Cellulase is a generic name of the enzyme produced by a wide-range of bacteria and fungi that hydrolyze cellulose to glucose. Cellobiohydrolases (CBH; EC 3.2.1.91), CBH I and CBH II, hydrolyze the cellulose chain from the reducing and non-reducing ends respectively and produce cellobiose. The endoglucanases (EG; EC 3.2.1.4) hydrolyze the internal glucosidic bonds within the cellulose. Fungi such as *Trichoderma reesei* are known to produce five kinds of EG (EG I-V). The third major enzyme group within what typically constitutes a “cellulase complex” is the β -glucosidases (EC 3.2.1.21), which typically hydrolyze cellooligosaccharides and cellobiose to glucose (Lynd et al. 2002).

T. reesei has a long history of safe use for industrial enzyme production and is currently used as an important model and commercial system for assessing lignocellulose degradation (Aimi et al. 2005; Zhang et al. 2006). The properties of cellulases from *T. reesei* are summarized in Table 2. The molecular weight of the cellulases varies from 23 to 75 kDa and the pI of most cellulases ranges from 3.5 to 7.8. In the case of the *T. reesei* cellulase complex, CBHI and CBHII are the major cellulase components, comprising 60% and 20% respectively of the total protein content (Goyal et al. 1991). Most cellulases from *T. reesei* (except EGIII which is < 3% total protein) consist of two domains, a catalytic domain (CD) and cellulose binding domain (CBD), or cellulose binding module (CBM) (Palonen et al. 2004). The CD works to hydrolyze cellulose into oligosaccharides or glucose, while the CBD or CBM is thought to increase the adsorption of cellulases onto the surface of cellulose (Tu et al. 2007b). The mechanism of cellulases adsorption on pure cellulose has been widely studied and it is

reported that the adsorption involves both hydrophobic and hydrogen bonding interactions (Baker et al. 2001; Reinikainen et al. 1995). Structure-based site directed mutagenesis studies have indicated that conserved aromatic amino acids are essential for the function of fungal CBD's (Reinikainen et al. 1992). It has also been shown that the CD is involved in adsorption of the enzyme onto lignin (Berlin et al. 2005). In addition to the CD, the CBD or the CBM is reported to also play a role in increasing the adsorption of the enzymes onto the lignin (Borjesson et al. 2007a; Palonen et al. 2004) . The papers cited above describe most of the work that has been carried out on this topic so far and indicate the limited extent of our knowledge with regard to cellulase interactions with solid surfaces. Therefore, subsequent discussion will focus on some of the other protein properties which have been shown to affect protein adsorption onto solid surfaces.

Table 2. The properties of cellulases from *T. reesei*.

Enzyme	Number of total amino acids	Amino acids core	Molecular weight (kDa)	Isoelectric Point (pI)	Position of CBM	Molar extinction coefficients (M ⁻¹)
CBHI (Cel7A)	497	430	59-68	3.5-4.2	C	78,800
CBHI core	-	-	-	<3.0		
CBHII(Cel6A)	447	367	50-58	5.1-6.3	N	92,000
EGI (Cel7B)	437	368	50-55	3.9-6.0	C	67,000
EGII (Cel5A)	397	327	48	4.2-5.5	N	78,000
EGIII (Cel12A)	218	218	25	6.8-7.5	-	38,200
EGV (Cel45A)	225	166	23	-	C	-
β-GI	713	713	75	7.4-7.8	-	-

Actual data was adapted from (Hui et al. 2001; Medve et al. 1997; Valjamae et al. 2001)

1.8 Effect of molecular weight of protein on adsorption

The affinity of proteins for synthetic polymers has been shown to increase with the increasing molecular mass of the protein (Zsom 1986). When Zsom measured the protein adsorption of several bovine serum albumin (BSA) samples containing different amounts of dimeric and/or polymeric BSA on polystyrene (PS) lattices, he found that the BSA dimer and/or polymers preferentially adsorbed compared with the BSA monomers. As the

molecular weights of cellulase components range from 23-75 kDa (Table 1), it is possible that the cellulases adsorb on lignocellulose differently because of their different molecular weights.

1.9 Effect of protein stability on adsorption

In addition to molecular weight, protein stability has also been shown to affect the tendency for proteins to adsorb to solid surfaces (Arai and Norde 1990). They examined the adsorption of proteins with similar molecular size and shape on various well-defined surfaces using lysozyme (LSZ), myoglobin (MGB), ribonuclease A (RNase), and α -lactalbumin (α -LA) as their protein controls. They found that the LSZ and MGB proteins, whose Gibbs energy of heat denaturation were high (-4.1 , -3.2 Jg^{-1} , respectively), resulted in the proteins behaving like hard particles. The LSZ and MGB proteins adsorbed onto hydrophobic interfaces under all conditions of charge interaction but only onto hydrophilic surfaces if electrostatically attracted. The RNase and α -LA, which are characterized by a lower structural stability (-2.8 , -1.5 Jg^{-1} , respectively) adsorbed onto hydrophobic and hydrophilic surfaces under both attractive and repulsive electrostatic conditions. Thus, it is possible that the cellulases adsorb on lignocellulose differently because of differences in protein stability.

To try to quantify the adsorption of proteins onto surfaces and the effect on their structural organization, various techniques have been evaluated. Differential scanning calorimetry (DSC) has been shown to be a powerful and very general method of studying the structural organization of proteins as it relates to their thermostability. The LSZ and MGB proteins, which have higher structural stability, were shown to have a higher T_m (74.2°C and 86°C , respectively) (Brandes et al. 2006; Hagerdal and Martens 1976), while the RNase and

α -LA, which have lower structural stability, have a lower T_m . For the RNase, the T_m was reported to be 52 °C (Karp and Shield 2008; Tsong et al. 1972) while for the α -LA it was shown to be 39.6 ± 0.2 °C (Boye et al. 1997). The cellulases seemed to have moderate thermostability except for EGII. The T_m of CBHI (Cel 7A) was 62 ± 2 °C (Baker et al. 1992; Wyman 1996) and that of CBHII (Cel6A) at pH 5 was about 65 °C (Baucher et al. 1998). When a more comprehensive examination of the thermostability of cellulases was carried out, the T_m of CBHI, CBHII, EGI, and EGII were found to be 64.4, 64.1, 64.6, and 75.1 °C, respectively (Baker et al. 1992). When the thermostability of the enzymes was assessed in the presence of cellobiose, it increased the T_m of CBHI to 65.7 °C (cellobiose, 1 mM) and 72.0 °C (cellobiose, 100 mM) respectively, indicating the positive effect that the presence of the product had on the thermostability of the cellulases.

Thus, it is possible that cellulases (except for EGII, which has a higher thermostability), behave like both higher and lower structural proteins in the absence of substrates, but alternatively behave like higher structural proteins in the presence of their substrates.

1.10 Hydrophobic interactions

Although protein stability may be an important factor, hydrophobic interactions seem to be the primary driving force that governs protein adsorption (Schmaier et al. 1984). As the hydrophobicity of both the protein and solid surface increase, there is a greater tendency for adsorption to occur. When protein molecules are dissolved in water, they tend to minimize the exposure of their hydrophobic groups to the aqueous environment (Norde 1986). However, the protein exterior is often partly hydrophobic. Dehydration of hydrophobic portions of the protein and the sorbent surface is driven by entropy gain and, therefore,

promotes adsorption to occur spontaneously (Norde 1986). As a rule, the amount of protein adsorbed is larger at hydrophobic surfaces. Azioune et al. (2002) measured the hydrophobic interaction between human serum albumin and polypyrrole powders doped with chloride (PPyCl), dodecyl sulfonate (PPyDS), and tosylate (PPyTS) to give varying hydrophobicities to the proteins (Azioune et al. 2002). They used van Oss-Good-Chaudhury (VOGC) theory to determine the dispersive, acidic, and basic components of the surface free energy (γ_s^d , γ_s^+ , and γ_s^- , respectively). The three surface free energy components were subsequently used to assess the absolute hydrophobicity of the substrates (ΔG_{1w1}) and the free energy of protein-PPy in water (ΔG_{1w2}), which is used to assess the extent of hydrophobic interaction forces. In the case of the PPyTS and PPyDS, the acid-base force contribution was much more important than was the van der Waals influence. In contrast, for the HSA-PPyCl system, the van der Waals forces predominantly contributed to the ΔG_{1w2} .

1.10.1 Hydrophobicity of cellulases

As the adsorption of protein onto substrates is generally enhanced by increasing the hydrophobicity of the protein (Hansen et al. 1988), it is reasonable to expect that cellulases with different hydrophobicities adsorb differently onto various lignocellulosic substrates. The hydrophobicity of cellulases can be calculated depending on their amino acid sequence (Yemshanov and McKenney 2008). The specific amino acids present within some CBM's are reported to promote the adsorption of cellulases on cellulose as well as onto lignin (Palonen et al. 2004). When Park et al. (2002) modified cellulases with copolymers containing polyoxyalkylene and maleic anhydride (Park et al. 2002), they showed that, as the hydrophilicity of modified cellulases increased, the amounts of free modified enzyme in the

supernatant also increased.

1.10.2 Hydrophobicity of the lignocellulosic substrate

In addition to the hydrophobicity of cellulases, the hydrophobicity of the “solid surface” i.e. the lignocellulosic substrate itself, will also affect the tendency of the enzymes to adsorb to lignin or cellulose. Consequently, the presence of lignin in the lignocellulose tends to increase the substrates hydrophobicity. When Hodgson and Berg (1988) measured the contact angle of various wood fibres (Hodgson and Berg 1988) they used the Wilhelmy technique (Collins 1947) to measure the contact angle. They showed that the contact angle of α -cellulose was 14.0° , while that of thermomechanical pulps was 42.8° and 51.2° , indicating that the hydrophobicity of lignin is higher than that of cellulose. When Maximova (2004) examined the effect of the wetting properties of cellulose fibres and mica (Maximova et al. 2004), which was a smooth, non-porous, and hydrophilic surface, they found that the adsorption of lignin onto cellulose fibres and mica increased the contact angle, indicating that the lignin has a higher hydrophobicity than does the cellulose. Hydrophobicity of lignin and cellulose was also measured using a thin film prepared by a spin coater (Norgren et al. 2007; Norgren et al. 2006; Notley and Norgren 2010). As a result, the contact angle of softwood kraft lignin, softwood MWL, and hardwood MWL between water was 46, 52.5, 55.5° respectively, while the contact angle of cellulose was from about 20 to 30° (Eriksson et al. 2007; Gunnars et al. 2002). Thus, pretreated pulps that have a high lignin content may be more hydrophobic when compared to cellulose in the absence of lignin.

1.10.3 Hydrophobic interactions between cellulases and lignocellulosic substrates

As mentioned earlier, hydrophobic interactions are an important factor that influences protein adsorption. The fact that the presence of lignin increases the non-productive adsorption of cellulases and that the hydrophobicity of lignin is higher than that of cellulose suggests that hydrophobic interactions are one of the main factors governing the adsorption of cellulases to lignin. There are some reports that mention the relationship between the cellulase hydrophobicity and non-productive binding of cellulases on lignin. Previously, Palonen et al. (2004) prepared three kinds of lignin, alkali-lignin, cellulolytic enzyme lignin (CEL), and acid-lignin from steam pretreated spruce (SPS). They then compared the adsorption properties of the intact enzymes and the CD from the CBHI and EGII cellulases to the SPS and isolated lignins (Palonen et al. 2004). They showed that the amount of CD adsorbed onto the substrates was lower than was observed for the intact enzymes, likely because of the higher hydrophobicity of the CBM component of the complete enzyme. As they noted that the adsorbed amounts of EGII on the lignins were higher than those of CBHI, they suggested that this difference was a result of the more open nature of the active site of EGII, which lead to greater adsorption through hydrophobic interactions. Borjesson (2007) used two cellulase component enzymes, Cel7A (CBHI) and Cel7B (EGI), to compare the interaction between cellulases and lignin (Borjesson et al. 2007a). When the full-length enzymes were used, about 50% of the Cel7A and about 65% of the Cel7B were adsorbed onto the isolated lignin from spruce sawdust, while about only 15% of the CDs were adsorbed onto the isolated lignin. This indicated that the CBMs contributed to the proteins adsorption onto the lignin. It was suggested that the difference in hydrophobicity between the CBM of Cel7A and Cel7B caused their different adsorption behavior on the lignin preparations. The CBM of

Cel7A has four aromatic residues, all of which are tyrosine, and three of these tyrosines form the flat surface of the CBM giving the enzyme its affinity for crystalline cellulose. The CBM of Cel7B has five aromatic residues, four of which are tyrosine and one is tryptophan. Borjesson et al. (2007) suggested that the presence of a tryptophan in the CBM of Cel7B instead of a tyrosine on the flat surface increases the hydrophobicity of this part of the protein, based on the hydrophobicity scale of amino acid side chains that has been proposed by Roseman (Roseman 1988). The difference in the hydrophobicity of the tyrosine is caused by the phenolic hydroxyl group in its side chain, while tryptophan has an indole ring.

In previous work, Berlin et al. (2006) isolated two types of organosolv lignin from Douglas-fir, with one fraction defined as dissolved lignin (DL), prepared from organosolv liquor, while another was defined as enzymatic residual lignin (ERL), prepared from the enzymatic hydrolysis of the organosolv pulp (Berlin et al. 2006). These workers compared the functional groups in the lignin preparations and correlated the amount of the reactive groups within the lignin with their inhibitory effects on cellulose hydrolysis. The low amounts of carboxyl and aliphatic hydroxyl groups found in the DL compared to the ERL lignin was presented as evidence for the higher hydrophobicity of the DL lignin which inhibited hydrolysis to a greater degree than did the ERL lignin (Table 3). However, this result still needs to be verified since the DL was dissolved in 50% (w/w) ethanol, while the ERL was not dissolved in the ethanol solvent. The empirical parameter, $E_T(30)$ of ethanol/water (80:20), which is similar to the chemical composition of the organosolv washing solvent, is 53.7 kcal/mol, while the $E_T(30)$ of dioxane, which is used for dissolving lignin, is 36.0 kcal/mol (Reichardt 1979). This means that the hydrophobicity of dioxane is higher than that of the ethanol/water (80:20) mixture. Although these workers did not

measure the hydrophobicity of the isolated lignins, it is possible that the hydrophobicity of the DL was lower than that of the ERL.

Table 3. Comparison of inhibitory effects on cellulases and amounts of reactive groups in isolated lignins from organsolv pretreated Douglas-fir.

	Dissolved	Enzymatic residual
	Lignin	lignin
Inhibitory effects on cellulases	>	
CO	>	
COOH	<	
Total OH	<	
Phenolic OH	>	
Aliphatic OH	<	

Actual data were adapted from Berlin et al (2006)

Other related work has reported that the addition of additives such as Bovine Serum Albumin (BSA), or polyethylene glycol to the reaction mixture can decrease the non-productive binding of cellulases onto lignin (Borjesson et al. 2007a; Borjesson et al. 2007b; Eriksson et al. 2002). Palonen et al. (2004) reported that adding an excess amount of BSA to the cellulose mixture that included CBHI or EGII, resulted in a decrease in the amount of cellulases that bound to the SPS and the CEL-lignin (Palonen et al. 2004). Other workers have shown that cellulases adsorbed to both cellulose and lignin, while BSA adsorbed only onto the lignin (Yang and Wyman 2006). The reasons for the difference in adsorption properties between the BSA and cellulases are not well understood, although Eriksson et al. (2002) suggested that the BSA has hydrophobic sites, which will bind with fatty acids and will readily adsorb onto hydrophobic surfaces (Eriksson et al. 2002).

The addition of surfactants into the reaction mixture has been shown to increase the saccharification of lignocellulosic materials and to decrease the non-productive binding of cellulases to lignin (Eriksson et al. 2002). These workers used steam-pretreated spruce substrates and compared the action of several non-ionic, anionic, and cationic surfactants for their potential to increase enzymatic hydrolysis (Eriksson et al. 2002). The addition of non-ionic or anionic surfactant (sodium dodecyl sulfate; SDS) to the reaction mixture reduced the adsorption amounts of Cel7A onto the SPS. In the related work, Borjesson et al. (2007b) showed that non-ionic surfactants and polymers containing poly(ethylene oxide) effectively increased the enzymatic hydrolysis of lignocellulosic substrates (Borjesson et al. 2007b). The adsorption of polyethylene glycol (PEG) onto the SPS was thought to be through hydrophobic interaction (the dominant action) as well as through hydrogen bond interactions between the PEG and the lignin component in the lignocellulose. The effect of surfactants on

hydrolysis was only evident when lignin was present in the substrate (Borjesson et al. 2007b; Eriksson et al. 2002).

It is apparent that hydrophobic interactions between cellulases and lignin play an important role in the non-productive binding of cellulases, which consequently contribute to the decrease in efficiency of lignocellulosic hydrolysis. However, comparative analysis using several types of lignins from different types of biomass (agricultural, hardwood and softwood biomass) and proteins varying in their hydrophobicity has not yet been carried out. Thus, the work in this thesis describes a comprehensive examination of lignin-cellulase interactions so that we can better understand the role of possible hydrophobic interaction between cellulases and lignins.

1.11 Electrostatic interactions

Compared to the effect of potential hydrophobic interactions between cellulases and lignin, the role of possible electrostatic effects has not been examined in any detail. In general, both the protein molecules and polymer surfaces carry charged groups and it is known that dissociation or association of the surface groups is one of the major mechanisms causing a surface charge that originates from carboxyl, amino, phosphate, imidazole groups, etc. If both acidic and basic groups are present, the surface may become positively or negatively charged, depending on the experimental conditions that are used. Typically, these materials have an electronic neutral point called the pI or point of zero charge. If both the protein molecule and polymer surface have the same charge, they repel each other. If they possess opposite charges, they adsorb. The maximum amounts of adsorbed protein onto polymers are sometimes the same as the pI of the protein or the pI of protein-polymer complex (Elgersma et al. 1990;

Fukuzaki et al. 1996). Several groups have looked at possible electrostatic interactions between proteins and solid surfaces. For example, Fukuzaki et al. (1996) examined the interaction between BSA and metal oxide surfaces: silicon dioxide (SiO₂, silica), titanium dioxide (TiO₂, titania), zirconium oxide (ZrO₂, zirconia), and aluminium oxide (Al₂O₃, alumina) (Fukuzaki et al. 1996). The pI of BSA was found to be pH 5.1, at which maximum adsorption was observed for all of the metal oxides. There was a direct correlation between the amount of adsorbed BSA and surface charge density. All of the adsorbed BSA on the metal oxides showed charge shifts towards the positive site, suggesting the possible involvement of the carboxylic acids groups on the BSA molecules. When Elgersma et al. (1990) examined the adsorption of BSA on positively and negatively charged polystyrene lattices with different surface charges (Elgersma et al. 1990), they found that maximum adsorption was attained at the pI of the protein-covered polystyrene (PS) particles rather than that of the pI of the protein itself. When the negatively charged PS ($\sigma_0 = -5.5$, and $-11.6 \mu\text{C cm}^{-2}$) was used, the plateau values of adsorption had a maximum of around pH 4.7. The zeta potential of PS ($\sigma_0 = -5.5 \mu\text{C cm}^{-2}$) and that of PS ($\sigma_0 = -11.6 \mu\text{C cm}^{-2}$) were about -130 mV and -90 mV, respectively. When the positively charged PS ($\sigma_0 = +7.8$, and $+13.2 \mu\text{C cm}^{-2}$) was used, the plateau values of adsorption had a maximum at pH 6.0. As mentioned above, Eriksson (2002) had shown previously that hydrophobic interactions occurred between BSA and lignin in pretreated substrates (Eriksson et al. 2002). Considering electric charges, it is possible that the BSA may be preferentially adsorbed onto lignin when compared to Cel7A, because the pIs of these proteins are quite different (Cel7A: 3.5-4.2, BSA: 4.7-5.3). This suggested that Cel7A carried a negative charge at hydrolysis condition (~pH 5.0) while BSA would carry a positive charge or be electrically neutral.

1.11.1 Electrostatic charges of cellulases

Enzymatic hydrolysis of lignocellulosic substrates is usually carried out under mildly acidic conditions. When the pI values of cellulases are considered (Table 2), it is apparent that, under the conditions normally employed for cellulose hydrolysis (pH 4.8-5.0), the CBHII, EGIII, and β -glucosidase components of *T. reesei* will carry a net positive charge and that CBHI, the CBHI core, EGI, and EGII components will carry a net negative charge. The β -glucosidase from *Aspergillus niger*, which is typically added to a reaction mixture to decrease productive inhibition caused by cellobiose, has a pI of 4.0 (McCleary and Harrington 1988). In addition to the net charge of the cellulases, it has been reported that the electric charges of specific amino acids may be involved in the adsorption onto cellulose. When Reinikainen et al. (1995) looked at the interaction between cellulose and the CBHI from *T. reesei* (Reinikainen et al. 1995), the pH dependency of the adsorbed cellulases indicated that electrostatic repulsion between the bound proteins greatly influenced protein adsorption. As the CBHI core was insensitive to pH, they thought that the observed pH-dependent adsorption of the native enzyme was caused by the CBM. When they replaced the amino acid residues in the CBM a significant reduction in adsorption of the protein to the cellulose was observed. They suggested that, at low pH (<6.2), the histidine (H) 465 which is part of an ionizing amino acid side chain (H465) in the CBM, became positively charged and preferably attracted the hydroxyl oxygen of the tyrosine (Y) 466 side chain. This consequently weakened the hydrogen bond with cellulose, significantly influencing the protein-carbohydrate interaction.

1.11.2 Electrostatic charges of lignocellulosic substrates

Both the cellulases and the pretreated lignocellulosic substrates components possess electrostatic properties which are governed by their aqueous environments. Lignocellulosic fibres carry a negative charge when suspended in water due to the presence and ionization of acidic groups in the hemicelluloses and lignin (Bhardwaj et al. 2004). In earlier work, Lin et al. (2008) measured the zeta potential of steam-exploded yellow poplar treated at 240 °C for 3 min and subsequently extracted with alkali (Lin et al. 2008). The resulting pulp was shown to have a zeta potential of -14 mV at pH 6.0, with the zeta potential decreasing with increasing pH. They attributed the decrease in the zeta potential to the dissociation of the carboxylic groups and the ionization of the phenolic hydroxyls in the remaining lignin. When Bhardwaj et al. (2004) measured the zeta potential of kraft pulps from *Eucalyptus* (Bhardwaj et al. 2004), the zeta potential of unbleached pulps was found to be -32.8 mV and that of the bleached pulp was -13.2 mV. In earlier work, when Dong et al. (1996) measured the zeta potential of kraft lignin from eucalyptus (Dong et al. 1996) the zeta potential of the kraft lignin was shown to be -40 mV at pH 4.5.

