SUBSTRATE PROPERTIES THAT INFLUENCE THE ENZYMATIC HYDROLYSIS OF ORGANOSOLV-PRETREATED SOFTWOODS AT LOW ENZYME LOADINGS

by

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Abstract

Lignocellulosic biomass is a potential source of sugars for the production of fuels and chemicals. However, its resistance to chemical and biological degradation poses a significant challenge. Consequently, a pretreatment is required to increase the accessibility of cellulose to cellulases. The organosolv process is one of the few pretreatments that can process softwoods to generate substrates that are readily hydrolyzed by cellulases. However, because the residual lignin and hemicelluloses can restrict cellulose accessibility, obtaining significant cellulose conversion at low enzyme loadings (< 5 FPU g\(^{-1}\) cellulose) remains a challenge. As complete delignification is not economically viable, we hypothesize that other substrate properties could be altered to reduce the recalcitrance of organosolv-pretreated softwoods.

The initial work reported in this thesis compared the effects of pretreatment severity on the physico-chemical properties and enzymatic hydrolysis of organosolv-pretreated lodgepole pine and hybrid poplar (a representative hardwood). As expected, hybrid poplar was less recalcitrant than lodgepole pine. However, unlike lodgepole pine, which remained unaffected by changes in pretreatment severity, the recalcitrance of hybrid poplar increased with pretreatment severity. Interestingly, it was found that this increased recalcitrance was due to non-productive binding between lignin and cellulases. In contrast the inhibitory effect of lignin on the enzymatic hydrolysis of lodgepole pine was due to a combination of restricted cellulose accessibility and hydrophobic interactions between lignin and cellulases.

Subsequent studies showed that rather than a single substrate characteristic such as lignin content, particle size or cellulose degree of polymerization, the overall cellulose accessibility is the key substrate characteristic that governs the susceptibility of the
organosolv-pretreated substrates to enzymatic hydrolysis. Surprisingly, attempts to increase cellulose accessibility through PFI-mill refining were unsuccessful. However, increasing the hydrophilicity of the residual lignin via neutral sulphonation resulted in significant increases in enzymatic hydrolysis at 5 and 2.5 FPU g\(^{-1}\) cellulose (from 80% to 95% and from 35% to 80%, respectively). This was likely due to decreased nonspecific interactions between the sulphonated lignin and cellulases. Overall, the results suggest that increasing the hydrophilicity of the residual lignin could be a viable strategy to reduce the recalcitrance of organosolv-pretreated softwoods.
Preface


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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>δ</td>
<td>delta (Hildebrand solubility parameter)</td>
</tr>
<tr>
<td>ACOS</td>
<td>acid-catalyzed organosolv saccharification</td>
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<tr>
<td>AFEX</td>
<td>ammonia fibre explosion</td>
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<tr>
<td>AIL</td>
<td>acid-insoluble lignin</td>
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<tr>
<td>Ara</td>
<td>arabinan</td>
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<tr>
<td>ASAM</td>
<td>alkaline sulphite anthraquinone methanol</td>
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<td>ASL</td>
<td>acid-soluble lignin</td>
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<td>ATR</td>
<td>attenuated total reflectance</td>
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<td>bdl</td>
<td>below detection limit</td>
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<td>BSA</td>
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<td>cal</td>
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<td>Ca(OH)(_2)</td>
<td>calcium hydroxide (lime)</td>
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<td>CBH</td>
<td>cellobiohydrolase</td>
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<td>CBM</td>
<td>carbohydrate binding module</td>
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<td>CD</td>
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<td>cellulolytic enzyme lignin</td>
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<tr>
<td>COSLIF</td>
<td>cellulose solvent and organic solvent lignocellulose fractionation</td>
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<td>DL</td>
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<td>DP</td>
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<tr>
<td>H$_3$PO$_4$</td>
<td>phosphoric acid</td>
</tr>
<tr>
<td>HMF</td>
<td>5-hydroxymethylfurfural</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>IL</td>
<td>ionic liquid</td>
</tr>
<tr>
<td>IU</td>
<td>international units</td>
</tr>
</tbody>
</table>
L litre
LCC lignin carbohydrate complex
LPP (mountain pine beetle-killed) lodgepole pine
LHW liquid hot water
kJ kilo joule
m meter
Man mannan
mg milligram
min minute(s)
 mL millilitre
mm millimeter
mmol millimole
 mM millimole per litre
 M mole per litre
 Na₂S sodium sulfide
NaClO₂ sodium chlorite
NAEM neutral alkaline earth metal
NaOH sodium hydroxide
NBSK northern bleached softwood kraft pulp
nm nanometer
ODW oven-dried weight
PAA peracetic acid
POP (hybrid) poplar
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSI</td>
<td>pound per square inch</td>
</tr>
<tr>
<td>RAC</td>
<td>regenerated amorphous cellulose</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>s</td>
<td>seconds (s)</td>
</tr>
<tr>
<td>SPORL</td>
<td>sulfite pretreatment to overcome the recalcitrance of cellulose</td>
</tr>
<tr>
<td>SO$_2$</td>
<td>sulphur dioxide</td>
</tr>
<tr>
<td>t</td>
<td>time</td>
</tr>
<tr>
<td>$T_g$</td>
<td>glass transition temperature</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>ultraviolet-Visible</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per volume</td>
</tr>
<tr>
<td>w/w</td>
<td>weight per weight</td>
</tr>
<tr>
<td>XPS</td>
<td>x-ray photoelectron spectroscopy</td>
</tr>
<tr>
<td>Xyl</td>
<td>xylan</td>
</tr>
</tbody>
</table>
Acknowledgements

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Dedication

This thesis is dedicated to my daughter Bella Areta Del Rio.
Chapter 1: Introduction

1.1 Cellulosic Ethanol

Fossil fuels account for over 40% of the world’s energy and their consumption continuously contributes to the atmospheric carbon dioxide burden. However, as the result of the increased energy requirements of emerging economies such as China and India, the world’s energy demand is projected to double within the next decade (Kerr, 2007). Moreover, disruption of oil supplies as a consequence of exclusive monopolies held by politically volatile nations, coupled with global dependency on fossil fuels has resulted in increased oil prices from less than US $10 per barrel 15 years ago to the current (November 2011 to February 2012) US$ 90-105 per barrel range (International Energy Agency, 2012).