Thus, it is highly likely that pretreated pulps and lignins carry a negative charge when suspended in water. This assumption is supported by several studies. When Berlin et al. (2006) measured the chemical groups in lignin from organosolv pulps they found that the isolated lignins contained carboxylic acids (Berlin et al. 2006). Similarly, when Ragnar et al. (2000) measured the pKa-values of guaiacyl- and syringyl-derived phenols (Ragnar et al. 2000), the pKa of the phenolic hydroxyl groups were shown to be above 7.0, indicating that the phenolic hydroxyl groups are hydroxylated under typical hydrolysis condition (pH 4.8). In related work, when the pKa of carboxylic acid groups were found to be below 5.0, it was

shown that the carboxylic acid groups were dissociated when dispersed in water (Sjostrom 1989). In order to determine the role that any potential electrostatic interaction between lignin and cellulases might have, the zeta potential of the isolated lignins needs to be measured to determine if an overall attractive or repulsive force between cellulases and their respective substrates occurs.

1.11.3 Electrostatic interaction between cellulases and lignocellulosic substrates

As mentioned above, it is highly likely that pretreated substrates will have a negative charge when dispersed in water, primarily because the substrates typically contain significant amounts of carboxyl groups. With the negative charge of the pulps, it is possible that there will be an electrostatic attraction between pretreated substrates and CBHII, EGIII and β -glucosidase from *T. reesei*. On the other hand, there may be an electrostatic repulsion between the substrate and CBHI and EGII from *T. reesei*. The occurrence of electric repulsion between CBHI (Cel7A) from *T. reesei* and an anionic surfactant has been reported previously by Eriksson et al. (Eriksson et al. 2002). They showed that the addition of an anionic surfactant, SDS, to the reaction mixture reduced the adsorption of Cel7A on the SPS substrate, while the addition of a cationic surfactant increased the Cel7A adsorption onto the SPS. Considering that the pI of the Cel7A is approximately 3.9, the Cel7A must be negatively charged under typical hydrolysis conditions (pH 4.8), so that the Cel7A and SDS electrostatically repelled each other. It has been reported that β -glucosidase adsorbs onto lignin more than do cellulases, due to their preferential absorbance by lignin (Yang and Wyman 2006). One possible explanation for the adsorption difference is that the β -glucosidase (pI 7.4-7.8) has a net positive charge resulting in a strong attraction to the

negatively charged lignin. In addition to the overall electric charge, which is determined by the pI or zeta potential, partial electric charge caused by specific amino acids has also been reported to influence the amount of cellulases adsorbed onto lignocellulosic substrates (Palonen et al. 2004). Berlin et al. (2006) proposed that ionic-type lignin-enzyme interactions played a major role in protein- substrate interactions as the occurrence of charged (COOH, OH) or partially charged (CO) functional groups on both the lignin and cellulase surfaces could mediate these interactions (Berlin et al. 2006).

Although there remains a possibility that electrostatic interaction is the major mechanism that determines the amount of adsorbed cellulases, these studies (Borjesson et al. 2007b; Palonen et al. 2004) did not consider the additional involvement of electrostatic interactions. For example, Palonen et al. (2004) assumed that the different adsorption properties of CBHI and EGII onto lignin were caused by the more “open nature” of the active site of EGII that may lead to adsorption through hydrophobic interactions (Palonen et al. 2004). However, it is still possible that the different electric properties between CBHI and EGII caused the observed differences in adsorption behavior. The CBHI would carry a negative charge under the hydrolysis condition because the pI of CBHI ranges from 3.5 to 4.2 (Table 1). The EGII would carry a negative (weaker electric charge than CBHI) or positive charge under the hydrolysis condition, because the pI of EGII ranges from 4.2 to 5.5 (Table 1). It is also possible that the different amino acid composition resulted in the different adsorption profiles, because the CBM of CBHI has a positive charged amino acid (His) and the CBM of EGII contains no charged amino acids. Borjesson et al. (2007b) attributed the different adsorption behavior between CBHI (Cel7A) and EGI (Cel7B) to the different hydrophobicities of the enzymes (Borjesson et al. 2007a). However, it is possible that

electrostatic interactions are also involved, because the pI of EGI (3.9-6.0) indicates that the EGI carries either a lesser negative charge or a positive charge than does the CBHI. In addition, it is apparent that the number of charged amino acids in the respective CBMs are different as there is one positive charged group in the CBM of CBHI, H465 (Reinikainen et al. 1995), while the number of positive and negative charged amino acids in the CBM of EGI are two and one, respectively (Gilkes et al. 1991).

Thus, it is clear that electrostatic interactions are an important factor that influence the amounts of protein adsorbed onto solid surfaces (Norde 1986). It is also recognized that the electric charge will vary according to the nature of each of the cellulase components. To determine the electrostatic interactions between cellulases and lignin, the measurement of zeta potential of isolated lignin will be necessary. The reported pI of cellulases varies widely (Table 1) and the use of cellulase complex mixtures preparations will not allow us to easily observe specific cellulase behavior. Therefore, a study on each of the individual cellulases will be necessary for us to be able to provide a more comprehensive understanding of enzyme adsorption behavior. Thus, if we are able to determine the adsorption isotherms of cellulases onto isolated lignins as a function of pH, this would greatly contribute to a better understanding of the role that electrostatic interactions might play between cellulases and lignin.

1.12 Hydrogen bonding interactions between cellulases and lignin

Another potential means of interaction between cellulases and lignin is via hydrogen bonding. Hydrogen bonding occurs when a hydrogen atom is attached to an electron-attracting atom, typically oxygen, fluorine, or nitrogen, so that the hydrogen is at the

positive end of an electric dipole and it is then attracted to an atom at the negative end of the dipole (Brey 1978). The energy associated with such a hydrogen bond may be as much as 10 kcal/mol, compared to 1 or 2 kcal/mol for a typical van der Waals interaction. It is, however, five to ten times less than that of an ordinary covalent interatomic bond. For the interaction between cellulases and lignocellulose, hydroxyl groups in cellulose and lignin were reported to be involved in hydrogen bonding (Pan 2008; Sewalt et al. 1997). Although carboxylic acid groups in lignin could be involved in the hydrogen bond interaction, only Berlin et al. (2006) mentioned this possibility (Berlin et al. 2006).

1.12.1 Phenolic hydroxyl group in lignin

Some groups mention that phenolic hydroxyl groups are involved in cellulase adsorption on lignin. When Sewalt (1997) compared the effect of hydroxypropylated organosolv lignin and steam pretreated lignin on filter paper digestion, they showed that the hydroxypropylation of lignin, which removed free phenolic sites, increased cellulose hydrolysis (Sewalt et al. 1997). Pan (2008) used five different isolated lignins: hardwood organosolv lignin, baggasse hydrolytic lignin, softwood kraft lignin, softwood organosolv lignin, and hardwood organosolv lignin to assess the effect of phenolic hydroxyl group on hydrolysis (Pan 2008). The addition of 20% (w/w) lignin to the reaction mixture containing microcrystalline cellulose (Avicel) resulted in a 10-23% reduction in hydrolysis, depending on the source of the lignin. The hydroxypropylation of the phenolic hydroxyl groups of the isolated lignin reduced the inhibitory effect of lignin, suggesting that the phenolic hydroxyl groups negatively affected the hydrolysis. These workers also used lignin model compounds to assess the role of functional groups within the lignin on enzymatic hydrolysis inhibition.

They showed that phenolic compounds resulted in 1-5% more inhibition than did non-phenolic compounds, suggesting that the interference caused by the phenolic hydroxyl groups on lignin during enzymatic hydrolysis played a more important role than did lignin's role in acting as a physical barrier and/or non-specific adsorption. Although hydroxypropylation is a good way to modify phenolic hydroxyl groups, the hydroxypropylation reaction will likely modify lignin in different ways. For example, the numbers of hydroxyl groups will not be changed because phenolic hydroxyl groups are changed into aliphatic hydroxyl groups. It is also possible that the hydrophobicity increases as a result of the hydroxypropylation reaction. When Berlin et al. (2006) compared the amounts of phenolic hydroxyl groups in the DL and the ERL lignin from Douglas-fir steam pretreated pulps by Nuclear Magnetic Resonance (NMR) (Berlin et al. 2006), the DL was shown to be more effective in decreasing hydrolysis efficiency than was the ERL. Similarly, the amount of phenolic hydroxyl groups was higher in the DL than in the ERL, showing that the phenolic hydroxyl groups negatively affected the hydrolysis.

One study that did not find a correlation between the amounts of phenolic hydroxyl groups in lignin and adsorption of protein was the work of Kawamoto et al. (1992) who examined the adsorption of BSA on five lignins: thioglignin, steam-explosion lignin, acetic acid lignin, organosolv lignin, and MWL (Kawamoto et al. 1992). Although the phenolic hydroxyl groups were present at almost the same level (about 0.3/C₆-C₃ unit) for each of the five lignin preparations, the amount of adsorbed of BSA was different. It was therefore concluded that more complex factors other than just the content of the phenolic hydroxyl groups influenced the protein-adsorbing ability of lignin.

1.12.2 Aliphatic hydroxyl groups in lignin

In addition to phenolic hydroxyl groups, aliphatic hydroxyl groups have also been shown to be involved in hydrogen bonding (Brey 1978). The CBM of cellulases are known to interact with the hydroxyl groups on cellulose, suggesting that aliphatic hydroxyl groups are also involved in the adsorption on lignin. However, the role of aliphatic hydroxyl groups in the binding between cellulases and lignin has yet to be reported. When Borjesson et al. (2006) used surfactant MEGA 10 containing aliphatic hydroxyl groups (Borjesson et al. 2007a) they were able to increase the hydrolysis of steam pretreatment spruce from 47% to 65%. When Berlin et al. (2006) compared aliphatic hydroxyl groups in the isolated two lignins from Douglas-fir (Berlin et al. 2006) the aliphatic hydroxyl groups content was higher in the ERL than in the DL, whereas the inhibitory effects on the cellulase preparations was greater with DL than with the ERL. This was likely due to the DL having a lower hydrophobicity when compared to the ERL.

1.12.3 Hydrogen bond interactions between cellulases and lignocellulose

It is known that hydroxyl groups are involved in hydrogen bonding (Brey 1978) and phenolic hydroxyl groups and aliphatic hydroxyl groups in lignin could, hypothetically, be involved in the hydrogen bonding. As mentioned earlier, Sewalt et al. (2008) and Pan (2008) reported that hydroxypropylation reduced the negative effect of lignin on the hydrolysis reaction (Pan 2008; Sewalt et al. 1997), while Kawamoto et al. (1992) reported that there was no correlation between the amount of phenolic hydroxyl groups and the adsorbed protein content (Kawamoto et al. 1992). The addition of surfactants with aliphatic hydroxyl groups has been shown to have no influence on enzymatic saccharification, suggesting that hydrogen

bonding does not influence the adsorption of cellulases on lignin. However, the total hydroxyl groups should be taken into consideration to better understand the role of hydrogen bonding in cellulase adsorption on lignin. The presence of carboxylic groups should be considered as it will increase the hydrogen bond interaction (Brey 1978). Changes in the functional groups will influence both the hydrophobicity and electric charges of the pretreated pulps and lignins. Therefore, the hydrophobicity of modified lignin should also be determined.

1.13 Thesis objectives

Although the conversion of lignocellulosic materials to ethanol is a promising approach to both reduce our dependency on fossil fuels and to lower the release of fossil fuel-derived carbon dioxide, currently there is little or no commercial production of ethanol from biomass in the so called second generation bioethanol approach. As well as investing in scale up and applied research there is still a need for considerably more fundamental work to help make the processes more economical. One of the difficulties associated with enzyme based biomass-to-ethanol process is the high cost of cellulase enzymes and their low specific activity. This is partly caused by the recalcitrance of the lignocellulosic substrate. One of the main restrictions to achieving efficient enzymatic hydrolysis is inhibition by lignin, which limits access to the cellulose, adsorbs cellulases and generally inhibits the action of the cellulase complex. By elucidating the influence that lignin might have on limiting the hydrolysis of pretreated lignocellulosic substrates, we hope to improve both the overall efficiency of the enzymatic hydrolysis of the cellulose step of the overall process while better understanding the substrate-enzyme interactions that occur during cellulose hydrolysis.

It is recognized that protein adsorption is a complex process that is influenced by a

number of parameters, such as electrostatic forces, hydrogen bonding, and the hydrophobic/hydrophilic characteristics of both the protein and the surface to which it is adsorbed (Quinn et al. 2008). Several protein properties such as their molecular weight, hydrophobicity, electrostatic interactions and protein stability are all known to influence protein adsorption (Norde 1986).

However, comparatively little work has looked at the mechanisms involved in cellulases-lignin interactions with the predominant view being that is primarily hydrophobic interactions that are involved (Eriksson et al. 2002). Although some researchers have mentioned the possibility of hydrophobic interactions (Eriksson et al. 2002; Ooshima et al. 1986), electrostatic interactions (Berlin et al. 2006), and hydrogen bonding (Berlin et al. 2006; Pan 2008; Sewalt et al. 1997) as possible mechanisms for the adsorption of cellulases on lignin, the relative contributions of each of these types of influences are still unknown.

When we initiated this research, we anticipated that lignins derived from various biomass sources, which had been pretreated by different processes over a range of conditions, would differ in their ability to negatively/positively affect the efficiency of enzymatic hydrolysis of the cellulosic substrates. We hypothesized that the degree to which the isolated lignins might influence hydrolysis might correlate with the chemical and physical properties of the lignin, such as hydrophobicity, electric charge and hydrogen bonding potential.

We looked at both steam and organosolv pretreated substrates to observe and compare the differences between the two pretreatment processes when agricultural (corn stover), hardwood (polar) and softwood (lodgepole pine and Douglas-fir) materials were used as substrates and to assess the effect of the pretreatment conditions on the lignins derived from

these substrates. In the initial work, the steam and organosolv processes were operated under conditions that were optimal for maximizing carbohydrate (hemicellulose and cellulose) recovery and digestibility (Ewanick et al. 2007) and the lignin was then isolated from the cellulose/lignin rich water or solvent insoluble fraction. The hydrophobicity and electric charge of the various isolated lignins were measured as they are known to be important factors that will influence protein adsorption. It was anticipated that these properties would differ according to the types of cellulases, pretreatment method and raw materials used. It was thought likely that any observed differences might provide useful information to help us elucidate some of the underlying factors responsible for the differences in behavior of the various cellulases when they encounter lignin from different biomass sources that have been pretreated at varying conditions.

Lodgepole pine was used as a representative of softwood in this study, because large amounts of beetle-killed lodgepole pine are currently available in British Columbia (BC) and much of the Pacific coast. In earlier work, it was found that steam treated lodgepole pine at high severity resulted in better enzymatic hydrolysis of the cellulose when compared with low and medium severity conditions (Ewanick et al. 2007). The use of high severity conditions resulted in the increased content of lignin in the pulp, primarily due to the removal of hemicellulose. However, the specific reason for the better hydrolysis achieved after high severity pretreatment was not clearly apparent as, during pretreatment, both the hemicellulose and lignin content and structure were changed. Poplar was selected as a representative of hardwoods because of its current availability in locations such as Alberta and Ontario and because its relatively rapid growth rate is conducive to its likely use as an energy crop. Corn stover was used as a representative of an agricultural substrate because it is currently being

studied intensively in the US for its potential as a feedstock to produce cellulosic ethanol.

It is assumed from the general theory of protein adsorption on solid surfaces that low hydrophobicity and negative charges of lignin will decrease the adsorption of negatively charged cellulases. It is known that the sulfonation of lignin decreases its hydrophobicity and imparts a negative charge to the lignin. Thus, as a result of the sulfonation of lignin, the amount of adsorbed cellulases on lignin will be reduced. It is also likely that the addition of carboxylic groups to the lignin would contribute to the negative charge on lignin as well, since carboxylic groups are dissociated under typical hydrolysis condition (Ragnar et al. 2000).

In summary, we hoped that by using two different pretreatment methods that are currently undergoing commercial evaluation and assessing their effectiveness on three likely biomass feedstock's, with a primary focus on cellulase-lignin interactions, we could both help increase the efficiency of the overall biomass-to-ethanol process, while further elucidating the enzyme-substrate interactions that occur during typical, enzyme mediated, cellulose hydrolysis.

2 MATERIALS AND METHODS

2.1 Enzymes

2.1.1 Cellulases

Spezyme CP (filter paper activity 55.3 FPU/ml, protein content 173.9 mg/mL; Genencore International, San Francisco, CA), which is a commercial cellulases preparations derived from *T. reesei* was used in this study. Novozyme 188 (cellobiase activity 1,063 IU/mL, protein content 271.3 mg/mL; Novozymes A/S, Bagsværd, Denmark), which is a commercial β -glucosidase preparations from *A. niger* was used.

2.1.2 Enzyme activity assays

The total cellulase activity of the enzyme preparation was measured using the filter paper assay according to Ghose (Ghose 1987). Enzymes (0.5 ml) diluted by Na-acetate buffer (pH 4.8, 50 mM) and Na-acetate buffer (pH 4.8, 50 mM) (1.0 ml) were added into a test tube with a Whatman No.1 filter paper strip (1.0 X 6.0 cm) and incubated for 1 h at 50 °C. After the incubation, dinitrosalicylic acid (DNS) (3.0 ml), was added into the tube and boiled for 5 min and then immersed into a cold water bath to stop the reaction. Adsorption at 540 nm was measured by a Bio50 UV-vis spectrophotometer (Varian Inc. Palo Alto, CA). The filter paper units (FPU) was determined the amount of enzymes that produces 2.0 mg of reducing sugar from the filter strip. Glucose was used for the standard.

Cellobiase activity was measured using cellobiose as a substrates according to Ghose (Ghose 1987). The dilution of enzyme that produces 1.0 mg of reducing sugar from cellobiose after 30 min at 50 °C was determined using DNS using the same method as the FPU. Glucose was used for the standard.

2.1.3 Protein content assay

The protein content was measured using ninhydrin according to reported methods (Starcher 2001). Diluted sample (40 μ l) and 1N HCl (100 μ l) were mixed in an ependorf tube (1.5 ml) and incubated in an oven (105 $^{\circ}$ C) over night. The incubated tube was centrifuged and 2% of ninhydrin reagent (200 μ l) (Sigma) was added into it and incubated at 100 $^{\circ}$ C for 20 min. After the incubation, 50% (v/v) of ethanol (1 ml) was added into the tube and absorption at 570 nm was measured using a Bio50 UV-vis spectrophotometer (Varian). Bovine Serum Albumin (BSA) was used as the standard.

2.2 Lignocellulose substrates and chemicals

Corn stover, poplar, lodgepole pine, and Douglas-fir were used for this study. Avicel PH 101, a microcrystalline cellulose, was purchased from Fluka (Switzerland). Coniferyl aldehyde and ferulic acid were purchased from Sigma.

2.3 Steam pretreatment

Corn stover and wood chips from poplar, Douglas-fir, and lodgepole pine (OD; oven-dry weight of 300 g) were impregnated with 4.0% (w/w) of anhydrous SO₂ in a plastic bag and kept over night. The samples (50 g) were loaded into a preheated 2 L Stake Tech III steam gun (Stake Technologies, Norvall, ON, Canada) in the Forest Product Biotechnology Laboratory at the University of British Columbia. The conditions used for steam pretreatment are shown in Table 4. For the pretreatment, optimum conditions to maximize monosachrides recovery from the initial lignocellulosic substrates were used. After the pretreatment, the solid fractions of the pretreated substrates were collected and washed with water and stored at

4 °C.

2.4 Organosolv pretreatment

Corn stover and wood chips from poplar, Douglas-fir, and lodgepole pine were organosolv pretreated using a 2 L rotating digester (Aurora Products Ltd., Savona, BC, Canada) (Table 4). For the pretreatment, optimum conditions to maximize monosachrides recovery from the initial lignocellulosic substrates were used. A 200 g (OD) of sample was pretreated. After the pretreatment, the substrate and spent liquor were separated using a nylon cloth and washed with water.

Table 4. Pretreatment conditions.

Pretreatment	Species	Temperature (°C)	Time (min)	SO ₂ (%, w/w)	Ethanol (%, v/v)	H ₂ SO ₄ (%)
Steam pretreatment	Corn stover	190	5	3.0	-	-
	Poplar	200	5	3.0	-	-
	Lodgepole pine	200	5	4.0	-	-
	Douglas-fir	190-210	5	4.0	-	-
Organosolv pretreatment	Corn stover	200	60	-	75	1.0
	Poplar	200	60	-	60	1.25
	Lodgepole pine	170	50	-	60	1.0

2.5 Characterization of substrates

The carbohydrate and lignin content/composition of lignocellulosic substrates and

isolated lignins were determined using sulfuric acid hydrolysis according to TAPPI methods (TAPPI method T249 cm-85 and TAPPI useful method UM250). The carbohydrate composition of acid hydrolysates was determined using a Dionex DX-3000 High Performance Liquid Chromatography (HPLC) on a CarboPac-1 column (Dionex, Sunnyvale, CA). The ash content was measured using a muffle furnace (Fisher Scientific, Canada) by keeping it in the muffle furnace at 550 °C for 4 h.

2.6 Enzymatic hydrolysis of pretreated substrates

Pretreated substrates were hydrolyzed using Spezyme CP and β -glucosidase at 50 °C and 150 rpm. Substrates were suspended at 2 or 5% consistency in 50 mM Na-acetate buffer (pH 4.8). The sugar content was measured by HPLC (Dionex DX-3000). The hydrolysis yield (%) of the substrate was calculated from the sugar content as a percentage of the theoretical sugar available in the substrates. The protein content was measured by the nihydrin method (Starcher 2001) and BSA was used as the protein standard.

2.7 Enzymatic hydrolysis of Avicel

Avicel was enzymatically hydrolyzed using Spezyme CP and β -glucosidase at 50 °C and 150 rpm. Avicel was suspended at 2% (w/v) in Na-acetate buffer (pH 4.8, 50 mM). Isolated lignins and DHPs were suspended at 0.4% (w/v) in Na-acetate buffer (pH 4.8, 50 mM), respectively. The sugar content was measured by HPLC (Dionex DX-3000). The hydrolysis yield (%) of the substrate was calculated from the sugar content as a percentage of the theoretical sugar available in the substrates. The protein content was measured by the nihydrin method (Starcher 2001) and BSA was used as the protein standard.

2.8 Protease treated lignin (PTL) isolation

Pretreated substrates (10 g) were hydrolyzed for 72 h in 500 mL of Na-acetate buffer (50 mM, pH 4.8) by cellulases (40 FPU/g cellulose) and β -glucosidase (80 IU/g cellulose) with tetracycline (40 μ g/mL) and cycloheximide (30 μ g/mL) to prevent microbial contamination. After the enzymatic hydrolysis, the lignin residue was recovered by centrifugation and washed three times by resuspension in 600 mL distilled water and sonication at 40 kHz for 60 min in a TP 680DH ultrasonic water bath (Elma Hans Schmidbauer GmbH & Co., Singen, Germany). The lignin residues were incubated overnight at 37 °C in phosphate buffer (50 mM, pH 7), containing 1 U/mL protease (Sigma, USA) to hydrolyze remaining cellulases. The remaining protease was deactivated by incubation at 90 °C for 2 h. The residue was washed extensively with phosphate buffer and distilled water and freeze-dried. After drying, the PTLs were screened through a 180 mesh screen and stored in sealed vials at room temperature in a desiccator.

2.9 Cellulolytic enzyme lignin (CEL) isolation

Pretreated substrates (10 g) were suspended in acetate buffer (100 ml, pH 4.8) and cellulases and β -glucosidase was added and incubated for 24 h at 50 °C at the ratio of 1:2. The reaction system was centrifuged, the supernatant was removed and the residue was again suspended in acetate buffer (50 ml, pH 4.9) and treated with cellulases and β -glucosidase for an additional 24 h at 50 °C. The residue was again collected by centrifugation, washed with distilled water (200 ml), centrifuged and freeze-dried. The freeze-dried residue was extracted twice (2 X 24 h) with 100 ml of dioxane/water (96:4, v/v) under a nitrogen atmosphere. After each extraction, the supernatant was collected, combined and concentrated using a rotary

evaporator and poured into water. The precipitate was filtrated using a Nylon membrane (0.2 μm , Millipore, Billerica, MA) and freeze-dried.

2.10 Residual enzyme lignin (REL) isolation

After the extraction of lignin by dioxane (96:4, v/v) to obtain CEL, the residue was washed with water and freeze-dried. This was designated as the residual enzyme lignin (REL).

2.11 Milled wood lignin (MWL) Isolation

Air-dried lodgepole pine and Douglas-fir were milled in a Wiley mill using a 40-mesh screen. The coarsely milled wood was extracted with acetone for 48 h using a Soxhlet apparatus. After the extraction, the wood was air-dried and stored in a vacuum desiccator. The dried Wiley wood was ground using a planetary ball mill (Retsch PM 200) with two 50 mL zirconium oxide jars. Each jar contained 10 g of extractive-free Wiley wood and 6 zirconium oxide balls (20 mm diameter). Samples were ground under an argon atmosphere at 650 rpm for 15 h. To prevent overheating and thermal changes to the wood, the samples were milled for 30 min intervals, between which the samples were allowed to cool for 30 min. After milling, the milled wood was dried in a vacuum desiccator. MWL was isolated according to the method of Björkman. Accordingly, 20 g of milled wood were put into a 500 mL capped centrifuge bottle and 200 mL of dioxane/water (96:4, v/v) was added. The solution was shaken at room temperature for 24 h under a nitrogen atmosphere. The mixture was centrifuged at 8000 rpm for 15 min and the supernatant was collected. The remaining solid was resuspended in 200 mL of dioxane/water (96:4, v/v) and the above procedure

repeated. The combined supernatants from centrifugation were concentrated and added dropwise to deionized water and then freeze-dried using a VirTis EX freeze dryer.

2.12 Synthesis of dehydrogenative polymer

Two dehydrogenative polymer (DHP) model lignin samples were prepared from coniferyl alcohol (CA) and ferulic acid (FA, $\geq 99.0\%$, Sigma-Aldrich) to obtain lignin with different chemical groups in γ -position (alcohol and carboxylic acid, respectively). CA was prepared by the reduction of coniferyl aldehyde (98%, Sigma-Aldrich) by NaBH_4 (Sigma-Aldrich) according to the Ludley et al (Ludley and Ralph 1996). The yield from coniferyl aldehyde to CA was 73.3%. The DHPs were prepared by endwise (Zutropf mode) polymerization condition with 0.05% of H_2O_2 (Wayman 1974). The yields of the DHPs from CA and FA were 60% and 28%, respectively. The nitrogen contents in the DHPs, which was measured by elemental analyzer (model 1108, Carlo Erba), were 0.66% and 0.81%, respectively. The DHPs were stored at room temperature in a vacuum desiccator.

2.13 Cellulase adsorption

The assessment of cellulases and β -glucosidase adsorption to isolated lignin was performed at 50 °C in 2.0 mL vials using a 50 mM acetate buffer (pH 4.8). The vials containing 1% (w/v) lignin and various loadings of cellulase (20-350 mg/lignin-g) were incubated for 3 h turning end-over-end on a rotator driven by a variable speed motor (Fine PCR, Gyenggi-do, Korea). The supernatants were centrifuged (13,000 g, 10 min) then filtrated using a low protein binding membrane with a pore size of 0.22 μm (Millex-GV, Millipore).

2.14 ATR FTIR spectroscopic analysis

To investigate and quantify the chemical groups in the isolated lignin, a FTIR system (Varian 3100, Varian Inc. Palo Alto, CA) with MIRacle Accessory (Pike technologies, Madison, WI) was used and mid-IR spectra were obtained by averaging 128 scans from 4000 to 600 cm^{-1} at a spectral resolution of 4 cm^{-1} .

2.15 Acetylation of lignin

Isolated lignin and DHPs were acetylated for physical and chemical analysis. Acetylation was performed using 20-100 mg of lignin and DHP, which was dissolved in 2 mL of pyridine/acetic anhydride (1:1, v/v), and the mixture was stirred for 48 h at room temperature. The reaction solution was added dropwise to 100 mL of ice-water with stirring. The precipitated lignin was collected by filtration through a Nylon membrane (0.20 μm , 47 mm), washed with ice-water and freeze-dried using a VirTis EX freeze dryer. This procedure was repeated to ensure complete acetylation of the samples.

2.16 Solution state NMR quantitative structural analysis

NMR spectra were recorded on a Bruker AVANCE 300 MHz spectrometer (Bruker AXS Inc., Madison, WI) at 300 K using 200 mg of sample in 600 μl of dimethyl- d_6 sulfoxide. Chemical shifts were referenced to tetramethylsilane (δ 0.0 ppm). Quantitative ^{13}C NMR employed a 90° pulse width, a 1.2 s acquisition time and a 1.7 s relaxation delay. Chromium (III) acetylacetonate (0.01 M) was added to the solution to provide complete relaxation of all nuclei, as previously discussed (Capanema et al. 2004b). A total of 20,000 scans per sample were collected. NMR spectra were recorded for non-acetylated lignins.

2.17 Determination of lignin surface area and particle size analysis

The surface area of lignin preparations was determined by nitrogen adsorption according to the single-point BET procedure (Satterfield 1991) using an Autosorb-1 surface area analyzer (Quantachrome instruments, FL).

Particle sizes of lignin were determined using a Mastersizer 2000 (Malvern Instruments Ltd., Worcestershire, UK.). Lignin samples were dispersed in water using 6 min ultrasonic treatment and volume weighted mean of the samples was measured.

2.18 Molecular mass determination

The molecular mass distribution of the acetylated lignin samples was determined by gel permeation chromatography (GPC, Agilent 1100, UV and RI detectors) using styragel columns (Styragel HR 4 and HR 2) at 35 °C, THF as the eluting solvent (0.5 ml/ min) and UV detection at 280 nm. The lignin concentration was 1 mg mL⁻¹ and the injection volume was 75 µL. The GPC system was calibrated using standard polystyrene samples with molecular weights ranging between 580 and 1,800,000 Daltons. The weight average molecular weight (M_w), number average molecular weight (M_n), and polydispersity index (M_w/M_n) of lignin preparations were determined.

2.19 Nitrogen contents in isolated lignin and DHPs

An elemental analysis of isolated lignin was performed using a Perkin Elmer series II CHNS/O 2400 analyzer (Norwalk, CT, USA).

2.20 Zeta potential of lignocelluloses

The Zeta potential values were determined by using a Zeta-Meter 3.0+ (ZETA-METER, INC., Staunton, VA) and a ZetaProbe Analyzer (Colloidal Dynamics, LLC, North Attleboro, MA). For measurements using the Zeta-Meter 3.0+, samples were dispersed in Na-acetate buffer (pH 4.8, 50 mM). Five replications were assessed for each treatment.

2.21 Calculation of maximum adsorption capacity

Adsorption parameters (maximum adsorption capacity $[\sigma]$ and equilibrium constants $[K_d]$) were determined according to the previously reported method (Kumar and Wyman 2009a; Lynd 1996) using the following equation:

$$[CE] = \frac{\sigma[S_t][E_f]}{K_d + [E_f]}$$

where $[CE]$ is the amount of adsorbed enzyme in mg/ml, $[E_f]$ the free enzyme concentration in mg/mL, σ the maximum adsorption capacity in mg/mg substrate, $[S_t]$ the substrate concentration in mg/mL, and K_d the equilibrium constant $= [C][E]/[CE]$ in mg of enzyme/ml. Affinity constants ($A=1/K_d$), and binding strength ($S=A \times \sigma$) were estimated.

2.22 Isoelectric focusing (IEF)

Cellulases (0.34 mg/ml) and β -glucosidase (0.09 mg/ml) were incubated with 5 mg of PTLs in 500 μ l of acetate buffer (pH 4.8, 50 mM) at 50 $^{\circ}$ C for 3 h. After centrifugation the

supernatants were collected, freeze-dried, and analyzed by Criterion IEF Gels (pH 5-8, Bio-Rad Laboratories, Inc., Hercules, CA).

2.23 Construction of pET plasmid to express CBM of CBH I from *T. reesei*.

The DNA that coded for the CBM of CBHI from *T.reesei* was amplified by PCR (Mastercycler ep gradient, Eppendorf, Hamburg, Germany; annealing temperature, 58 °C; extension time, 20 sec; and extension temperature, 72 °C) using genomic DNA from *T.reesei* QM 9414 (ATCC 26921D-2) as a template, a Phusion Master Mix (New England Biolabs Ltd. Ipswich, MA), and the following oligomers (5'>CCAGCCACTACCACTGGAAGCTCTCCC<3';5'>CCTTAATTAATTACAGGCAC TGAGAGTAG <3'). After the PCR reaction, the reaction mixture was purified by a PCR purification kit (Qiagen, Germantown, MD), and digested by PacI (New England Biolabs Ltd.). A pET- 45b(+) vector (EMD Chemicals Inc. Gibbstown, NJ) was digested with PmlI and Pac I (New England Biolabs Ltd.) and purified by a Gel extraction kit (Qiagen). The digested PCR reaction mixture and the pET vector were ligated using a Quick Ligation Kit (New England Biolabs Ltd.), followed by transformation into a One shot TOP 10 competent cells (Invitrogen, Carlsbad, CA). The plasmid containing the CBM of CBHI was purified from the transformants of the TOP 10 using a QIAGEN Plasmid Mini Kit (Qiagen) and the insert in the plasmid was confirmed by sequencing. The plasmid containing the CBM of CBHI was transformed into Rosetta gami B (DE3) pLysS (EMD). The transformant was incubated using LB medium at 20 and 37 °C at 150 rpm and after the O.D. reached 0.6. The production of the CBM was induced using 1 mM of isopropyl β -D-1-thiogalactopyranoside (IPTG). After the incubation, the broth was centrifuged at 3000 rpm for 5 min. The

supernatant was freeze-dried and the precipitate was homogenized using Fast PROTEIN BLUE (speed, 6.0; time 20 sec, MP Biomedicals, Solon, OH). The homogenized cell suspension was purified using His Spin Trap (GE Healthcare, Waukesha, WI). The supernatant and homogenized broth and purified fraction were analyzed by SDS-PAGE (Biorad Laboratory Inc., Hercules, CA)

2.24 Preparation of thin film

Avicel was dissolved in 50% (w/w) water/ *N*-methylmorpholine-*N*-oxide (NMMO) (1%, w/w) using a rotary evaporation at 80 °C. Dimethyl sulfoxide (DMSO) was added to adjust the concentration and viscosity of the polymer (0.05%) in the mixture (Turon et al. 2008). The cellulose solution was spin-coated onto a microscope cover glass (12 mm, Fisher Scientific Company, Ontario, Canada) using a spin coater (Laurell Technologies model WS-400B-6NPP, North Wales, PA) spinning at 2500 rpm. The substrate was then removed from the spin-coater and dried in an oven at 80 °C for 2 h. The cellulose-coated substrate was then washed thoroughly with Milli-Q water and stored at room temperature in a clean container. Isolated lignin was dissolved in pyridine (2%, v/v) and filtrated (0.45 µm, Millex-FH filter unit, Millipore, Tokyo, Japan) to remove the insoluble fraction. The soluble fraction (30 µl) was put on a microscope cover glass (12 mm, Fisher Scientific Company, Ontario, Canada). The thin film was made by use of a spin coater (400 rpm, 5 sec, and 2500 rpm, 30 sec).

2.25 Atomic force microscopy (AFM)

Atomic force microscopy (AFM, Veeco, Santa Barbara, CA) was used to observe the

roughness of the thin films made from the cellulose and lignin using a cantilever SCANASYST-AIR (Veeco).

2.26 Contact angle

The contact angle of the prepared thin film from the cellulose and lignin was measured using a KSV contact angle measurement system (KSV Instruments Ltd., Helsinki, Finland). The initial equilibrium contact angle between the water drop and the surface was evaluated and reported as an average of 4 measurements.

2.27 Surface analysis by X-ray photoelectron spectra (XPS)

The X-ray photoelectron spectra (XPS) of the lignin and cellulose film were obtained by using a spectrometer, Leybold MAX 200, with an AlK α source operated at 15 kV, 20 mA. The pass energy used for measuring the survey scan was 192 eV and the pass energy used for measuring the narrow scan was 48 eV. The base pressure of the chamber was 1×10^{-9} torr. A fitting program- Peakfit 4.1 (Systat Software Inc.) was used to deconvolute the C1s signal into Gaussian components having equal full width at half maximum (FWHM). The C1s signal at 285 eV was used as an internal standard. The chemical shifts relative to C-C used in the deconvolution were 1.7 ± 0.2 eV for C-O, 3.1 ± 0.2 eV for C=O, or O-C-O and 4.2 ± 0.3 eV for O=C-O (Palonen and Viikari 2004).

3 RESULTS AND DISCUSSION

3.1 The characterization and effect of isolated lignins, obtained from a range of pretreated lignocellulosic substrates, on enzymatic hydrolysis

3.1.1 Background

As mentioned previously, it has been suggested that cellulases are adsorbed on lignin via either hydrophobic, ionic bond or hydrogen bond interactions (Berlin et al. 2006; Pan 2008; Sewalt et al. 1997). In previous work, cellulolytic enzyme lignin (CEL), where the substrate is enzymatically hydrolyzed to remove carbohydrates and then subsequently extracted with aqueous dioxane, was found to be structurally similar to milled wood lignin (MWL) (Chang et al. 1975). Alternatively, rather than using a dioxane extraction, protease treated lignin (PTL) could be produced where the lignin remaining after the complete enzymatic hydrolysis of the carbohydrate component of the lignocellulosic substrate was subsequently treated by proteases, to remove any adsorbed proteins, with the assumption that this fraction would more closely resemble the lignin-rich fraction remaining after enzymatic hydrolysis (Berlin et al. 2006).

In our initial work, we first looked at the influence that the residual lignin remaining in the pretreated lignocellulose might have on the enzymatic hydrolysis of cellulose. Twelve lignin preparations were isolated from three types of biomass substrate using two pretreatment and two lignin isolation methods. Corn stover, poplar, and lodgepole pine were used as representatives of agricultural residue, hardwood, and softwood, respectively and two pretreatment processes (steam and organosolv pretreatment) were compared. Lignin was isolated using the two PTL and CEL methods described briefly above and the yields of the

lignin and the properties of the isolated lignin were compared. Finally, the isolated lignin preparations were added to reaction mixtures containing crystalline cellulose (Avicel) to observe their possible influence on enzymatic hydrolysis.

3.1.2 Chemical composition of substrates

Despite various attempts to reduce the amount of lignin in the pretreated substrates, because of their potential detrimental influence such as non-productive binding and steric hindrance of cellulases (Berlin et al. 2006; Palonen et al. 2004; Sewalt et al. 1997), virtually all pretreated substrates will always contain some amount of acid insoluble lignin (AIL). It was apparent that the amount of AIL remaining on each of the substrates ranged from 18.2 to 42.8% (Table 5). The AIL remaining in the steam pretreated lodgepole pine was the highest (42.8%), while the amounts of AIL in the other substrates were about 20%. The glucose content (Glu) of the steam pretreated substrates was lower than those in the organosolv pretreated substrates, indicating that organosolv pretreatment is better able to separate the lignin from the cellulose, as previously reported (Tu et al. 2007b). As previously described (Ohgren et al. 2007; Tu et al. 2007b) the xylose (Xyl) and ash content of the pretreated corn stover was higher than that obtained from other substrates. It was anticipated that the different chemical composition of the pretreated substrates was likely due to the hydrolysis yields and cellulases adsorption properties (Kurabi et al. 2005; Tu et al. 2007b).

Table 5. Characterization of prepared substrates obtained by Klason analysis.

Pretreatment	Species	Neutral sugar compositions					Lignin		Ash
		(%)					analysis		(%)
							(%)		
		Ara	Gal	Glu	Xyl	Man	AIL	ASL	
Steam	CS	0.2	0.4	50.7	15.4	0.7	23.3	3.4	7.2
pretreatment		(0.0)	(0.1)	(0.9)	(0.1)	(0.0)	(0.3)	(0.2)	(0.0)
	Poplar	0.3	0.3	58.2	7.7	2.0	28.5	2.8	0.8
		(0.0)	(0.0)	(0.2)	(0.1)	(0.1)	(0.2)	(0.1)	(0.0)
	LPP	0.1	0.1	53.4	0.2	0.4	42.8	0.6	0
		(0.0)	(0.0)	(1.0)	(0.0)	(0.1)	(0.2)	(0.0)	
	Organosolv	CS	0.4	0.2	60.3	12.2	1.0	26.2	1.6
pretreatment		(0.0)	(0.0)	(0.6)	(0.5)	(0.1)	(0.7)	(0.2)	(0.3)
	Poplar	0	0	78.2	4.5	2.2	18.2	1.0	0.1
				(0.3)	(0.0)	(0.1)	(0.5)	(0.1)	(0.0)
	LPP	0	0.1	77.7	1.5	2.0	25.4	0.3	0.1
			(0.0)	(0.7)	(0.0)	(0.0)	(0.1)	(0.0)	(0.0)

Ara: arabinose, Gal: galactose, Glu: glucose, Xyl: xylose, Man: mannose, AIL: acid insoluble lignin, ASL: acid soluble lignin, CS: corn stover, LPP: lodgepole pine. Values in parentheses are standard deviation.

3.1.3 Hydrolysis of pretreated substrates

To compare the possible influence of the lignin on hydrolysis rates and yields, the pretreated substrates were initially enzymatically hydrolyzed at 10 FPU/g-cellulose (1:1 FPU:CBU) at 50 °C, 5% (w/v) consistency. The reaction mixture was periodically collected and the glucose and protein contents in the supernatant were measured (Figures 5 and 6). The initial hydrolysis rates were high and then reached a plateau as time proceeded, which is a typical enzymatic hydrolysis profile for nearly all biomass substrates (Mansfield et al. 1999). The hydrolysis yields obtained from the steam pretreated substrates were lower than those obtained from the organosolv pretreated substrates (Kurabi et al. 2005). Lodgepole pine was the most recalcitrant to hydrolysis when compared to corn stover and poplar. The hydrolysis yields of the pretreated lodgepole pine were 39.4% (steam pretreatment) and 53.1% (organosolv pretreatment) after 72 h, while those of corn stover and poplar were above 75% (Figure 5).

The amount of protein detected in the supernatant initially decreased and then gradually increased (Figure 6) as hydrolysis proceeded. It has been shown that cellulases are slowly liberated into the supernatant as the reaction proceeds, after the initial adsorption of the enzymes onto the substrates during the hydrolysis of pure cellulose. It was also found that the lignin present in the substrates decreased the rate of cellulase desorption from the substrate (Tu et al. 2007a). In the work reported here, the same protein adsorption/desorption patterns were also observed. The amount of protein detected in the supernatant during hydrolysis varied depending on the substrate that was used. For example, when using organosolv pretreated corn stover and poplar the protein detected in the supernatant increased by 17.0% and 32.2% respectively during the hydrolysis, while it increased by less than 8%

with the other substrates. The pretreated lodgepole pine and steam pretreated corn stover retained significantly higher amounts of protein. It was apparent that the different cellulase desorption properties were largely caused by the different lignin content and properties in each of the pretreated substrates. However, the lignin content in the substrates did not correlate particularly well with protein detected in the supernatant during hydrolysis. For example, the organosolv pretreated lodgepole pine substrate, which was composed of 25.4% AIL, had a lower free protein content than did the organosolv pretreated corn stover, which had a 26.2% AIL content. It was apparent that the different protein adsorption profiles observed with the different pretreated substrates could not be explained just by the differences in their lignin content. For example, Pan et al. (2005b) showed that the removal of only a further 7% of the lignin from a steam pretreated Douglas-fir substrate using a cold NaOH treatment resulted in a 30% improvement in hydrolysis.

As the pretreated substrates from lodgepole pine were more recalcitrant to enzymatic hydrolysis, as compared to those obtained from corn stover and poplar, we next determined if the nature of the lignin was a major factor resulting in these observed differences during hydrolysis. Lignin was isolated from the pretreated substrates and the effects of the isolated lignins on hydrolysis and on the adsorption of cellulases were assessed.

Figure 5. Cellulose conversion of pretreated substrates by steam (A) and organosolv (B) pretreatment. Legends: corn stover (●) , poplar (○) , lodgepole pine (■). Hydrolysis conditions: 10 FPU Spezyme CP and 10 IU β -glucosidase g-1cellulose at 50 °C and 150 rpm. Substrates were suspended at 5% consistency in 50 mM Na-acetate buffer (pH 4.8).

Figure 6. Time courses of protein content in the supernatant during enzymatic hydrolysis of steam (A) and organosolv pretreated (B) substrates. Legends: corn stover (●) , poplar (○) , lodgepole pine (■). Hydrolysis conditions: 10 FPU Spezyme CP and 10 IU β -glucosidase g^{-1} cellulose at 50 °C and 150 rpm. Substrates were suspended at 5% consistency in 50 mM Na-acetate buffer (pH 4.8).

3.1.4 Comparison of mass and lignin yields

The comparison of mass and lignin yields of the two extraction methods is summarized in Table 6. The PTL showed higher mass and lignin yields than did the CEL. The lower yields of the CEL were likely caused by the partial solubility of the lignin in the dioxane: water (96:4) mixture. As a part of the lignin becomes water soluble during enzymatic hydrolysis, and would therefore not be recovered (Capanema et al. 2004a), the different PTL yields likely relate to the differences in the water solubility of the remaining lignin after pretreatment. It has been suggested that the hydrophobic interaction between the lignin and cellulases decreases hydrolysis yields (Eriksson et al. 2002; Ooshima et al. 1986). Thus, a comparison of the PTL yields as it relates to the hydrophobicity of the remaining lignin in the pretreated substrates was of interest to determine. The PTL yields decreased in the order of lodgepole pine, poplar, and corn stover, suggesting that the hydrophobicity of lignin in the pretreated substrates also decreases in this order. If the hydrophobic interaction is the main cause of the non-productive binding of cellulases, the higher content of low hydrophobic lignin in the pretreated substrates should contribute to a decrease in the non-productive binding of cellulases during enzymatic hydrolysis. A possible explanation for the high water solubility of the lignin would be its high carbohydrate contents and its low molecular mass (Capanema et al. 2004a; Duarte et al. 2000). Although the water soluble lignins were not analyzed, it was apparent that the initial substrates and the isolated lignins in corn stover contained a higher amount of carbohydrate than did the poplar and lodgepole pine (Table 7), indicating that the water soluble lignin from corn stover also contained more carbohydrates than did the poplar and lodgepole pine substrates. In addition to a higher carbohydrate content and low molecular mass, an increase in the hydrophilically-functionalized groups would increase the

hydrophilicity of lignin. The lignin present in steam pretreated softwood is known to have free carboxylic groups (Berlin et al. 2006; Shevchenko et al. 2001). Thus, it is likely that the lignins in the pretreated substrates in this study would also contain carboxylic groups, resulting in increased water solubility.

Table 6. Comparison of mass and lignin recovery during lignin isolations.

Sample			Mass yield (%)			Lignin yield (%)		
			Hydrolysis	Extraction	Total	AIL	ASL	Total
Corn stover	SP	PTL	13.7	n.d.	13.7	39.8	8.5	35.8
		CEL	27.3	27.6	7.5	27.6	4.0	24.6
	OS	PTL	19.4	n.d.	19.4	50.6	13.3	48.4
		CEL	36.8	28.6	10.5	35.7	17.7	34.7
Poplar	SP	PTL	22.8	n.d.	22.8	66.0	15.5	61.5
		CEL	36.5	32.2	11.8	38.5	10.1	36.0
	OS	PTL	17.1	n.d.	17.1	86.4	13.7	82.7
		CEL	20.1	74.9	15.1	79.2	11.9	75.7
Lodepole pine	SP	PTL	41.5	n.d.	41.5	78.9	69.2	78.8
		CEL	38.4	17.9	8.0	17.5	20.0	17.5
	OS	PTL	28.0	n.d.	28.0	99.5	21.8	98.6
		CEL	38.3	48.4	18.5	70.4	67.8	70.3

PTL: protease treated lignin, CEL: cellulolytic enzymatic lignin, SP: steam pretreatment, OS: organosolv pretreatment. n.d. not determined. For other abbreviations see Table 5.

3.1.5 Chemical properties of the isolated lignins

As was mentioned earlier, the pretreated substrates were extensively hydrolyzed by cellulases to reduce the amount of cellulose associated with the residual lignin, as it was shown previously that the cellulose would interact with the cellulases (Tu et al. 2007a), making it difficult to assess the role of lignin. Since the substrates from corn stover contained higher amounts of hemicelluloses (Table 5), both cellulases and xylanase were used for hydrolysis of this pretreated substrate. The protease treated lignin (PTL) was prepared by protease treatment to hydrolyze the remaining enzymes in the residues (Berlin et al. 2006). The cellulolytic enzyme lignin (CEL) was prepared by a dioxane: water (96:4) extraction of the enzymatically hydrolyzed residues (Hu et al. 2006).

The isolated lignin preparations contained a higher AIL content when compared to the respective initial substrates (Tables 5, 7). However, the isolated lignin fractions also contained small amounts of carbohydrates and ash, depending on the biomass feedstock, pretreatment, and isolation method used. It was apparent that the dioxane extraction method increased the purity of the lignin when compared to the protease treatment, due to the lower carbohydrate and ash content of these lignin fractions. The carbohydrate content of the lignins isolated from corn stover was higher than those fractions obtained from poplar and lodgepole pine. During the hydrolysis of the pretreated substrates to obtain the isolated lignin, an excess of cellulases, β -glucosidase, and xylanase (for corn stover) were used. It was probable that the remaining carbohydrates could not be hydrolyzed by the enzymes because of the steric hindrance caused by the lignin or, alternatively, by the recalcitrance of the bond between the cellulose and lignin in the form of a lignin-carbohydrate complex (LCC).

The nitrogen content of the isolated lignins was measured using an elemental analyzer as the nitrogen content in the substrates has been shown to act as a good indicator of the amount of residual enzymes associated with the substrates after the hydrolysis of the sample to obtain the isolated lignin (Berlin et al. 2006). The results indicated that the dioxane extraction was better than the protease treatment in decreasing the nitrogen content of the isolated lignins (Table 7). Although the nitrogen content of the isolated lignin from corn stover was higher than that from poplar and lodgepole pine, this is probably because of the high nitrogen content in the initial corn stover substrates rather than due to any cellulase associated nitrogen bound to the isolated lignin. The pretreated substrates from poplar and lodgepole pine had a nitrogen content of less than 0.20%, while steam and organosolv pretreated corn stover contained 0.66% and 0.59% nitrogen respectively.

Table 7. Chemical composition of isolated lignins from pretreated substrates.

Pretreatment	Sample		Neutral sugar compositions					Lignin analysis		Ash (%)	N (%)
			(%)					(%)			
			Ara	Gal	Glu	Xyl	Man	AIL	ASL		
Steam pretreatment	CS	PTL	1.0	0.4	17.6	4.9	0.4	67.7	2.1	14.8	1.66
			(0.1)	(0.0)	(0.5)	(0.0)	(0.0)	(0.3)	(0.1)	(0.2)	
		CEL	0.6	0.1	1.5	3.7	0.4	85.8	1.8	1.0	0.84
			(0.0)	(0.0)	(0.1)	(0.0)	(0.0)	(0.5)	(0.1)	(0.0)	
	Poplar	PTL	0	0	6.4	0.8	1.0	82.5	1.9	6.2	0.74
			(0.0)	(0.0)	(0.0)	(0.4)	(0.1)	(0.1)			
		CEL	0	0	0.5	0.3	0.4	93.1	2.4	0.4	0.33
			(0.0)	(0.0)	(0.0)	(0.3)	(0.0)	(0.0)			
	LPP	PTL	0	0	8.5	0.2	0.8	81.4	1.0	6.3	1.10
			(0.2)	(0.0)	(0.0)	(0.5)	(0.0)	(0.1)			
CEL		0.1	0.1	0.6	0.3	0.6	93.6	1.5	0	0.29	
		(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.6)	(0.1)			
Organosolv pretreatment	CS	PTL	0.2	0.1	18.9	4.3	0.8	68.3	1.1	22.1	1.13
			(0.0)	(0.0)	(1.0)	(0.0)	(0.0)	(0.3)	(0.0)	(0.3)	
		CEL	0.3	0.1	1.0	1.8	0.1	89.2	2.7	0.7	0.86
			(0.1)	(0.0)	(0.0)	(0.1)	(0.0)	(0.4)	(0.1)	(0.0)	
	Poplar	PTL	0.3	0	2.2	0.4	0.5	92.0	0.8	0.8	0.62
			(0.1)	(0.0)	(0.0)	(0.0)	(0.5)	(0.1)	(0.0)		
		CEL	0	0	0.4	0.2	0.2	95.4	1.4	0.2	0.28
			(0.0)	(0.0)	(0.0)	(0.0)	(0.8)	(0.0)	(0.0)		
	LPP	PTL	0	0	4.9	0.4	1.0	90.3	0.2	1.6	0.88
			(0.1)	(0.1)	(0.1)	(0.2)	(0.0)	(0.1)			
CEL		0.3	0.1	0.5	0.1	0.2	96.6	1.1	0	0.29	
		(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.6)	(0.0)			

Standard deviation of nitrogen contents were ± 0.05 . For other abbreviations see Table 5, 6. Values in parentheses are standard deviation.

3.1.6 Influence of isolated lignin on cellulose hydrolysis

In order to assess the effect of different types of lignin on cellulose hydrolysis, the isolated lignin preparations were added to the reaction mixture at a concentration of 20% of the weight of Avicel. The hydrolysis results were compared to that of pure Avicel as a control. Both the glucose and protein content in the supernatant were measured periodically to examine the extent of hydrolysis and protein adsorption. At high enzyme loadings (20 FPU and 40 CBU /g of cellulose), no differences in the hydrolysis yield between the control and lignin-containing samples were noted. However, at low enzyme loadings (5 FPU and 10 CBU/g of cellulose), the lignin was found to have a range of effects on hydrolysis. The degree by which the lignin reduced the hydrolysis yield varied considerably depending on the source of biomass, type of pretreatment and method of isolation (Figure 7).

Regardless of the isolation method and pretreatment used, lignin from corn stover did not affect the hydrolysis of Avicel. Alternatively, the degree of the lignin inhibitory effect from poplar and lodgepole pine varied considerably depending on the type of pretreatment and isolation method used. The addition of the PTL and the CEL from steam pretreated poplar decreased the hydrolysis yield of Avicel by 8.6 and 11.0%, respectively (Figure 7 A, B). It should be noted that when the hydrolysis was supplemented with the PTL from the lodgepole pine, the reduction in the yield (17.9%) was high compared to that obtained with the poplar lignin (Figure 7A). However, the CEL isolated from the steam pretreated lodgepole pine had a negligible effect on hydrolysis (Figure 7B). The organosolv lignin from lodgepole pine was found to be inhibitory resulting in a 23% and 25% decrease respectively for both the CEL- and PTL-supplemented lignin samples. As a significant decrease in the cost of cellulases is necessary for the economic conversion of lignocellulose-to-ethanol (Aden

2008), high hydrolysis yields at a low dosage of cellulases are desirable.

When we compared the two lignin isolation methods for their effect on hydrolysis, there were not obvious differences, with the exception of the lignin isolated from steam pretreated lodgepole pine. The PTL from steam pretreated lodgepole pine had a substantial, negative effect on hydrolysis, while the CEL was found to have no effect (Figure 7A, B). One significant difference between these two lignin samples is their yield during extraction (Table 6). It was apparent that the PTL lignin consisted of the majority of the lignin originally present in the pretreated substrate and that the dioxane did not extract that component of the lignin that had a more inhibitory effect on hydrolysis. The residual material remaining after dioxane extraction, characterized as milled wood residue (MWR) is known to contain more neutral carbohydrates than does milled wood lignin (MWL) (Aimi et al. 2005; Furuno et al. 2006). In this study, the neutral sugar content of the CEL from steam pretreated lodgepole pine was also lower when compared to those of the PTL suggesting that the lignin with the higher carbohydrate content had the greatest detrimental effect on the hydrolysis of cellulose. It has been reported that the chemical structures of the CEL and residual enzyme lignin (REL) from loblolly pine were different (Hu et al. 2006). These workers showed that the REL likely originated from the middle lamella, which typically has a lower methoxyl and phenolic hydroxyl content and is richer in *p*-hydroxyphenyl-propane units (H) when compared to the secondary wall (S2) from which the CEL mainly originated. Although further analyses are required, these results suggested that lignin components with carbohydrate lignin covalent bonds and H units in the pretreated steam substrates, such as those from lodgepole pine, have a negative effect on cellulose hydrolysis.

To determine the effect of lignin on the cellulase adsorption profile during hydrolysis

of Avicel, the protein content in the hydrolysis supernatant was measured. This provided an indirect indication about the amount of protein adsorbed to the solid fraction (Figure 8). It was apparent that the adsorption profile of the cellulases onto the substrates was influenced significantly by lignin. While more than 90% of the original protein was detected in the supernatant after 72 h of hydrolysis of Avicel, only relatively small amounts of the originally added protein was detected in the supernatants of the lignin containing substrates other than those supplemented with the lignin from organosolv pretreated corn stover. It is likely that the cellulase adsorption properties of the isolated lignins will be different depending on the types of biomass, pretreatment methods, and lignin isolation methods used.

Our work indicated that the hydrolysis yields of pretreated substrates correlated with the hydrolysis yields of Avicel in the presence of isolated lignins. High correlation ($r=0.88$) was observed when the PTLs were used (Figure 9A), while low correlation ($r=0.58$) was observed when the CELs were used (Figure 9B). The reason for the different correlations is likely due to the structural and chemical differences of the isolated lignins. As the lignins isolated by the protease method were prepared from the hydrolysis residues of the pretreated pulps, it should be structurally and chemically similar to the residues left after the hydrolysis of the pretreated pulps. In the case of the dioxane extraction method, part of the lignin in the enzymatic residual lignin was extracted by dioxane, as indicated by the lower total mass and lignin yields (Table 6). The better relationship between the hydrolysis yields of pretreated substrates and those of Avicel with the isolated lignin by the protease method indicated that the hydrolysis residues generated during hydrolysis of substrates affected the hydrolysis. As the lignin contents in the isolated lignin were increased compared to the initial substrates (Tables 5 and 7), it is likely that the lignin in the pretreated substrates affected the hydrolysis

yields and protein content detected in the supernatant.

3.1.7 Conclusions

A comparative analysis of twelve isolated lignins showed that the CEL contained lower amounts of carbohydrates and protein than did the PTL and that the isolated lignin from corn stover contained more carbohydrates than did the lignin derived from the poplar and lodgepole pine. The lower yields of AIL obtained from the corn stover when using the PTL method indicated that the lignin from the corn stover had a higher hydrophilicity than did the lignin from the poplar and lodgepole pine. The isolated lignin preparations were added to the reaction mixture containing crystalline cellulose (Avicel) and their possible effects on enzymatic hydrolysis were assessed. It was apparent that the lignin isolated from lodgepole pine and steam pretreated poplar decreased the hydrolysis yields of Avicel, whereas the other isolated lignins did not appear to decrease the hydrolysis yields significantly, likely because of the different adsorption properties of the isolated lignins. The hydrolysis yields of crystalline cellulose in the presence of isolated lignin preparations and those of pretreated substrates showed a good correlation, confirming the detrimental effects of residual lignin on the effective hydrolysis of pretreated lignocellulosic substrates.

Figure 7. Effect of isolated lignins on Avicel hydrolysis: in the presence of isolated lignins. PTLs from steam pretreatment (A), CELs from steam pretreatment (B), PTLs from organosolv pretreatment (C), CELs from organosolv pretreatment (D). Legends: Avicel (●), corn stover (○), poplar (□), lodgepole pine (Δ). Hydrolysis conditions: 50 °C and 5 FPU/g-cellulose, 10 IU β -glucosidase/g-cellulose. Substrates and isolated lignin were suspended at 2% (w/v) and 0.4% (w/v) consistency in Na-acetate buffer (pH 4.8).

Figure 8. Effect of isolated lignins on protein content in the supernatant during Avicel hydrolysis in the presence of isolated lignins. PTLs from steam pretreatment (A), CELs from steam pretreatment (B), PTLs from organosolv pretreatment (C), CELs from organosolv pretreatment (D). Legends: Avicel (●), corn stover (○), poplar (□), lodgepole pine (Δ). Hydrolysis conditions: 50 °C and 5 FPU/g-cellulose, 10 IU β-glucosidase/g-cellulose. Substrates and isolated lignin were suspended at 2% (w/v) and 0.4% (w/v) consistency in Na-acetate buffer (pH 4.8), respectively.

Figure 9. Correlation of Avicel conversion yields and pretreated substrates conversion yields using the PTL (A) and the CEL (B). For abbreviations see Table 5 and 6.

3.2 Enhancing the enzymatic hydrolysis of lignocellulosic biomass by increasing the carboxylic acid content of the associated lignin

3.2.1 Background

As mentioned earlier, it is recognized that the residual lignin, which is inevitably associated with the cellulose after pretreatment and post-treatment, significantly influences subsequent enzymatic hydrolysis (Chandra et al. 2007; Grethlein 1985; Grous et al. 1986; Mooney et al. 1999; Shevchenko et al. 2000). Two of the possible mechanisms by which lignin likely decreases the yield of cellulose hydrolysis are, a “physical” steric hindrance of the cellulose (Mooney et al. 1998) and the reversible or, more problematic, irreversible adsorption of cellulases onto the lignin rather than the cellulose (Berlin et al. 2006; Palonen et al. 2004; Sewalt et al. 1997). Cellulases have been thought to adsorb to lignin via hydrophobic interactions (Eriksson et al. 2002; Ooshima et al. 1986), ionic bond interactions (Berlin et al. 2006) and hydrogen bond interactions (Berlin et al. 2006; Pan 2008; Sewalt et al. 1997). However, the exact mechanisms by which cellulases interact with lignin and become inhibited have yet to be fully resolved. The elucidation of these possible inhibitory effects of lignin during hydrolysis should contribute to the refinement of current pre-and post-treatment processes, improve enzymatic hydrolysis of the cellulose and the economic viability of the overall biomass-to-ethanol process.

As shown in previous chapter, it was apparent that the isolated lignins varied in their inhibitory effects on the enzymatic hydrolysis of pure cellulose (Avicel). The isolated lignin from lodgepole pine softwood (except for the CEL obtained after steam pretreatment) and steam pretreated poplar decreased the hydrolysis yields of Avicel by ~25% after 72 h,

whereas the addition of the other lignin preparations did not result in any significant decrease in hydrolysis yields (Nakagame et al. 2010).

In this chapter, the physical and chemical properties of the isolated lignins were characterized to try to determine which lignin properties or characteristics influence the adsorption of cellulases on lignin and, consequently, decrease the enzymatic hydrolysis of cellulose. We also synthesized two dehydrogenative polymers (DHP), from coniferyl alcohol (CA) and ferulic acid (FA), to obtain lignin models with different chemical groups in the γ -position (alcohol and carboxylic acid, respectively) to examine the possible effect of the DHPs on cellulase adsorption and enzymatic hydrolysis of Avicel.

3.2.2 Cellulase adsorption onto lignin

The PTL and CEL lignin fractions which were described in the previous chapter, were isolated from steam and organosolv pretreated corn stover, poplar, and lodgepole pine (Nakagame et al. 2010), and were used to determine the possible effect of the physicochemical characteristics of lignin on the interaction between lignin and cellulases. The isolated lignins from lodgepole pine (except for the CEL from steam pretreatment) and steam pretreated poplar were shown to decrease the hydrolysis yields of Avicel, whereas the other isolated lignins did not significantly affect the hydrolysis yields (Nakagame et al. 2010). As it is unlikely that the surface of Avicel would be completely covered by lignin prior to and during enzymatic hydrolysis, we had assessed the effectiveness of the enzymatic hydrolysis of Avicel in the presence of the isolated lignin preparations. In this way, we hoped to gain an insight into the possible effects that non-productive binding of cellulases to lignin might have on hydrolysis rather than examining lignin as a physical barrier that restricts enzyme access to

the cellulose (Nakagame et al. 2010; Pan 2008).

The adsorption properties (maximum adsorption capacity, affinity, and binding strength) of cellulases to the isolated lignin preparations were assessed by measuring the protein concentration in the supernatant after incubation of each lignin with cellulase at 50°C for 3 h. The enzyme adsorption to the PTL and CEL lignin fractions (Table 8 and Figure 10) were in alignment with the Langmuir isotherms ($R^2 > 0.85$), as previously reported (Furuno et al. 2006; Tu et al. 2009).

The maximum adsorption capacities of the PTL fractions were higher than those of the CEL fractions, suggesting that the PTLs had a greater amount of adsorption sites when compared to the CEL lignin preparations. The maximum adsorption capacities obtained in this chapter were higher than those reported previously by Tu et al. (2009), for both the steam (10.20 mg/g-lignin) and organosolv (2.73 mg/g-lignin) lodgepole pine samples (Tu et al. 2009). The difference between our results and those reported earlier is likely due to the previous use of a surfactant (Tween 80), which is known to reduce non-productive binding of cellulases to lignin, to help in the isolation of the lignin from the pretreated lodgepole pine. The use of Tween 80 for lignin isolation would likely result in a lower maximum adsorption capacity, as shown previously (Eriksson et al. 2002), when compared to the lignin preparations isolated in the current study. The maximum adsorption capacities of the isolated lignin from our steam pretreated corn stover and poplar were also about 20 mg/g-lignin higher than the lignins isolated in other work (Kumar and Wyman 2009b). As it is known that the maximum amount of adsorbed protein increases with increasing temperature of incubation (Tu et al. 2009), the higher values for maximum protein adsorption in this current study may have resulted from the higher temperature (50 °C) employed. This is the same

temperature as that employed during the enzymatic hydrolysis of the pretreated substrates.

The affinity constants of cellulases to lignin ranged from 0.16 to 1.69 L/g-protein. The isolated lignins from lodgepole pine had higher affinity constants when compared with the other biomass substrates when using the same pretreatment process. The lignin isolated from the organosolv pretreatments had higher affinity constants than did the lignin obtained from the steam pretreatment substrates. However, the CEL from steam pretreated poplar did not follow the same trend (Table 8). As mentioned earlier, the binding strength is a value obtained when the maximum adsorption capacity is multiplied by the affinity values, which has been reported to correlate with the degree of desorption from the lignin (Kumar and Wyman 2009a). The correlation coefficient ($R^2 = 0.52$) between the binding strength and Avicel hydrolysis in the presence of the isolated lignin indicated that the desorption properties of cellulases may have affected the efficiency of Avicel hydrolysis (Figure 11). The variations in the adsorption properties and the effects of the different isolated lignin preparations from the three feedstocks and the two pretreatment processes on hydrolysis was likely related to their chemical and physical properties. Therefore, we next tried to determine how the chemical composition, various chemical functionalities and physical properties of each of the lignin preparations might influence hydrolysis.

Previously, it was shown that both the chemical and physical properties of lignin can affect cellulase adsorption properties (Berlin et al. 2006). It has also been shown that the chemical composition of the isolated lignins differed depending on the type of biomass or isolation method used (Nakagame et al. 2010). Although the PTL fractions contained a higher amount of neutral sugars than did the CEL fractions, the chemical composition of the lignin preparations did not correlate with either the adsorption properties of cellulases to the isolated

lignin or to the hydrolysis yields of Avicel in the presence of each of the lignin preparations. Thus, we next compared the physical properties and/or other chemical groups in the isolated lignin preparations to try to elucidate the factors responsible for the non-productive binding of cellulases to lignin and to try to develop strategies for reversing the observed decreased hydrolysis yields of Avicel when lignin was added.

Figure 10. Relationship between the amount of adsorbed Spezyme CP (mg/g lignin) and the free cellulases in the supernatant (mg/mL) for (A) Lodgepole pine steam pretreatment and (B) Corn stover organosolv pretreatment. Legend: PTL (●), CEL (○). For the other abbreviations, refer to Table 1. Spezyme CP was performed in 2.0 mL vials using a 0.5 ml of 50 mM acetate buffer (pH 4.8). The vials containing 1% (w/v) lignin and various loadings of cellulase (20-350 mg/lignin-g) were incubated for 3 h at 50 °C. The incubation mixtures were centrifuged (13,000 g, 10 min) followed by filtration using a low protein binding membrane with a pore size of 0.22 μ m.

Table 8. Cellulase adsorption parameters determined for the PTL and CEL lignin fractions.

Pretreatment	Sample		Maximum Adsorption capacity, σ_{lignin} (mg/g lignin)	Affinity Constants, A (L/g protein)	Binding Strength, $S_{\text{lignin}}=A \times \sigma$ (ml/g lignin)	R^2
Steam pretreatment	CS	PTL	88.95	0.35	31.50	0.85
		CEL	79.45	0.16	12.74	0.97
	Poplar	PTL	102.5	0.25	25.29	0.93
		CEL	46.76	1.69	78.95	0.98
	LPP	PTL	96.36	0.81	77.92	0.98
		CEL	39.88	0.86	34.46	0.87
Organosolv pretreatment	CS	PTL	60.87	1.30	79.16	0.97
		CEL	9.11	1.29	11.79	0.97
	Poplar	PTL	38.28	0.84	32.13	0.92
		CEL	34.80	1.06	36.72	0.92
	LPP	PTL	74.68	1.35	100.61	0.98
		CEL	34.91	1.43	50.05	0.95

PTL, protease treated lignin; CEL, cellulolytic enzyme lignin; CS, corn stover, LPP, lodgepole pine.

Figure 11. The relationship between binding strength and hydrolysis yields of Avicel, when supplemented with lignin, after 72 h hydrolysis.

3.2.3 Physical properties of isolated lignin

The particle size, surface area, and molecular weight of the isolated lignin preparations were next determined (Table 9) as it had been suggested that these physical properties could affect the adsorption properties of cellulases (Berlin et al. 2006). Despite their similar particle sizes, which ranged from 13.0 to 37.4 μm , the specific surface areas of the PTLs were from 1.4 to 15.0 times higher than those of the CELs, suggesting that the PTLs had a greater number of accessible fine pores. As mentioned earlier, the PTLs were prepared using cellulases to hydrolyze the carbohydrates in the pretreated substrates, followed by protease to solubilise and remove the absorbed, residual cellulases. Alternatively, the CELs were prepared using dioxane extraction and precipitation into water, which could result in an alteration of the physical properties of the lignin preparations (Nakagame et al. 2010). Although the maximum adsorption capacities in the PTLs were higher than those in the CELs, when compared with the lignin isolated from the same pretreatment and biomass, the maximum adsorption capacities did not correlate with the observed specific surface areas of the lignins.

The weight average molecular weight (M_w) and the number average molecular weight (M_n) of the acetylated CEL's were measured using gel permeation chromatography (GPC) (Berlin et al. 2006). Although the NMI/DMSO system has been reported to be effective for the dissolution of woody plants into organic solvents (Lu and Ralph 2003), the PTLs did not dissolve when using this solvent system. The M_n of the CELs ranged from 984 to 1661 and the M_w of the CELs ranged from 2646 to 8875 (Table 9). The degree of polydispersity of the samples was approximately 3.0 with the exception of the lignin isolated from the organosolv pretreated lodgepole pine, which had a polydispersity of 5.35 (Table 9). Although a lower

polydispersity value has been hypothesized to favor the adsorption of protein (Berlin et al. 2006), this tendency was not observed here. These results suggested that a chemical mechanism could be involved in the interaction between the cellulases and the isolated lignin preparations. To test this hypothesis the various chemical functionalities of the lignin samples were next investigated.

Table 9. Comparison of the physical properties of the isolated lignin preparations.

Pretreatment	Biomass	Lignin	Particle size (μm)	Specific surface area (m^2/g)	Molecular weights		
					Mn	Mw	D
Steam pretreatment	CS	PTL	29.4	21.1	n.d.	n.d.	n.d.
		CEL	27.4	4.9	1526	4515	2.96
	Poplar	PTL	37.4	19.8	n.d.	n.d.	n.d.
		CEL	20.8	14.1	1118	3699	3.31
	LPP	PTL	21.3	45.3	n.d.	n.d.	n.d.
		CEL	22.4	3.3	984	2646	2.69
Organosolv pretreatment	CS	PTL	36.6	25.0	n.d.	n.d.	n.d.
		CEL	28.9	7.4	1029	2815	2.74
	Poplar	PTL	23.0	6.3	n.d.	n.d.	n.d.
		CEL	26.5	1.9	1103	3111	2.82
	LPP	PTL	13.0	46.6	n.d.	n.d.	n.d.
		CEL	29.3	3.1	1661	8875	5.35

Mn: number average molecular mass, Mw: weight average molecular mass, D: polydispersity,
n.d.: not determined. For other abbreviations see Table 8.

3.2.4 ATR FTIR analysis of isolated lignins

ATR (attenuated total reflectance) FTIR analysis was employed to measure the amount of key chemical groups in the isolated lignin preparations (Figure 12) and to assess the possible effects of specific chemical functionalities within the lignin on the enzymatic hydrolysis of the pretreated lignocellulosic substrates. The relative absorbance was defined as the ratio of the intensity of different lignin bands to the intensity of the C-H vibration of the aromatic ring at 1500 cm^{-1} (Tables 10 and 11) (Guo et al. 2009; Nada et al. 1998). The relative absorbance of the various bands, due to the presence of the different chemical groups in the isolated lignin, was compared with hydrolysis yields of Avicel in the presence of the isolated lignin. It was apparent that the relative absorbance at 1710 cm^{-1} , which corresponds to the carboxylic acid functionality exhibited good correlation with the hydrolysis yields of Avicel ($R = 0.78$) (Figure 13). This indicated that carboxylic acid groups in the lignin may reduce the inhibitory effect of lignin on the cellulases. The isolated lignin from corn stover contained 1.3 to 5.9 times greater amounts of carboxylic acid groups than did those from poplar and lodgepole pine when the same pretreatment and lignin isolation methods were used (Tables 10 and 11). The greater amount of carboxylic acids in the corn stover was likely due to the higher amounts of in *p*-coumarate and ferulate groups found in the corn stover lignin when compared to softwoods and hardwoods (Kim and Ralph 2010). This could also contribute to the observed increased carboxylic content of the isolated lignin from corn stover.

Figure 12. FT-IR spectra of isolated lignin from organosolv (OS) pretreated substrates. 1, hydroxyl groups; 2, aldehyde; 3, carboxylic acid; 4, ketone; 5, ester; 6, ether. For abbreviations, see Table 8.

Figure 13. The relationship between the relative absorbance of carboxylic acid in the ATR-FTIR values of isolated lignin preparations and hydrolysis yields of Avicel after 72 h in the presence of each of the isolated lignin preparations.

Table 10. Summary of the chemical groups in the steam pretreated lignins.

		Steam pretreatment						
No.	Assignment	cm ⁻¹	PTL			CEL		
			CS	Poplar	LPP	CS	Poplar	LPP
1	Hydroxyl groups	3300-	0.83	0.53	0.49	0.47	0.34	0.40
	OH	3400	(0.07)	(0.02)	(0.02)	(0.02)	(0.03)	(0.02)
2	Aldehyde	1720	0.48	0.34	0.29	0.66	0.34	0.54
	C=O		(0.02)	(0.02)	(0.01)	(0.01)	(0.01)	(0.01)
3	Carboxylic acid	1710	0.64	0.42	0.33	0.81	0.42	0.64
	HO-C=O		(0.02)	(0.03)	(0.01)	(0.01)	(0.01)	(0.01)
4	Ketone	1650	1.28	0.62	0.68	0.76	0.33	0.46
	C=O		(0.00)	(0.02)	(0.00)	(0.01)	(0.01)	(0.00)
5	Ester	1250	2.17	1.30	1.37	1.90	1.45	1.64
	-O=C-O-		(0.05)	(0.01)	(0.02)	(0.02)	(0.02)	(0.02)
6	Ether	1120	4.05	2.21	1.56	2.29	2.66	1.49
	-O-		(0.18)	(0.07)	(0.01)	(0.09)	(0.05)	(0.01)

The relative intensity of chemical groups to the intensity of 1500 cm⁻¹ was shown. CA, coniferyl alcohol; FA, ferulic acid. For other abbreviations, see Table 8. Values in parentheses are standard deviation.

Table 11. Summary of the chemical groups in the organosolv pretreated lignins.

No.	Assignment	cm ⁻¹	Organosolv pretreatment					
			PTL			CEL		
			CS	Poplar	LPP	CS	Poplar	LPP
1	Hydroxyl groups	3300-	0.93	0.34	0.42	0.43	0.40	0.33
	OH	3400	(0.04)	(0.02)	(0.02)	(0.00)	(0.01)	(0.02)
2	Aldehyde	1720	0.62	0.27	0.10	0.69	0.35	0.14
	C=O		(0.01)	(0.03)	(0.01)	(0.00)	(0.01)	(0.01)
3	Carboxylic acid	1710	0.78	0.36	0.13	0.89	0.45	0.17
	HO-C=O		(0.00)	(0.02)	(0.01)	(0.02)	(0.00)	(0.01)
4	Ketone	1650	1.07	0.39	0.43	0.86	0.38	0.24
	C=O		(0.01)	(0.02)	(0.01)	(0.01)	(0.01)	(0.01)
5	Ester	1250	2.14	1.42	1.30	1.84	1.40	1.42
	-O=C-O-		(0.06)	(0.03)	(0.01)	(0.01)	(0.02)	(0.01)
6	Ether	1120	4.79	2.64	1.39	2.14	2.51	1.37
	-O-		(0.22)	(0.11)	(0.01)	(0.01)	(0.06)	(0.01)

The relative intensity of chemical groups to the intensity of 1500 cm⁻¹ was shown. CA, coniferyl alcohol; FA, ferulic acid. For other abbreviations, see Table 8. Values in parentheses are standard deviation.

Table 12. Summary of the chemical groups in the DHP from coniferyl alcohol and ferulic acid.

No.	Assignment	cm ⁻¹	DHP	
			CA	FA
1	Hydroxyl groups	3300-	0.34	0.26
	OH	3400	(0.02)	(0.05)
2	Aldehyde	1720	0.07	0.74
	C=O		(0.01)	(0.02)
3	Carboxylic acid	1710	0.10	0.81
	HO-C=O		(0.00)	(0.03)
4	Ketone	1650	0.34	0.59
	C=O		(0.03)	(0.01)
5	Ester	1250	1.04	1.55
	-O=C-O-		(0.01)	(0.01)
6	Ether	1120	1.05	1.59
	-O-		(0.02)	(0.09)

The relative intensity of chemical groups to the intensity of 1500 cm⁻¹ was shown. CA, coniferyl alcohol; FA, ferulic acid. For other abbreviations, see Table 8. Values in parentheses are standard deviation.

3.2.5 Solution state NMR

In addition to ATR FTIR analysis, quantitative ^{13}C NMR of the CELs from steam and organosolv pretreated lodgepole pine were measured to characterize and compare the isolated lignins (Table 13, Figures 14 and 15) (Berlin et al. 2006; Capanema et al. 2004b; Zhang and Gellerstedt 1999). We chose to study these two isolated lignin preparations for quantitative ^{13}C NMR analysis because the CEL from the steam pretreated lodgepole pine did not appear to influence the hydrolysis of Avicel while the CEL from the organosolv pretreated lodgepole pine decreased the hydrolysis yields by 25% (Nakagame et al. 2010). Milled wood lignin (MWL) was isolated from lodgepole pine and compared with the CEL from SP and OS pretreated lodgepole pine. Decreasing amounts of the alkyl-O- (90-58 ppm) showed cleavage of the lignin internal bonding, including the β -O-4 linkage, by the pretreatment. Organosolv pretreatment decreased the degree of condensation, while steam pretreatment increased it, indicating that steam pretreatment resulted in both degradation and condensation reactions (Shevchenko et al. 2001). It was apparent that the CEL from the steam pretreated lodgepole pine was enriched in aliphatic carbon (30-10 ppm), aliphatic COOR (carboxyl/ester groups, 175-168 ppm) and spirodienone (D)/quinone structures (182-180 ppm) when compared to the organosolv pretreated lignin (Table 13, Figures 14 and 15). Thus, it was possible that these groups were largely responsible for the observed differences in the behavior of the two lignin preparations during enzymatic hydrolysis. The higher amount of carboxylic groups in the steam pretreated lodgepole pine lignin corresponded with the results obtained from the ATR FTIR analysis (Tables 10 and 11). It has been shown that aliphatic carbons can be found in biomass in the form of extractives and lignin (Sievers et al. 2009). In this past study, which looked at the acid hydrolysis of loblolly pine wood, it was concluded that aliphatic carbon

must be formed during the pretreatment, and that the formation of aliphatic groups from carbohydrates involves the redistribution of hydrogen and oxygen (Sievers et al. 2009). In earlier work on steam pretreatment of *Pinus radiata*, resin and fatty acid extractives were reported to be included in the *P. radiata* lignin, which had been isolated from the pretreated substrates using acetone (Hemmingson 1987). As some of the extractives could not be removed by repetitive extraction with hexane, they concluded that some of the extractives had been incorporated into the isolated lignin (Hemmingson 1987). In the work reported here, further analysis is required to determine the derivation of the aliphatic carbons in the isolated lignins. The greater amount of quinone groups in the steam pretreated isolated lignin suggested that the phenolic group in lignin, which has also reported to be involved in the adsorption of cellulases, was decreased by steam pretreatment (Pan 2008; Sewalt et al. 1997).

Figure 14. ^{13}C NMR spectra of isolated lignin from lodgepole pine. (A) MWL, (B) CEL from steam pretreated lodgepole pine, (C) CEL from organosolv pretreated lodgepole pine. Numbers in figure are identical to those in Table 11.

Table 13. Signal assignment in the NMR spectrum of non-acetylated of isolated lignin.

no	range (ppm)	assignment	amount (per Ar)		
			LPP SP	LPP OS	MWL
1	210-200	nonconjugated CO	0.12	0.08	0.02
2	200-196	α -CO except A	0.05	0.04	0.04
3	196-193	CO in α -CO/ β -O-4 (A), B	0.04	0.03	0.04
4	193-191	Ar-CHO (C)	0.02	0.02	0.02
5	182-180	C-4 in D , quinone	0.04	0.01	0.01
6	175-168	aliphatic COOR	0.12	0.06	0.05
7	168-166	conjugated COOR	0.03	0.02	0.02
8	162-160	C-4 in h-units	0.04	0.02	0.02
9	157-151	C-3 in E _{et} , C-3,5 in F _{et} , C- α in B , C-3,6 in I , C-4 in conjugated CO/COOR etherified units	0.29	0.17	0.43
10	144.5-142.5	C-3 in G , C-4 in E _{ne} , C-4 in conjugated F , unknown	0.20	0.26	0.18
11	58-54	OMe, C- β in H , C-1 in D , C- γ in I	0.75	0.84	1.05
12	54-52	C- β in G and J	0.08	0.12	0.11
13	35-34	C- β in K , C- α in L	0.16	0.04	0.03
14	32.5-31.5	C- α in K	0.11	0.03	0.02
		clusters			
	162-142	"C _{Ar-O} "	1.98	1.72	2.14
	142-125	"C _{Ar-C} "	2.00	1.92	1.60
	125-102	"C _{Ar-H} "	2.14	2.48	2.38
	90-58	Alk-O-	0.91	1.59	2.11
	90-77	Alk-O-Ar, α -O-Alk	0.29	0.55	0.76
	77-65	γ -O-Alk, OH _{sec}	0.33	0.49	0.64
	65-58	OH _{prim}	0.29	0.55	0.56
		Degree of condensation	0.73	0.46	0.56

LPP, lodgepole pine; SP, steam pretreatment; OS, organosolv pretreatment; MWL, milled wood lignin.

Figure 15. Lignin substructures R=H, R'=CHO, CH=CH-CHO, COOH (Capanema et al. 2004b).

3.2.6 The possible effect of carboxylic groups present in lignin on enzymatic hydrolysis and on the adsorption of cellulases to lignin

The enzymatic hydrolysis yields of Avicel in the presence of the isolated lignin and the amounts of carboxylic acid in the isolated lignin measured by ATR FTIR showed good correlation (Figure 13), suggesting that the presence of carboxylic acids might reduce the negative effects of lignin on enzymatic hydrolysis. The results from solution state NMR also showed that the aliphatic carboxylic group content was higher in the steam pretreated lodgepole pine lignin than in the organosolv pretreated lodgepole pine lignin (Table 13, Figures 14 and 15). To assess the possible effects of the carboxylic acid groups present in the lignin preparations on the enzymatic hydrolysis of lignocellulose, DHPs were prepared from coniferyl alcohol (CA) and ferulic acid (FA), using previously reported methods (Wayman 1974). The molecular weights (Mw) of acetylated DHP from the CA and FA were 3176 and 1170, respectively. As expected, the ATR-FTIR analysis showed that the synthesized DHP from FA contained a greater amount of carboxylic acids when compared with the DHP from CA (Table 12). The adsorption isotherms of cellulases to the DHPs showed a good correlation with the Langmuir isotherm ($R^2 > 0.90$) (Table 14, Figure 16). The maximum adsorption capacity of DHP from CA was 64.9 mg/g-lignin, while the FA's capacity was 26.29 mg/g-lignin. The binding strength, which showed a moderate correlation with the hydrolysis yields of Avicel in the presence of the isolated lignin preparations, was 100.67 ml/g lignin for the DHP from CA and 56.93 ml/g lignin for the DHP from FA. The hydrolysis yields of Avicel in the presence of the DHPs showed that the DHP from CA decreased the hydrolysis yield of Avicel from 68.5 to 60.1% at an enzyme loading of 5 FPU and 10 CBU/g-cellulose after 72 h, while the DHP from FA did not affect the hydrolysis yield (Figure 17). Previous

work reported that the addition of 20 mM (3.88 mg/ml) of ferulic acid decreased the enzymatic hydrolysis of Avicel by 10% after 48 h (Pan 2008), and it was suggested that, if the phenolic hydroxyl groups and phenolic acids occupy one or more of the binding sites in the catalytic tunnel in cellulases, this could decrease cellulose hydrolysis. As the DHP from FA (5 mg/ml) did not appear to decrease the enzymatic hydrolysis of Avicel, it is possible that it did not access the catalytic tunnel in the cellulase due, possibly, to the higher molecular weight of its polymerized form. It is also possible that the increase in the carboxylic content of the lignin contributed both to an increase in the hydrophilicity and negative charge of the lignin, influencing the non-productive binding of the enzymes to the lignin. In addition, the negative charge imparted by additional carboxylic acid groups on the lignin may decrease enzyme binding by electrostatic repulsion due to the negative charge of the cellulase enzyme components at pH 4.8, which is typically employed during hydrolysis.

As mentioned earlier, steam pretreated softwood substrates have been reported to require subsequent post-treatment to increase hydrolysis yields (Pan et al. 2004b). The post-treatment was reported to increase hydrolysis yields by decreasing the lignin content and modifying the lignin structure. The positive correlation between the carboxylic acid contents in the lignin and hydrolysis yields of Avicel in this study suggested that oxidative agents such as ozone and hydrogen peroxide, which can increase the carboxylic content of lignin, could also enhance the solubilization/degradation of lignin (Kadla and Chang 2002; Lind et al. 1997). Previously it was found that post-treatment of steam pretreated Douglas-fir by alkali-oxygen increased enzymatic hydrolysis, although the substrate still contained more than 10% (w/w) lignin (Pan et al. 2004b). One possible explanation for the increase in hydrolysis yields is that the carboxylic acid content of the residual lignin was increased by

oxygen-delignification (Zhang et al. 2007). By increasing the hydrophilicity of the lignin and by increasing its carboxylic content, this may facilitate the solubilization of lignin in water and likely enhances the enzymatic hydrolysis of the pretreated substrates. We found that the addition of 1% (w/v) of liginosulfonic acid sodium salt, (which is known to be water soluble), to the reaction mixture containing steam pretreated lodgepole pine, resulted in an increase in the hydrolysis yield from 39.4 to 50.2% after 72 h hydrolysis at an enzyme loading of 10 FPU and 10 CBU/g-cellulose (Figure 18). This indicated that the water soluble lignin can, in some cases, increase enzymatic hydrolysis. Previously we had shown that some of the residual lignin was released during enzymatic hydrolysis of the pretreated substrates (Nakagame et al. 2010) indicating that these water soluble lignins might work to increase, rather than decrease, the hydrolysis yields of pretreated lignocellulosic substrates. Even if the lignins cannot be completely dissolved in water, the addition of hydrophilic groups such as carboxylic acid to the lignin would likely increase their hydrophilicity, thus reducing the non-productive binding of cellulases to lignin. In addition to chemical modification of lignin, genetic modification of lignin could, possibly, also be used to increase the carboxylic content of the lignin. Other workers have shown that lignin could be modified in this way by using monolignols such as coniferyl alcohol, and synapyl alcohol that were produced from the reduction of ferulic acid and sinapic acid (Ferrer et al. 2008; Ralph et al. 2004). By increasing the carboxylic acid content of the lignin of a woody plant by, for example increasing the amount of ferulic acid, will likely increase the hydrophilicity of lignin and should consequentially improve the enzymatic hydrolysis of the lignocellulosic substrates.

Table 14. Adsorption parameters determined for DHP from Coniferyl alcohol (CA) and Ferulic acid (FA).

		Maximum	Affinity	Binding	R^2
		Adsorption capacity,	Constants,	Strength,	
		σ_{lignin}	A (L / g protein)	$S_{\text{lignin}} = A \times \sigma$	
		(mg/g lignin)		(ml/g lignin)	
DHP	CA	64.91	1.55	100.67	0.99
	FA	26.29	2.17	56.93	0.90

Figure 16. Adsorption isotherm of cellulases to the DHP from coniferyl alcohol (○) and ferulic acid (●). Hydrolysis using Spezyme CP was performed in 2.0 mL vials using a 0.5 ml of 50 mM acetate buffer (pH 4.8). The vials containing 1% (w/v) lignin and various loadings of cellulase (20-350 mg/lignin-g) were incubated for 3 h at 50 °C. The incubation mixtures were centrifuged (13,000 g, 10 min) followed by filtration using a low protein binding membrane with a pore size of 0.22 μ m.

Figure 17. Effect of DHP from CA and FA on Avicel hydrolysis. Hydrolysis conditions: 50°C and 5 FPU/g-cellulose, 10 IU β -glucosidase/g-cellulose. Substrates and DHPs were suspended at 2% (w/v) and 0.4% (w/v) consistency in Na-acetate buffer (pH 4.8), respectively.

Figure 18. Effect of lignosulfonic acid sodium salt on cellulose conversion of steam pretreated lodgepole pine. Hydrolysis conditions: without lignosulfonic acid sodium salt (●), with lignosulfonic acid sodium salt 1% (w/v) (○). 10 FPU Spezyme CP and 10 IU β -glucosidase g^{-1} cellulose at 50 °C and 150 rpm. Substrates were suspended at 5% consistency in 50 mM Na-acetate buffer (pH 4.8).

3.2.7 Conclusions

Protease treated lignin (PTL) and the cellulolytic enzyme lignin (CEL), which had been isolated from steam and organosolv pretreated corn stover, poplar, and lodgepole pine, were characterized. They were then used to determine which physical and chemical properties of lignin might affect the enzymatic hydrolysis of lignocellulose. The adsorption of cellulases to the isolated lignins followed the Langmuir isotherm. Dehydrogenative polymer (DHP) lignins from FA, which were enriched in carboxylic acid groups when compared with the DHP from CA, adsorbed lower amounts of cellulases and did not decrease hydrolysis yields of Avicel when compared with the DHP from CA. Increasing the carboxylic acid content of the lignin appeared to reduce the non-productive binding of cellulases and consequently increased the enzymatic hydrolysis of the substrate. The carboxylic acid content of the isolated lignins, as measured by FTIR and NMR spectroscopy, was shown to play a key role in influencing the enzymatic hydrolysis of cellulose.

3.3 Characterization and effect on enzymatic hydrolysis of lignin isolated from steam pretreated Douglas-fir with different severity

3.3.1 Background

Douglas-fir, which is a valued commercial softwood in the Pacific coast, has been the subject of a great deal of bioconversion research (Boussaid et al. 2000b; Saddler et al. 1982; Saddler et al. 1983). However, even under optimized conditions, a post-treatment step which involved delignification after steam pretreatment, was necessary in order to obtain good hydrolysis of the cellulose. Typically, oxygen-alkali, hydrogen peroxide and alkaline have been used for the delignification step (Pan et al. 2004a; Pan et al. 2005d; Yang et al. 2002). After steam pretreatment, the lignin content of the water insoluble, cellulosic rich substrate increased when compared with the initial biomass, mainly due to the solubilization of the hemicelluloses into the liquid stream (Shevchenko et al. 2001). At higher pretreatment severities, the hemicellulose and some of the cellulose was degraded, whereas the vast majority of the lignin remained associated with the water insoluble, cellulosic rich fraction (Ewanick et al. 2007; Kabel et al. 2007; Shevchenko et al. 2001). It has been shown that, as the severity of the pretreatment increased, the proportion of lignin in the water insoluble fraction increased, primarily because of the loss of cellulose through acid hydrolysis. During SO₂-catalyzed steam pretreatment, the β -5 and β -O-4 aryl ethers (Hemmingson 1986; Hemmingson and Newman 1985) in the lignin tend to decrease, likely caused by acid catalyzed condensation reactions (Robert et al. 1988; Shevchenko et al. 2001).

During the saccharification step, the pretreated substrates are hydrolyzed to glucose by cellulases and β -glucosidase. It has been shown that the residual lignin, which is inevitably

associated with cellulose after the pretreatment, significantly influences enzymatic hydrolysis through both its influence on the physical properties of the pretreated substrates such as the surface area, pore size, crystallinity, and degree of polymerization (Chandra et al. 2007; Grethlein 1985; Grous et al. 1986; Mooney et al. 1999; Shevchenko et al. 2000) and directly through enzyme-lignin interactions (Nakagame et al. 2010; Berlin et al. 2006). As mentioned earlier, lignin is an essential structural component in nearly all plant material, and it is made up from a polymer of phenylpropane units produced through oxidative coupling of 4-hydroxyphenyl propanoid compounds (Humphreys and Chapple 2002). Previous work has suggested two possible mechanisms by which the presence of lignin decreases the yield of cellulose hydrolysis. These are through a physical, steric hindrance of the cellulose (Mooney et al. 1998) and through the reversible/irreversible adsorption of cellulases onto lignin rather than cellulose (Berlin et al. 2006; Palonen et al. 2004; Sewalt et al. 1997). Cellulases have been proposed to be adsorbed to lignin via hydrophobic (Eriksson et al. 2002; Ooshima et al. 1986), electrostatic (Berlin et al. 2006) and hydrogen bond interactions (Berlin et al. 2006; Pan 2008; Sewalt et al. 1997). However, as mentioned earlier, the exact mechanisms by which cellulases interact with lignin and become inhibited have yet to be fully elucidated.

In the previous chapter, we described the production and characterization of different types of lignin. Cellulolytic enzyme lignin (CEL), where the substrate is enzymatically hydrolyzed to remove carbohydrates with a subsequent extraction with aqueous dioxane, was been found to be structurally similar to milled wood lignin (MWL). However, it could be obtained in higher yields with less degradation occurring (Chang et al. 1975). Alternatively, rather than using dioxane extraction, the protease treated lignin (PTL) method provided lignin that remained after the complete enzymatic hydrolysis of the carbohydrate component of the

lignocellulosic substrate with subsequent treatment by proteases to remove any adsorbed proteins. The assumption was that this fraction more closely resembles the lignin-rich fraction that remained after enzymatic hydrolysis (Berlin et al. 2006).

One of the more puzzling observations from our previous studies on the steam pretreatment of softwoods is that, although an increase in pretreatment severity resulted in an increase in the lignin content of the water insoluble, cellulosic rich fraction, the ease of enzymatic hydrolysis of these more severely treated substrates increased (Ewanick et al. 2007). Although the lignin in the pretreated substrates is known to, usually, negatively affect enzymatic hydrolysis, it was apparent that other factors such as the redistribution, nature and charge of the lignin also plays a role such that the increased amount of lignin may be incidental to the role it plays in influencing the enzymatic hydrolysis of the residual cellulose. For example, increasing steam pretreatment severity might change the adsorption properties of cellulases to lignin, thus explaining the observation that higher pretreatment severities resulted in better enzymatic hydrolysis of the cellulose. The goal of the work described here was to help elucidate the factors that influence the cellulase and lignin interactions during enzymatic hydrolysis when using lignin preparations isolated from steam pretreated Douglas-fir chips treated at increasing severities.

3.3.2 Hydrolysis of the pretreated substrates

This study was undertaken to investigate how the interaction between lignin and cellulases might change over a range of steam pretreatment severities. The lignin associated with biomass substrates has been implicated as both a chemical and physical impediment to enzymatic hydrolysis (Berlin et al. 2006). When softwoods are pretreated by acid catalyzed

steam pretreatment, most of the hemicellulose is removed into the water soluble stream. Lower severities typically provide greater recovery of hemicelluloses while substrates pretreated at increased severity usually result in higher cellulose hydrolysis yields on subsequent enzymatic hydrolysis, despite the fact that these substrates contain a greater amount of lignin (Ewanick et al. 2007). In the current study, Douglas-fir wood chips were pretreated over a range of severities and we then assessed the effect that the lignin component might have on both enzymatic hydrolysis and substrate adsorption. We hoped to determine which lignin characteristics of the samples pretreated at elevated severity influenced the ability of cellulases to hydrolyze the substrates.

When SO_2 -catalyzed steam pretreatment was carried out at three different severities (190, 200, and 210 $^{\circ}\text{C}$), as the severity increased, the acid insoluble lignin (AIL) content increased from 38.4 to 45.0%, with a concomitant decrease in the glucose content from 59.4 to 54.4% (Table 15). The amount of hemicellulose remaining in the substrates ranged from only 2.3% in the substrate pretreated at 190 $^{\circ}\text{C}$, to an undetectable level at 210 $^{\circ}\text{C}$. We next hydrolyzed the steam pretreated Douglas-fir samples over a range of enzyme loadings from 5 to 80 FPU/g-cellulose (Figure 19). As is typical of the enzymatic hydrolysis most lignocellulosics hydrolysis, there was an initially rapid rate of hydrolysis followed by a slowing down and an eventual plateau was reached (Mansfield et al., 1999). As has been shown before (Ewanick et al. 2007), the initial hydrolysis rates and the extent of final cellulose hydrolysis of the pretreated substrates increased as the severity of the pretreatment increased (Figure 19). Although more than 95% of the cellulose was hydrolyzed in the substrates pretreated at 200 and 210 $^{\circ}\text{C}$ after 72 h, only 65% of the cellulose in the 190 $^{\circ}\text{C}$ treated samples was hydrolyzed at the higher enzyme loadings (Figure 19). It was apparent

that the lignin content of the substrates increased with increasing pretreatment severity while the ease of hydrolysis of the substrates also increased, suggesting that the lignin content *per se* did not affect enzymatic hydrolysis of the steam pretreated Douglas-fir.

One of the main factors which has been shown to influence the ease of enzymatic hydrolysis of pretreated lignocellulosic biomass is the “availability” of the cellulose contained in the substrate that is accessible to cellulolytic enzymes. Or as it has been termed the “accessible cellulose surface area” to the enzyme (Chandra and Saddler, 2009). It is likely that the cellulose accessible surface area which might have been reduced by the by steric hindrance of cellulose by lignin, is lower in the substrates pretreated at 190 °C than those in the substrates pretreated at 200 and 210 °C. This was thought possible as, in addition to the different enzymatic hydrolysis yields observed with each of the substrates (Figure 19), the amount of adsorbed protein to the substrate pretreated at 190 °C was significantly lower than was obtained with the substrates pretreated at 200 and 210 °C (Figure 20). In previous work where the enzymatic hydrolysis of refiner mechanical pulp (RMP) and delignified RMP from Douglas-fir were compared (Mooney et al. 1998), it was found that the delignified RMP was almost completely hydrolyzed at a cellulase loading of 60 FPU/g-cellulose, while only 20% of the cellulose in the un-delignified RMP was hydrolyzed, even at a cellulase loading of 240 FPU/g-cellulose. These workers attributed the observed differences in the enzymatic hydrolysis yields of the two substrates to differences in the accessible cellulose surface area which was estimated by comparing the adsorption capacity of the cellulases to the substrates. The absorption capacity of the RMP was approximately 25% which increased to nearly 100% upon delignification of the RMP substrate. Similar results were obtained in the current study where the substrates pretreated at 200 and 210 °C showed higher protein adsorption as the

protein loading was increased, whereas the protein adsorbed on the substrate pretreated at 190 °C did not show an increase in protein adsorption. This indicated that accessible surface area in the substrates pretreated at 190 °C was lower than that of the substrates pretreated at 200 and 210 °C.

It has also been shown that, in addition to differences in the accessibility of the substrates, changing the properties of the associated lignin via treatments such as sulphonation could also influence the degree of enzyme adsorption to the substrate (Mooney et al. 1998). In the previous chapter, we showed that by increasing the carboxylic acid of the lignin component, the non-productive binding of cellulases to lignin could be alleviated and, consequently, hydrolysis yields increased. Earlier work has shown that, by increasing the steam pretreatment severity, the structure of substrate lignin was changed (Shevchenko et al. 2001). Thus it is likely that the cellulase adsorption profile to the lignin might also be changed by differences in the steam pretreatment severity.

As previous work had shown that the interaction of cellulases with isolated lignins could help gain us a better insight into the effect of lignin on enzymatic hydrolysis (Berlin et al. 2006; Nakagame et al. 2010; Tu et al. 2009) the lignins from the substrates pretreated at elevated severity were isolated. They were then used to assess the effects that increasing the severity of steam pretreatment had on the structure of the resulting substrate lignin and the consequential effects that the differences in lignin structure might have on enzymatic hydrolysis.

Table 15. Composition of differentially pretreated Douglas-fir upon Klason analysis of the water insoluble component

Pretreatment Severity		Neutral sugar compositions					Lignin	Ash	
temperature (°C)	(log R ₀)	(%)					analysis	(%)	
							(%)		
		Glu	Xyl	Man	Gal	Ara	AIL	ASL	
190	3.34	59.4	0.9	1.2	0.1	0	38.4	0.5	0.1
		(0.7)	(0.1)	(0.1)	(0.0)		(0.1)	(0.0)	(0.0)
200	3.64	56.5	0.4	0.8	0	0	41.6	0.5	0.1
		(1.4)	(0.0)	(0.1)			(0.4)	(0.0)	(0.0)
210	3.93	54.4	0	0	0	0	45.0	0.5	0.1
		(0.5)					(0.5)	(0.0)	(0.0)

Glu: Glucose, Xyl: xylose, Man: mannose, Gal: galactose, Ara: arabinose, AIL: acid insoluble lignin, ASL: acid soluble lignin. Values in parentheses are standard deviation.

Figure 19. Cellulose hydrolysis of Douglas-fir steam pretreated at increasing (A) 190 °C, (B) 200 °C, (C) 210 °C severities. Legends: 5 FPU/10 CBU (●), 10 FPU/20 CBU (■), 20 FPU/40 CBU (▲), 40 FPU/80 CBU (○), 60 FPU/120 CBU (□), 80 FPU/160 CBU (△). Pretreated substrates were suspended in 50 mM Na-acetate buffer (pH 4.8) at 2% consistency and hydrolyzed by (Spezyme CP) and β -glucosidase (Novozymes 188) at a ratio of 1 FPU/g-cellulose to 2 CBU/g-cellulose at 50 °C and 150 rpm.

Figure 20. Relation between protein loading and adsorbed protein to the pretreated substrates after 1 h. Legend: Douglas-fir steam pretreated at 190 °C (●), 200 °C (○), 210 °C (■). Pretreated substrates were suspended in 50 mM Na-acetate buffer (pH 4.8) at 2% consistency. Cellulases (Spezyme CP) and β -glucosidase (Novozymes 188) were added at a ratio of 1 FPU/g-cellulose to 2 CBU/g-cellulose at 50 °C and 150 rpm. After 1 h, the samples were centrifuged, and protein contents in the supernatant were measured.

3.3.3 Lignin isolation

To determine the effect of lignin on the enzymatic hydrolysis of the pretreated substrates in greater detail, lignins were isolated using the two methods that have been described in detail in the previous chapters (Nakagame et al. 2010). It was desirable to have lignin samples containing as low amounts of carbohydrates as possible to decrease the influence the residual carbohydrate might have on enzymatic hydrolysis. However, the dilemma was that, by using higher enzyme concentrations to obtain better removal of this residual cellulose, we may in fact be adding more protein to the lignin and further “contaminating” the lignin. In effect, decreasing the carbohydrate at the expense of adding extraneous protein (Capanema et al. 2004a). Therefore, we first wanted to determine the minimum enzyme loading that could be used to achieve effective lignin isolation. To do this the pretreated substrates were hydrolyzed over a range of enzyme dosages at a cellulases to β -glucosidase ratio of (1:2) (Figure 19). As mentioned above, although almost 100% hydrolysis was obtained after 72 h with the substrates pretreated at 200 and 210 °C at enzyme loadings of 60 FPU and 120 CBU/g-cellulose, only 65% of the substrate pretreated at 190 °C was hydrolysed, even at high enzyme loadings. Thus to compromise and minimize both carbohydrate and protein contamination of the isolated lignin, enzyme loadings of 60 FPU and 120 CBU/g-cellulose were assessed for their effectiveness in isolating lignin from the pretreated substrates.

As mentioned earlier, the protease treated lignin (PTL) refers to the lignin remaining after intensive enzymatic hydrolysis of the carbohydrate component in the lignocellulosic substrate with subsequent treatment by proteases to remove any adsorbed proteins on the lignin-rich residues, with the assumption that this fraction more closely resembles the

lignin-rich fraction remaining after enzymatic hydrolysis (Berlin et al. 2006; Nakagame et al. 2010). It is recognized that part of the lignin becomes water soluble during enzymatic hydrolysis and is therefore not recovered (Capanema et al. 2004a; Nakagame et al. 2010). Thus, the differences in the PTL yields likely indicate the differences in the water solubility of the substrate lignin after pretreatment. The PTL yields obtained from the three steam pretreated Douglas-fir samples was about 95% and this yield did not change over the different severities (Table 16). In the previous chapter, the PTL yields from steam pretreated lodgepole pine were found to be about 78%, whereas those from steam pretreated corn stover were about 35% (Nakagame et al. 2010). The higher yields of the PTLs from Douglas-fir indicated that the lignin in the steam pretreated Douglas-fir was likely more hydrophobic as less of the material was lost in solution during enzymatic hydrolysis. The acid insoluble lignin (AIL) content of the PTL increased from 71.6 to 91.4% as the severity of the pretreatment increased, likely due to the lower amount of neutral sugars present in the lignins from substrates pretreated at the higher severities (Table 17).

As mentioned earlier, the cellulolytic enzyme lignin (CEL) refers to the lignin from the steam pretreated substrates which have been enzymatically hydrolyzed to remove residual carbohydrates, then the lignin-rich fraction is subsequently extracted with aqueous dioxane. The yield of CELs was significantly lower than was that of the PTLs, probably due to their lower extractivity in dioxane (96%, v/v) (Table 16). This had also been found earlier when trying to make CEL from steam pretreated lodgepole pine (Nakagame et al. 2010). With increasing pretreatment severity, the CELs yields increased from 10.9 to 19.8% (Table 16). The CELs also contained more than 95% acid insoluble lignin, probably due to the lower amount of carbohydrate and ash in these samples (Table 17). The RELs, which refers to the

residual lignin after extraction with dioxane (96%, v/v), contained higher amounts of neutral sugars compared to the PTLs and CELs (Table 17). The chemical composition of the RELs was similar to that of the PTLs.

Having established the chemical composition of these samples we next wanted to assess the effect that the three types of isolated lignins (PTL, CEL, and REL) might have on enzymatic hydrolysis.

Table 16. Comparison of mass and lignin recovery during lignin isolations.

Pretreatment temperature (°C)		Mass yield (%)			Lignin yield (%)		
		Hydrolysis	Dioxane	Total	AIL	ASL	Total
		extraction					
190	PTL	65.9	n.d.	65.9	95.7	45.3	95.1
	CEL	72.5	4.7	3.4	11.2	4.2	10.9
200	PTL	51.3	n.d.	51.3	95.3	34.1	94.6
	CEL	58.9	8.9	5.2	15.8	7.0	15.5
210	PTL	49.5	n.d.	49.5	88.6	31.8	94.5
	CEL	59.0	12.4	7.3	20.2	6.3	19.8

PTL: protease treated lignin, CEL: cellulolytic enzyme lignin. For other abbreviations, refer to Table 15. Initial substrate weight was defined as 100%.

Table 17. Chemical composition of isolated lignins from pretreated substrates.

Pretreatment temperature (°C)		Neutral sugar compositions					Lignin		Ash	N
		(%)					(%)		(%)	(%)
		Glu	Xyl	Man	Gal	Ara	AIL	ASL		
190	PTL	23.8	0.4	0.9	0	0	71.6	0.4	1.4	0.87
		(0.4)	(0.0)	(0.0)			(1.1)	(0.0)		
	CEL	1.2	0	0.4	0	0	95.5	1.6	0.4	0.36
		(0.1)		(0.0)			(0.3)	(0.0)		
	REL	27.4	0.3	1.3	0	0	72.0	0.6	0	1.73
		(0.4)	(0.0)	(0.1)			(0.4)	(0.0)		
200	PTL	9.3	0	0.5	0	0	87.6	0.3	0.5	0.71
		(0.1)		(0.1)			(0.6)	(0.0)		
	CEL	0.7	0	0	0	0	95.6	1.4	0.2	0.26
		(0.1)					(0.4)	(0.0)		
	REL	12.3	0	0.9	0	0	89.6	0.6	0.4	1.68
		(0.2)		(0.0)			(0.3)	(0.0)		
210	PTL	7.9	0	0.5	0	0	91.4	0.3	1.2	0.58
		(0.1)		(0.0)			(0.9)	(0.0)		
	CEL	0.9	0	0	0	0	95.3	0.9	0.4	0.35
		(0.1)					(0.4)	(0.0)		
	REL	10.3	0	0.8	0	0	88.0	0.5	0	1.62
		(0.2)		(0.0)			(0.9)	(0.0)		

PTL: protease treated lignin, CEL: cellulolytic enzyme lignin, REL: Residual enzyme lignin,
 For other abbreviations, see Table 15. Values in parentheses are standard deviation. Standard
 deviation of nitrogen contents was ± 0.05 .

3.3.4 Influence of the isolated lignins on cellulose hydrolysis

In order to assess the effect of lignin on cellulose hydrolysis, the isolated lignin preparations were added to reaction mixtures containing pure cellulose (Avicel) at a ratio of 1:5 of lignin to Avicel. It was apparent that all of the lignin preparations decreased the hydrolysis yields from 15.2 to 29.0% when compared with that of the Avicel control (Figure 21). The possible negative effects of residual lignin present in delignified steam pretreated Douglas-fir substrates on the enzymatic hydrolysis has also been studied (Kumar et al. 2010; Lu et al. 2002). A similar trend was observed when using these isolated lignins, with a decrease in the hydrolysis of Avicel observed by the addition of the isolated lignins.

The lignin preparations isolated from the samples treated at higher pretreatment severities tended to decrease the enzymatic hydrolysis of Avicel to a greater degree while the PTL and CEL fractions isolated from the substrate pretreated at 190 °C affected the hydrolysis of the substrate the least. For the PTL fractions, the Avicel hydrolysis yields after 72 h were decreased with increasing pretreatment severity by 45.5% (190 °C), 40.9% (200 °C), and 39.2% (210 °C) respectively. The 72 h Avicel hydrolysis yields decreased in the presence of the CEL isolated from the pretreated substrate by 49% (190 °C), 45% (210 °C), and 42% (200 °C) respectively. The RELs, which are the residual lignins remaining after dioxane extraction when isolating the CELs, had the least negative effect on the Avicel hydrolysis. It was apparent that varying the pretreatment severity did not affect the enzymatic hydrolysis of Avicel when combined with the RELs (about 53% after 72 h). This suggested that the lignin rather than carbohydrates remaining in the enzymatically hydrolyzed residues had more of a negative effect on the enzymatic hydrolysis yields of Avicel as the CELs contained higher amounts of acid insoluble lignin (AIL) than did the

RELs (Table 17).

As mentioned earlier, the enzymatic hydrolysis yield of the steam pretreated substrate at 190°C was lower than those obtained after pretreatment at 200 and 210°C (Figure 19). However, in contrast to the enzymatic hydrolysis of the starting substrates, combining the isolated lignins from the substrate pretreated at 190°C, resulted in the highest hydrolysis yields when compared to Avicel hydrolysis obtained when adding lignin preparations isolated from the substrates pretreated at 200 and 210°C. Therefore, the Avicel hydrolysis yields obtained in the presence of the isolated lignins could not clearly explain the differences in enzymatic hydrolysis yields of the steam pretreated substrates pretreated at the different severities. As previous work (Pan 2008) had indicated the decreasing hydrolysis yields of Avicel in the presence of the lignin isolated from the substrates pretreated at higher severity might be the result of cellulases adsorption to lignin, we next compared the adsorption of cellulases to the PTL, CEL and REL lignins.

Figure 21. The influence of isolated lignins from substrates pretreated at temperatures of (A) 190 °C, (B) 200 °C, (C) 210 °C, on Avicel hydrolysis. Legends: Avicel (●), PTL (○), CEL (■), REL (□). Hydrolysis conditions: 50 °C and 10 FPU/g-cellulose, 20 IU β -glucosidase/g-cellulose. Substrates and isolated lignin were suspended at 2% (w/v) and 0.4% (w/v) consistency in Na-acetate buffer (pH 4.8).

3.3.5 Adsorption isotherms of cellulases to lignin

The non-productive binding of cellulases to lignin is known to be detrimental to enzymatic hydrolysis (Berlin et al. 2006; Palonen et al. 2004; Sewalt et al. 1997). Thus, to determine the amount of non-productive binding of cellulases to the isolated lignin, adsorption isotherms of cellulases to the isolated lignin were measured by incubation of cellulases with the isolated lignins at 50 °C for 3 h. The maximum adsorption capacity, affinity, and binding strength were estimated by measuring the amount of protein present in the supernatant.

The adsorption of cellulases to the isolated lignin followed the Langmuir isotherm ($R^2 > 0.83$). The maximum adsorption capacity of the isolated lignins ranged from 32.03 to 81.99 mg/g (Table 18, Figure 22). The PTLs and the CELs showed higher maximum adsorption capacities when compared to the RELs. The maximum adsorption capacities of the CELs and the RELs increased with increasing pretreatment severities, while for the PTLs, the substrate pretreated at 200 °C showed the highest maximum adsorption capacity (70.80 mg/g). The differences between the maximum adsorption of the PTLs and the CELs were not as pronounced when compared to earlier work with steam pretreated lodgepole pine (pretreated at 200 °C) where the maximum adsorption of the PTL's and CEL's were 96.36 and 39.88 mg/g , respectively (Nakagame et al. 2010). The affinity of the isolated lignin varied from 0.48 to 4.19 L/g-protein (Table 18). Binding strength, which was reported to correlate with the desorption of cellulases (Kumar and Wyman 2009a), increased for the PTLs and the CELs with increasing pretreatment severity. The binding strength of the cellulases to the lignin fractions paralleled the enzymatic hydrolysis yields when the lignins were supplemented to the Avicel ($R^2=0.87$) (Figure 23) indicating that the

enzymatic hydrolysis of Avicel in the presence of lignin was determined by both the adsorption and desorption of cellulases to lignin. The CELs, which contained a higher concentration of AIL than did the RELs (Table 17), exhibited a higher affinity and binding strength for cellulases, suggesting again that the lignin in the pretreated substrates extracted by dioxane (96%, v/v) bound non-productively to the cellulases. It was evident that the lignin isolated from the samples at higher severities had a higher affinity for cellulases and this resulted in the non-productive binding of the enzymes to these lignins when they were added to Avicel. As it was likely that changes in lignin structure occurred as the pretreatment severity was raised and these changes were responsible for the increased adsorption of the enzymes to lignin, we next wanted to characterize the different lignins to see if we could better understand these enzyme-lignin interactions.

Figure 22. Relationship between amount of adsorbed cellulases to lignin (mg/g-lignin) and free cellulase in supernatant (mg/mL) for (A) pretreated at 190 °C, (B) 200 °C, and (C) 210 °C. Legend: PTL(●), CEL(○), REL(■). SP: steam pretreatment, OS organosolv pretreatment. For other abbreviations, refer to Table 1. Spezyme CP was performed in 2.0 mL vials using a 0.5 ml of 50 mM acetate buffer (pH 4.8). The vials containing 1% (w/v) lignin and various loadings of cellulase (20-350 mg/lignin-g) were incubated for 3 h at 50 °C. The incubation mixtures were centrifuged (13,000 g, 10 min) followed by filtration using a low protein binding membrane with a pore size of 0.22 μ m.

Table 18. Cellulase adsorption parameters determined for PTL, CEL, REL.

Pretreatment	Sample	Maximum	Affinity,	Binding Strength,	R^2
temperature		Adsorption capacity, $A(L / g \text{ protein})$		$S_{\text{lignin}} = A \times \sigma$	
($^{\circ}\text{C}$)		$\sigma_{\text{lignin}}(\text{mg/g lignin})$		(ml/g lignin)	
190	PTL	58.88	2.57	151.15	0.99
	CEL	33.15	2.87	95.31	0.98
	REL	32.03	0.48	15.23	0.84
200	PTL	70.80	3.19	226.01	0.99
	CEL	79.27	1.86	147.49	0.98
	REL	34.21	1.30	44.49	0.95
210	PTL	63.59	4.19	266.33	0.99
	CEL	81.99	2.36	193.09	0.98
	REL	49.95	0.76	37.92	0.83

For abbreviations, refer to Table 15.

Figure 23. Relationship between binding strength and cellulose conversion.

3.3.6 Spectroscopic analysis of lignin structure

The chemical structures of the isolated lignin were determined by using ATR FTIR and ^{13}C NMR to try to characterize the different chemical groups in the isolated lignins. All of the isolated lignins were analyzed by ATR FTIR. However, only the CELs were analyzed by ^{13}C NMR due to the limited solubility of the PTLs and the RELs in organic solvents.

For ATR FTIR analysis, the relative amounts of the chemical groups in the isolated lignins were determined by calculating the ratio of the intensity of the different lignin bands in comparison to the intensity of C-H vibration of the aromatic ring at 1500 cm^{-1} (Nada et al. 1998) (Table 19, Figure 24). We could see that the amount of hydroxyl groups (3300 cm^{-1}) decreased with increasing pretreatment severity, which could result from both structural changes in the lignin and the lower carbohydrate content within the lignin sample. Similarly, the higher amounts of hydroxyl groups detected in the PTLs and RELs samples could also be caused by the higher carbohydrate content of these lignins preparations (Table 17). It was observed that the aldehyde (1720 cm^{-1}) and carboxylic acid (1710 cm^{-1}) contents of the steam pretreated lignins were higher than those in the MWL, suggesting that lignin was oxidized during the steam pretreatment. When the relative amounts of the chemical groups in the isolated lignin were compared with the enzymatic hydrolysis yields of Avicel, it was evident that there was no significant correlation between the relative amounts of specific chemical groups and the hydrolysis yields of Avicel obtained in the presence of the lignins. This was in contrast to previous work that showed that the amount of carboxylic acid groups in the pretreated substrates correlated positively with enzymatic hydrolysis of Avicel (Nakagame et al. 2010). However, the same trend was not observed when the isolated lignins from the steam pretreated Douglas-fir were plotted. In the current case, the coefficient of correlation

did not change ($R^2=0.61$) while it did in the previous cases, which included the isolated lignins from steam and organosolv pretreated corn stover, poplar and lodgepole pine. The likely reason for the lower coefficient of correlation in this study is smaller range of variation in the amounts of carboxylic acid groups in the lignin isolated from the steam pretreated Douglas-fir samples. As the earlier work utilized corn stover, poplar and lodgepole pine substrates pretreated by both steam and organosolv pretreatments a much greater variation was obtained.

When the ^{13}C NMR spectrum of non-acetylated CELs were quantitatively measured and compared with the results from milled wood lignin (MWL) (Table 20, Figure 25 and 26) the decrease in alkyl-O- (90-58 ppm) after steam pretreatment clearly indicated a cleavage of internal bonds within lignin including the β -O-4 linkages. It was apparent that with increasing pretreatment severity, the degree of lignin condensation (DC), (which was calculated according to (Capanema et al. 2004b) (see below)) increased from 0.51 (MWL) to 0.87 (210 $^{\circ}\text{C}$), confirming that steam pretreatment caused both degradation and condensation reactions, as described previously (Li et al. 2007; Shevchenko et al. 2001).

The degree of condensation was determined as follows:

$$\text{DC} = (3.00 - \text{H}) - [(\text{I}_{125-103}) \text{C} + 2 \times \text{D}]$$

where $\text{I}_{125-103}$ is the integral value at δ 125–103 ppm calculated based on the spectra of the non-acetylated lignins; H is the amount of H-units; C and D correspond to chemical structures in Figure 26. The non-conjugated CO (210-200 ppm) was also higher in the steam pretreated substrates, which corresponded with the results obtained previously from the ATR FT IR analysis.

From the spectroscopic analysis of lignin structure, it was apparent that the lignin in Douglas-fir was degraded and condensed by steam pretreatment. As the hydroxyl and carboxylic acid content of the residual lignin tended to decrease with increasing pretreatment severity, it was presumed that the hydrophobicity of the lignin would also increase. Since hydrophobic interactions have been reported to be one of the main factors involved in the non-productive binding of cellulases (Berlin et al. 2006; Palonen et al. 2004; Sewalt et al. 1997), an increase in the steam pretreatment severity could increase both the hydrophobicity of the lignin and consequently the non-productive binding of the cellulases to the residual lignin.

Figure 24. FT-IR spectra of the MWL and CELs from steam pretreated Douglas-fir. 1, hydroxyl groups; 2, aldehyde; 3, carboxylic acid; 4, ketone; 5, ester; 6, ether.

Table 19. Summary of chemical groups in the isolated lignin fractions.

			Pretreatment temperature (°C)									MWL
			190			200			210			
No.	Assignment	cm ⁻¹	PTL	CEL	REL	PTL	CEL	REL	PTL	CEL	REL	
1	Hydroxyl groups	3300-	0.50	0.32	0.58	0.36	0.30	0.41	0.32	0.29	0.43	0.35
	OH	3400	(0.03)	(0.01)	(0.00)	(0.01)	(0.03)	(0.03)	(0.01)	(0.02)	(0.01)	(0.01)
2	Aldehyde	1720	0.19	0.43	0.23	0.22	0.38	0.24	0.22	0.34	0.18	0.17
	C=O		(0.02)	(0.01)	(0.02)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)
3	Carboxylic acid	1710	0.21	0.44	0.26	0.24	0.41	0.26	0.24	0.38	0.25	0.19
	HO-C=O		(0.02)	(0.00)	(0.03)	(0.01)	(0.02)	(0.00)	(0.01)	(0.01)	(0.00)	(0.01)
4	Ketone	1650	0.44	0.39	0.68	0.42	0.36	0.59	0.38	0.35	0.59	0.41
	C=O		(0.03)	(0.02)	(0.05)	(0.02)	(0.02)	(0.00)	(0.02)	(0.01)	(0.01)	(0.01)
5	Ester	1250	1.25	1.31	1.16	1.25	1.37	1.19	1.25	1.36	1.23	1.23
	-O=C-O-		(0.05)	(0.03)	(0.01)	(0.01)	(0.02)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)
6	Ether	1120	1.62	1.30	1.53	1.24	1.34	1.47	1.29	1.34	1.48	1.26
	-O-		(0.01)	(0.00)	(0.05)	(0.00)	(0.01)	(0.01)	(0.01)	(0.02)	(0.01)	(0.02)

Intensity of 1500 cm⁻¹ was set as 1 and relative intensities of specific chemical were calculated.

For abbreviations, see Table 16. Values in parentheses are standard deviation.

Figure 25. ^{13}C NMR spectra of isolated lignin. Numbers in the figure are identical to those listed in Table 20.

Table 20. Signal assignment in the NMR spectrum of non-acetylated of isolated lignin.

no	range (ppm)	assignment	amount (per Ar)			
			190 °C	200 °C	210 °C	MWL
1	210-200	nonconjugated CO	0.11	0.12	0.09	0.02
2	200-196	α -CO except A	0.07	0.06	0.04	0.06
3	196-193	CO in α -CO/ β -O-4 (A), B	0.06	0.06	0.04	0.05
4	193-191	Ar-CHO (C)	0.03	0.03	0.02	0.03
5	182-180	C-4 in D, quinone	0.02	0.02	0.01	0.01
6	175-168	aliphatic COOR	0.10	0.12	0.09	0.04
7	168-166	conjugated COOR	0.03	0.03	0.02	0.02
8	162-160	C-4 in h-units	0.03	0.04	0.03	0.01
9	157-151	C-3 in E _{et} , C-3,5 in F _{et} , C- α in B, C-3,6 in I, C-4 in conjugated CO/COOR etherified units	0.30	0.32	0.29	0.42
10	144.5-142.5	C-3 in G, C-4 in E _{ne} , C-4 in conjugated F, unknown	0.21	0.22	0.33	0.19
11	58-54	OMe, C- β in H, C-1 in D, C- γ in I	0.94	0.88	0.82	1.03
12	54-52	C- β in G and J	0.13	0.09	0.14	0.10
13	35-34	C- β in K, C- α in L	0.04	0.06	0.07	0.04
14	32.5-31.5	C- α in K	0.05	0.06	0.06	0.03
		clusters				
	162-142	"C _{Ar-O} "	2.04	2.13	2.09	2.14
	142-125	"C _{Ar-C} "	1.78	1.75	1.97	1.55
	125-102	"C _{Ar-H} "	2.29	2.24	2.06	2.43
	90-58	Alk-O-	1.39	1.12	0.96	1.90
	90-77	Alk-O-Ar, α -O-Alk	0.37	0.33	0.30	0.70
	77-65	γ -O-Alk, OH _{sec}	0.52	0.40	0.17	0.55
	65-58	OH _{prim}	0.50	0.40	0.32	0.65
Degree of condensation			0.61	0.65	0.87	0.51

For abbreviations, see Table 16.

Figure 26. Lignin substructures R=H, R'=CHO, CH=CH-CHO, COOH (Capanema et al. 2004b).

3.3.7 Physical properties of isolated lignin

It is likely that during steam pretreatment, in addition to changes in the lignin's chemical structure they also undergo changes in their physical properties. Previously it was hypothesized that the physical properties of the lignin such as their particle size, surface area and molecular weight all have the potential to affect the adsorption of cellulases (Berlin et al. 2006). When the particle sizes of the isolated lignins were assessed, they ranged from 24.6 to 55.7 μm (Table 21) with the particle size of the PTL's decreasing and specific surface increasing with increasing pretreatment severity. The specific surface area, which was determined by the BET method and which should correlate with the lignins binding strength ($R^2=0.90$), showed that the lignin surface area, which increased with increasing pretreatment severity, had a detrimental effect on enzymatic hydrolysis. In previous work (Donaldson et al. 1988), the lignin was shown to flow at temperatures above its T_g , resulting in its agglomeration during steam pretreatment and the formation of droplets of lignin on the surface of the pretreated substrates (Donaldson et al. 1988; Selig et al. 2007). The particle size or droplets of the isolated lignin used in this current study were found to be about ten times larger than those reported previously. It was also apparent that the molecular weight of the CELs decreased with increasing severity (Table 21).

Table 21. Comparison of particle size and specific surface area among the isolated lignin preparations.

Pretreatment temperature (°C)	Lignin	Particle size (µm)	Specific surface area (m ² /g)	Molecular weights (Mw)
190	PTL	34.3	34.2	n.d.
	CEL	32.9	36.4	3780
	REL	40.7	9.7	n.d.
200	PTL	30.3	64.4	n.d.
	CEL	55.7	49.7	2765
	REL	45.5	17.6	n.d.
210	PTL	24.6	61.6	n.d.
	CEL	45.7	50.5	1371
	REL	40.5	25.0	n.d.

PTL: protease treated lignin, CEL: cellulolytic enzymatic lignin, Mw: weight average molecular mass, n.d.: not determined.

3.3.8 Interaction between PTLs and cellulases

The non-productive binding of cellulases to lignin has been reported to be due to various factors such as hydrophobic, electrostatic and hydrogen bonding interactions (Berlin et al. 2006; Palonen et al. 2004; Sewalt et al. 1997). Steam pretreatment was shown to result in increased lignin condensation at increasing severities while the adsorption isotherm values suggested that hydrophobic interactions were a major mechanism involved in the cellulose-lignin interaction. To try to obtain further evidence of major mechanisms involved, the electrostatic interactions between the enzymes and the isolated lignins were determined. The PTLs were shown to have a negative charge at pH 4.8: -21.5 mV (190 °C), -13.5 mV (200 °C), and -17.5 mV (210 °C), likely caused by the dissociation of carboxylic acid groups (Table 19) (Lin et al. 2008). It is known that cellulases possess different electric charges depending on the type of enzyme used (Chirico and Brown 1987; Hui et al. 2001; Medve et al. 1998; Vinzant et al. 2001). To assess the extent of any electrostatic interactions between the cellulases and the PTLs, isoelectric focusing (IEF) was conducted after the cellulases had been incubated with the PTLs at 50 °C for 3 h (Figure 27). Those cellulases which possessed a positive charge at pH 4.8, which included β -glucosidase I (pI 8.5), EG III (Cel 12A, pI 6.8-7.4), EGII (Cel5A, pI 5.5), and CBHII (Cel6A, pI 5.2, 5.9), were much more adsorbed than were the cellulases that had a negative charge at pH 4.8. These included EGI (Cel7B, pI 3.9, 4.5, 4.7), EGII (Cel5A, pI 4.2), CBHI (Cel7A, pI 3.6-3.9) and β -glucosidase (*Aspergillus* sp., pI 4.0). This suggested that the positively charged cellulases were preferentially adsorbed when compared to the negatively charged cellulases. As at least two negatively charged cellulases (arrows in Figure 27) were adsorbed to the PTLs, other interactions, such as hydrophobic interactions likely also contributed to the adsorption between these cellulases

and the PTLs. The IEF adsorption pattern did not change as a result of increasing the steam pretreatment severity. As the positively charged cellulases were adsorbed more strongly than were the negatively charged cellulases this suggested that, by altering the electrostatic properties of cellulases to a more negative charge, we might potentially decrease the non-productive binding of cellulases to the PTLs.

Figure 27. Cellulases adsorption to PTLs isolated from different pretreatment severities. M, IEF markers; lane 1, control (without the PTLs); lane 2, PTL 190 °C; lane 3, PTL 200 °C; lane 4, PTL 210 °C. Cellulases (0.34 mg/ml) and β-glucosidase (0.09 mg/ml) were incubated with 5 mg of PTLs in 500 µl of Na-acetate buffer (pH 4.8, 50 mM) at 50 °C for 3 h. Supernatants after centrifugation were collected, freeze-dried, and analyzed by IEF (pH 5-8). pI of each cellulase component was adapted from (Chirico and Brown 1987; Hui et al. 2001; Medve et al. 1998; Vinzant et al. 2001)

3.3.9 Conclusion

Due to their increased recalcitrance, softwoods generally require steam pretreatment at higher severities when compared with hardwoods or agricultural residues. However, although this resulted in more effective enzymatic hydrolysis of the cellulosic component, an increase in pretreatment severity also resulted in an increase in the lignin content of the pretreated substrate. To elucidate what mechanisms were involved, three types of lignin, protease treated lignin (PTL), cellulolytic enzyme lignin (CEL), and residual enzyme lignin (REL), with different chemical and physical properties were isolated from steam treated Douglas-fir which had been pretreated at three different severities (190, 200 and 210 °C). In contrast to the hydrolysis yields observed with the cellulose rich, water-insoluble materials for which the lignin had been extracted substrates, the isolated lignin from the lowest severity substrate (190 °C) had the least pronounced effect on the hydrolysis yields of Avicel and adsorbed the lowest amounts of cellulases. Characterization of the chemical structure in the isolated lignin using FTIR and ^{13}C NMR showed that the lignin in the steam pretreated substrates became more condensed with increasing pretreatment severity. This suggested that cellulases were adsorbed to the lignin by hydrophobic interaction. Isoelectric focusing (IEF) of supernatant after incubation of cellulases with the PTLs showed that positively charged rather than negatively charged cellulases were preferentially adsorbed to the lignin preparations, indicating that electrostatic interactions were also involved in the adsorption of cellulases to the PTLs. These results in combination with the lower adsorption of cellulases to the substrate pretreated at the lowest severity (190 °C), suggest that the accessible cellulose surface area of the cellulose played a greater role in the observed differences in enzymatic hydrolysis yields among the pretreated substrates rather than enzyme-lignin interactions.

3.4 Effect of hydrophobic and electrostatic interaction on cellulases adsorption to lignin

3.4.1 Background

In the previous chapters, the hydrophobicity of lignin was estimated by comparing lignin yields during isolation of the PTLs from the pretreated substrates. As a result, the lignin in the pretreated corn stover was shown to have a higher hydrophilicity compared with the lignin obtained from poplar, lodgepole pine, and Douglas-fir. The use of lignosulfate, which is a water soluble lignin, increased the hydrolysis yields of the steam pretreated lodgepole pine suggesting that water soluble lignin produced during enzymatic hydrolysis of the substrates could increase enzymatic hydrolysis. It was recognized that the water solubility of lignin could relate to the hydrophilicity of lignin. Thus, we hoped to determine the degree of hydrophobicity of the residual lignin after enzymatic hydrolysis by preparing thin films from the CELs, DHPs, MWL, and cellulose. Thin films of the isolated lignins were made by using a spin coater (Holmgren et al. 2009; Norgren et al. 2006; Notley and Norgren 2010; Turon et al. 2008) and the contact angle of each of the thin films was measured. The chemical groups on the surfaces was characterized using an X-ray photoelectron spectroscopy (XPS)

Although the electrostatic interaction between the PTLs from Douglas-fir and cellulases was determined earlier using IEF, to assess the electrostatic interaction between the cellulase and lignin in more detail, the zeta potential of the isolated lignins and DHPs were measured at pH 4.8. This is the pH at which enzymatic hydrolysis is normally carried out.

It was hoped that the use of thin film would reduce the influence of physical properties such as the surface area and molecular weight of the lignins on the attractive force between the cellulases and lignin. The cellulose binding module (CBM) was used for this experiment, as it is known that the presence of the CBM increased adsorption of cellulases to lignin (Palonen et al. 2004). Atomic force microscopy (AFM) was used previously to measure the attractive force between cellulose and a chemically modified tip (Bastidas et al. 2005) as well as a tip containing the cellulose binding domain (Nigmatullin et al. 2004). For the modification of the AFM tip by this protein, a His tag was used to orient the protein in a specific direction on the surface of the AFM tip through the use of Ni(II) and a self-assembled monolayer (SAM) (Sigal et al. 1996). Previously, the CBM from *Cellulomonas fimi* with a His tag was expressed using *E. coli* and the attractive force between the CBM and cellulose was measured (Yokota et al. 2008). It was found that the modified tip increased the attractive force between the CBM and cellulose when compared with that of the unmodified tip and cellulose. However, a similar interaction between the CBM from *T. reesei* and lignin has not yet been conducted.

In the work described in this chapter, we hoped to clarify which properties of the lignin most influenced the non-productive binding of cellulases by preparing thin films from isolated lignin. The contact angle, XPS analysis and Zeta potential of the isolated lignin was measured. In addition, we tried to prepare an AFM tip which had been modified with CBM from *T. reesei* CBH I to try to measure the atomic force between the CBM and the thin films.

3.4.2 Roughness of CBM based thin films

We initially produced thin films on the surface of microscope cover glass slides using a

spin coater and the root-mean square (RMS) roughness was determined by AFM. It was apparent that the roughness of the thin film ranged from 1.7 to 6.4 nm (Table 22, Figures 28 and 29), which was higher compared with the previous study (Holmgren et al. 2009). One of the reasons could be that they used a silicon wafer for making the thin film, while in this study a microscope cover glass was used (Sudam and Nichols 1994).

Figure 28. An AFM image of a lodgepole pine steam pretreated CEL film spin coated on a glass. The smoothness, as expressed by the rms-value from the height image, was 3.8 nm at $1 \mu \text{m}^2$.

Figure 29. An AFM image of a lodgepole pine MWL film spin coated on a glass. The smoothness, as expressed by the rms-value from the height image, was 2.1 nm at $1 \mu\text{m}^2$.

Table 22. Roughness of thin films measured by AFM.

Sample		Roughness (nm)
Corn stover	SP	6.4 (0.7)
Poplar	SP	3.6 (0.6)
Lodgepole pine	SP	3.8 (0.9)
Douglas-fir (200 °C)	SP	3.1 (1.5)
Corn stover	OS	1.8 (0.8)
Poplar	OS	4.5 (0.9)
Lodgepole pine	OS	2.4 (1.3)
DHP from CA		4.8 (0.6)
DHP from FA		4.0 (0.8)
Cellulose		1.7 (0.5)
Douglas-fir MWL		2.1 (0.3)
Lodgepole pine MWL		2.1 (0.5)

SP, steam pretreatment; OS, organosolv pretreatment, DHP, dehydrogenative polymers; CA, coniferyl alcohol, FA, ferulic acid; MWL, milled wood lignin. Values in parentheses are standard deviation.

3.4.3 Surface composition of thin films from lignin

X-ray photoelectron spectroscopy (XPS) was used to analyze the chemical composition of the thin films prepared from lignin, DHP and MWL. The XPS data are summarized in Table 23 while the XPS spectrum for the steam pretreated corn stover is presented in Figure 30. For the lignin sample, four categories of carbon bonds can be identified by XPS (Table 23): C1 carbons to other carbons or hydrogen (C-C, C-H), C2 carbons bonded to one oxygen atoms (C-O), C3 carbons attached to two oxygen atoms or a carbonyl group (C=O, O-C-O), and C4 carbons from carboxyl groups (O=C-O) (Palonen and Viikari 2004). The deconvolution process was employed and summarized in Table 24 and Figure 30. The O/C values ranged from 0.17 to 0.38. The DHPs and MWLs contained higher amounts of C2, which can be assigned to the carbon attached to oxygen, when compared to the isolated lignin fractions.

Table 23. Carbon and oxygen content in the thin film measured by XPS.

Sample		C1s	O1s	O/C
CS *	SP	79.5	20.5	0.26
Poplar	SP	83.5	16.5	0.20
LPP	SP	85.4	14.6	0.17
DF	SP	80.4	19.6	0.24
CS	OS	83.9	16.1	0.19
Poplar	OS	80.1	19.9	0.25
DHP CA		74.6	25.4	0.34
DHP FA		72.2	27.8	0.38
DF MWL		74.4	25.6	0.34
LPP MWL		75.1	24.9	0.33

CS, corn stover; LPP, lodgepole pine; DF, Douglas-fir; DHP, dehydrogenative polymers; CA, coniferyl acid; FA, ferulic acid; MWL, milled wood lignin; SP, steam pretreatment; OS, organosolv pretreatment. *corn stover was pretreated at 190 °C, other substrates were pretreated at 200 °C.

Figure 30. XPS spectrum of CEL from steam pretreated corn stover: (A) survey spectra; (B) high resolution spectra of the resolved carbon 1s signal (included is the deconvolution spectra).

Table 24. Carbon content in the thin film from lignin measured byXPS.

Sample		Peak area (%) by carbon type			
		C1	C2	C3	C4
CS	SP	61.9	30.0	6.2	1.9
Poplar	SP	57.8	31.4	7.3	3.4
LPP	SP	65.0	25.1	7.0	2.9
DF (200 °C)	SP	64.9	29.1	4.7	1.3
CS	OS	63.0	29.9	5.3	1.8
Poplar	OS	67.8	21.9	6.7	3.7
DHP CA		50.4	40.1	7.1	2.4
DHP FA		50.4	34.7	9.0	5.9
DF MWL		45.8	43.5	8.6	2.0
LPP MWL		47.8	43.1	7.6	1.5

C1s signal at 285 eV was used as an internal standard. The chemical shifts relative to C1(C-C) used in the deconvolution were 1.7 ± 0.2 eV for C2 (C-O), 3.1 ± 0.2 eV for C3 (C=O or O-C-O) and 4.2 ± 0.3 eV for C4 (O=C-O). For other abbreviations, see Table 23.

3.4.4 Contact angle of isolated lignin, DHP, MWL, and cellulose

The contact angles of thin films prepared from isolated lignin, DHP, MWL, and cellulose were measured using water (Table 25). The contact angles of the isolated lignin, DHP, MWL were higher than that of cellulose. The isolated lignin from corn stover had a lower contact angle when compared with those from poplar and lodgepole pine. The MWLs from Douglas-fir and lodgepole pine, which are similar to intact lignin, showed a lower contact angle when compared with the lignin from the steam and organosolv pretreated substrates, suggesting that pretreatment could increase the hydrophobicity of lignin. The contact angles of the DHP from CA (52.7°) and FA (54.0°) were lower than those values in the previously reported work which were 58 and 63°, respectively (Holmgren et al. 2009). However, the contact angle of the MWL (58.7, 60.1°) was higher than the previously reported value of 52.5° (Notley and Norgren 2010). When the ratio of O1s to C1s was plotted against the contact angle (Figure 30) it was apparent that, with increasing O/C, the contact angle decreased, which was same trend as was reported previously (Kumar et al. 2009b).

3.4.5 The zeta potential of isolated lignin, DHP, MWL, and cellulose

As was expected, the zeta potential of the isolated lignin showed a negative charge (Table 25). It ranged from -10.0 to -22.5 mV, which could be caused by dissociation of groups such as carboxylic acid, as mentioned previously. The zeta potential of the DHP from CA and FA was -31.4 and -38.8 mV, respectively. The higher zeta potential of the DHP from FA could be caused by the carboxylic groups in the γ -position instead of hydroxyl groups. In the previous chapter, the DHP from FA showed a lower adsorption of cellulases. As the contact angles of the DHP from CA and FA were similar, the different adsorption properties

might be the result of different zeta potentials rather than differences in wettability.

Table 25. Contact angle and zeta potential of isolated lignin and cellulose.

		Contact angle (°)	Zeta potential (mV)
CS	SP	59.2 (0.4)	-19.0 (2.4)
Poplar	SP	76.5 (1.8)	-19.0 (1.5)
LPP	SP	70.7 (2.7)	-22.5 (3.5)
DF (200 °C)	SP	79.9 (1.8)	-17.5 (1.5)
CS	OS	63.1 (1.9)	-13.5 (1.8)
Poplar	OS	77.5 (1.7)	-16.8 (2.6)
LPP	OS	73.5 (2.5)	-10.0 (1.9)
DHP CA		52.7 (1.9)	-31.4 (2.2)
DHP FA		54.0 (3.0)	-38.8 (3.2)
Cellulose		36.4 (1.9)	n.d.
DF MWL		58.7 (2.8)	n.d.
LPP MWL		60.1 (1.4)	n.d.

For abbreviations, see Table 23. Values in parentheses are standard deviation.

Figure 31. Relation between contact angle and O/C of isolated lignin, DHP, and MWL measured by XPS.

3.4.6 The influence of contact angle and zeta potential on the binding strength of cellulases to lignin

The possible influences of the contact angle and zeta potential on the binding strength of cellulases to lignin, which was previously shown to correlate well with the hydrolysis yields of Avicel in the presence of lignin, were next assessed (Figure 32). It was apparent that the binding strength increased with increasing contact angle, while it was not affected with changes in zeta potential. This again indicated that the hydrophobicity of the lignin was a major influence in determining the degree of cellulase adsorption.

Figure 32. Effect of contact angle and zeta potential on binding strength of cellulases to lignin.

3.4.7 Expression of CBM from *T.reesei* by *E. coli*

To produce the CBM of CBHI with His tag, which was needed to observe any attractive force between the CBM and lignin, the CBM from CBHI of *T. reesei* was cloned from a genomic DNA of *T. reesei* by PCR and a CBM expression vector was constructed. The constructed vector was successfully transformed into *E. coli* (Rosetta gami B (DE3) pLysS), which is designed to enhance the expression of eukaryotic proteins that contain codons rarely used in *E. coli*. It also greatly enhances disulfide bond formation in the cytoplasm. We then tried to determine the optimum conditions for expression of the CBM. Although we tried to express the CBM at 20 and 37 °C in the presence of IPTG (1 mM), we were unable to get expression at these conditions (Figure 32). Further work is required, such as a better choice of host strain, plasmid, temperature of incubation, concentration of inducer, etc, in order to get good expression of the CBM of CBHI. In future work, the AFM tip will be modified with synthesized CBM of CBHI with His tag and SAM.

Figure 33. SDS-PAGE of Rosetta gami B (DE3) pLysS, which was transformed by CBM expression vector. M, marker; Lane 1-4, control; Lane 5-8, induced with 1 mM of IPTG; Lane 1 and 5, medium; Lane 2 and 6, whole cell; Lane 3 and 7; His tag column unbounded fraction, Lane 4 and 8; His tag column bonded fraction.

3.4.8 Conclusion

The contact angles and zeta potentials of isolated lignins were measured to try to determine the effect of hydrophobicity and electrostatic interactions on non-productive binding of cellulases. To determine the contact angle of the lignins, thin films were prepared by use of a spin coater. The contact angle of the isolated lignin from corn stover was lower compared to those from poplar, lodgepole pine, and Douglas-fir. The zeta potentials of all of the isolated lignins showed negative charges. It was apparent that the contact angle of the isolated lignin was more influential than was the zeta potential of the isolated lignin when their influence on the binding strength of cellulases to the isolated lignins was considered. This indicated that a reduction in the hydrophobicity of lignin should decrease the non-productive binding of cellulases to lignin.

4 CONCLUSION AND FUTURE WORK

4.1 Conclusion

We hypothesized that the degree to which isolated lignins might influence hydrolysis might correlate with the chemical and physical properties of the lignin such as its hydrophobicity as measured by contact angle, electric charge as measured by zeta potential and hydrogen bonding potential. It appears that the hydrophobicity and electric charge of the lignin are the most influential factors that affect the non-productive binding of cellulases during hydrolysis of pretreated substrates.

Six different substrates: steam and organosolv pretreated substrates from softwood (lodgepole pine), hardwood (poplar), and an agricultural residue (corn stover), were prepared. In addition, steam pretreated Douglas-fir was pretreated at increasingly severe conditions. The lignin from all of these pretreated substrates was isolated by two methods. In the first method, after extensive enzymatic hydrolysis of the pretreated substrates, the residues were treated with protease to hydrolyze the enzymes remaining on the surface of the hydrolysis residues. This lignin preparation was termed protease treated lignin (PTL). In the alternate method, the residues, after similar pretreatments and extensive enzymatic hydrolysis, were extracted by dioxane. These fractions were designated as cellulolytic enzyme lignins (CEL). The residue after the isolation of CEL was designated as residual enzyme lignin (REL).

The isolated lignins from lodgepole pine, Douglas-fir, and steam pretreated poplar had a negative effect on the hydrolysis of Avicel, whereas the other isolated lignins had little effect. The hydrolysis yields of Avicel in the presence of the isolated lignin correlated with the hydrolysis yields observed with the pretreated substrates, suggesting that the lignin in the pretreated substrate decreased the hydrolysis yields. To assess the effects of the physical and

chemical properties of the isolated lignins on the enzymatic hydrolysis of pretreated lignocellulose, the chemical and physical properties of the PTLs, CELs, and RELs were characterized. It was apparent that the purity of the CELs was higher than that of the PTLs. The adsorption of cellulases to the isolated lignin preparations corresponded with the Langmuir adsorption isotherm. Rather than the physical properties and chemical composition of the isolated lignin preparations, the carboxylic acid functionality of the isolated lignin measured by FTIR and NMR spectroscopy seemed to show a significant influence on the enzymatic hydrolysis yields from lignin supplemented pure cellulose (Avicel) as an increase in the carboxylic content of the lignin preparation resulted in an increased hydrolysis yield. This suggested that the carboxylic acids within the lignin partially alleviated the non-productive binding of cellulases to lignin.

To determine the possible effect of the carboxylic acid component of the lignin on hydrolysis, dehydrogenative polymers (DHP) of monolignols were synthesized from coniferyl alcohol (CA) and ferulic acid (FA) respectively. The DHP from FA, which was enriched in carboxylic acid groups, when compared with the DHP from CA, adsorbed a lower amount of cellulases. It also had little effect on the hydrolysis yields when supplemented to the Avicel hydrolysis experiment when compared with the DHP from CA, which decreased the hydrolysis of Avicel by about 8-9%.

It was apparent that the hydrophobicity of the lignin was a highly influential factor in the non-productive binding of cellulases to lignin. Lignin recovery during preparation of PTL was higher for the softwood (lodgepole pine and Douglas-fir), hardwood (poplar), and agricultural residue (corn stover) in this order, suggesting that hydrophobicity was higher in the softwood, hardwood, agricultural residue in this order. The water-soluble lignin

produced during the enzymatic hydrolysis could increase enzymatic hydrolysis, which was demonstrated previously by the positive effect that lignosulfonates had on the hydrolysis of steam pretreated lodgepole pine. In addition, the hydrophobicity of lignin was measured after making thin films using a spin-coater. It was apparent that the isolated lignin from corn stover had a lower hydrophobicity when compared to lignin from poplar, lodgepole pine, and Douglas-fir. Electrostatic attraction also influenced the interaction between lignin and cellulases as the zeta potential of isolated lignin were shown to possessed negative charges, likely caused by the dissociation of groups in the lignin such as carboxyl groups. The IEF analysis of the PTLs from the steam pretreated Douglas-fir showed that cellulases possessing positive charges preferentially adsorbed the lignin when compared with cellulases with a negative charge.

Although not covered in detail in the work described in the thesis, the results from steam pretreated Douglas-fir with different severities showed that steric hindrance of cellulose by lignin appeared to be a major limitation with all of the pretreated substrates, likely have a greater influence than does the non-productive binding of cellulases to the lignin.

4.2 Future work

4.2.1 Post-pretreatment

This study showed that decreasing the hydrophobicity and adding negative charges to the lignin could decrease the non-productive binding of cellulases to lignin. As mentioned above, post-pretreatment is required especially for steam pretreated softwood. The options of post-pretreatment would include oxidation and modification of lignin to decrease the hydrophobicity of the lignin using methods such as oxidation and sulfonation as well as removing lignin from the pretreated substrates. It would be interesting to know how a change in the hydrophobicity and electric charges of the lignin after post-pretreatment might affect the non-productive binding of cellulases and enzymatic hydrolysis.

4.2.2 Observation of interaction between purified enzyme and lignin

In this work, cellulase “complex” commercial preparation was used to assess the interaction between cellulases and lignin. As mentioned earlier, each cellulase component has a different hydrophobicity and electrostatic charge, which would likely influence their adsorption to lignin. A comparison of the adsorption and desorption behavior between the different cellulases might help us find a way to improve cellulase preparations so we can decrease their non-productive binding to lignin. Comparison of adsorption and desorption properties of cellulase to lignin using different cellulases preparations, purified cellulases and cellulase components would elucidate their adsorption and desorption properties.

4.2.3 Genetic modification of cellulases

This study showed that positive charged cellulases adsorbed to lignin more than those

negative charged cellulases. Changing the electric charge of cellulases from positive to negative under the hydrolysis condition could decrease the non-productive binding of cellulases. The electric charges of CBHII, EGII, and EGIII, which have a positive charge under the enzymatic hydrolysis condition, could be altered to negative charge by genetic modification. It would be interesting to know how the alternation of the electric charge of the individual cellulases might affect their adsorption and desorption to lignin.

4.2.4 Genetic modification of lignin biosynthesis

In this study, we showed that the addition of hydrophilic groups such as carboxylic acid to the lignin would likely increase their hydrophilicity, thus reducing the non-productive binding of cellulases to lignin. In addition to the chemical modification of lignin, genetic modification of lignin could, possibly, also be used to increase the carboxylic content of the lignin. By increasing the carboxylic acid content of the lignin of a woody plant by, for example increasing the amount of ferulic acid, this will likely increase the hydrophilicity of lignin and should consequentially improve the enzymatic hydrolysis of the lignocellulosic substrates.

4.2.5 Expression of CBM by *E. coli* and *T. reesei*

In this study, we tried to express the CBM of CBHI from *T. reesei* using *E. coli* as a host strain. One of main reasons we selected *E. coli* as a host strain was that we could not use *T. reesei* as a host strain. We selected the CBM of CBHI because the CBM of CBHI from *T. reesei* is not glycosylated even by homogeneous expression, which means the expression of the CBM by *E. coli* should result in the production of a CBM which has the same properties as

that produced by *T. reesei*. In addition to finding the optimum conditions for expression of the CBM using *E. coli*, the expression of cellulases from *T. reesei* using homogeneous expression and consequently measuring the attractive forces between cellulases and lignin should give us more insight in how the cellulases associate with lignin. As amino acid alternation of the CBM in CBHI is known to change the adsorption properties of CBHI to cellulose (Linder et al. 1999), the amino acid alternation of the CBM of CBHI should also change the adsorption properties of the CBHI to lignin. This might then indicate how we might decrease the non-productive binding of cellulases to lignin.

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Appendix A. Experimental data.

Table A. Chemical composition of lignocellulosic substrates.

Biomass	Neutral sugar compositions					Lignin analysis		Ash
	(%)					(%)		(%)
	Ara	Gal	Glu	Xyl	Man	AIL	ASL	
Corn stover	4.2	1.4	34.4	22.8	0.6	17.2		n.d.
Poplar	0.6	1.0	43.8	14.9	3.9	29.1		n.d.
LPP	1.4	2.2	50.5	7.2	13.1	24.8	0.3	0.3
Douglas-fir	1.2	2.2	47.3	4.4	11.7	29.8	0.5	n.d.

Data was adapted from (Bura et al. 2009; Kumar et al. 2010; Pan et al. 2007). Ara: arabinose, Gal: galactose, Glu: glucose, Xyl: xylose, Man: mannose, AIL: acid insoluble lignin, ASL: acid soluble lignin, LPP: lodgepole pine

Figure A1. FT-IR spectra of isolated lignin from DHPs from ferulic acid (FA) and CA coniferyl alcohol. 1, hydroxyl groups; 2, aldehyde; 3, carboxylic acid; 4, ketone; 5, ester; 6, ether.

Figure A2. Langmuir linear regression using cellulases and PTLs from organosolv pretreated corn stover (A), poplar (B), and lodgepole pine (C).

Figure A3. Langmuir linear regression using cellulases and CEL from steam pretreated Douglas-fir at 190 °C (A), 200 °C (B), and 210 °C (C).