In addition to these factors, concerns over diminishing oil supplies and emission of greenhouse gases (GHG’s) from fossil fuels, are some of the factors that are driving the interest in renewable energy sources (International Energy Agency, 2008).

While stationary energy (such as heat and electricity) needs can be addressed by a variety of renewable technologies such as nuclear, solar, wind and geothermal power, sustainable and cost effective production of transportation (i.e. liquid) fuels poses a challenge that needs to be addressed. A possible strategy to address this challenge is the development of biofuels such as (bio)ethanol from plant feedstocks, which are renewable, sustainable, and emit less GHG’s during consumption (Lynd, 1996; Wyman et al, 2005; Carroll and Somerville, 2009). Moreover, fuels derived from plant (biomass) resources are considered carbon neutral because the carbon dioxide emitted during their consumption is equivalent to that absorbed by the plants during photosynthesis (Lynd et al, 1991).
Current commercial ethanol production in North America is largely based on the use of starch in grains such as maize/corn and wheat to produce ethanol while in other parts of the world such as Brazil, sugars such as sucrose from crops such as beet and sugar cane are used in place of starch (Schnepf, 2006). The ethanol produced from starch or simple sugars is known as “conventional” or “first generation ethanol” and is a mature commercial process with well understood process economics (Langan et al, 2011) that is capable of producing significant amounts of fuel ethanol. For example in 2010, fuel ethanol production in North and Central America, South America and the European Union (EU) was 13.7, 7.1, and 1.2 billion gallons respectively (Renewable fuels association, 2012). It is likely that these volumes will continue to increase due to global governments recognizing the importance of decreasing their dependency on fossil fuels. For instance, the U.S. Department of Energy intends to replace 30% of petroleum-derived transportation fuels with biofuels, and 25% of industrial organic chemicals with biomass-derived chemicals by 2025 (Ragauskas et al, 2006). Similarly, the European Union has a target that 25% of its transportation fuels will be derived from biofuels by 2030 (Biofuels Research Advisory Council, 2006).

While capable of producing relatively large volumes of ethanol, under current conditions starch to ethanol technologies are only capable of displacing a small (10%) proportion of gasoline. On the other hand, increasing ethanol production by shifting land use or maize utilization towards ethanol and away from other uses would have severe consequences for the agricultural markets and food prices (Langan et al, 2011). The “food vs. fuel dilemma” coupled with the need to meet renewable fuel targets has led to efforts in developing the so called “advanced” or “second generation” biofuels, which are based on
lignocellulosic biomass such as agricultural (corn stover, straw) and forest (sawdust, logging waste) residues (Sims et al, 2010).

The fibrous material derived from the plant cell wall is known as (lignocellulosic) biomass and is found in a wide range of materials such as agricultural residues, forest waste and municipal solid waste (Galbe and Zacchi 2002; Mabee and Saddler 2010).

Lignocellulosic biomass is composed of cellulose, hemicelluloses and lignin. However, its exact chemical composition and morphological properties vary depending with the source of the lignocellulosic material (Baucher et al, 1998). Cellulose and hemicelluloses comprise 65-75 % of the overall biomass dry weight and can be enzymatically hydrolyzed to their component sugars for fermentation into ethanol (Himmel et al, 2007; Wyman 1996). In addition to its renewable nature and potential to reduce greenhouse gas emissions, biomass is an attractive source of sugars for the production of second generation ethanol because it is relatively cheap, abundant and can be grown on marginal or non-arable lands and therefore does not compete with food production (Lin and Huber, 2009; Somerville et al, 2010). Other work (Nonhebel, 2005) has also shown that biomass has the potential to meet the United States’ needs for liquid transportation fuels, food, animal feed and fibre provided that advanced processing technologies are developed alongside certain land-use changes that would not require more land. However, strategies for feedstock supply, handling and processing and the technologies required for the large scale production of lignocellulosic biomass into fuels and chemicals have not yet been fully developed (Somerville, 2006; Himmel et al, 2007). As a result, the commercialization of biofuels and chemicals produced from lignocellulosic feedstocks has been hindered and virtually all of the suggested processes
require further research and development to improve their overall economics and efficiency (Banerjee et al, 2010).

1.2 Biomass Recalcitrance

Besides the logistic challenges mentioned above, a technical challenge to the cost-competitive production of second generation biofuels is the difficulty associated with accessing the polysaccharide components of lignocellulosic biomass. This is known as biomass recalcitrance and has evolved as a mechanism to protect the plant cell wall from biological, chemical and physical attacks by the microbial and animal kingdoms (Himmel et al, 2007). Biomass recalcitrance is a complex phenomenon that is governed by physicochemical properties spanning several orders of magnitude from the macroscopic to the molecular scale and by the hierarchical structure of the plant cell wall. These properties include the heterogeneity of cell wall constituents, the low accessibility of cellulose to cellulolytic enzymes and water due to strong interchain hydrogen bonding and hydrophobic interactions between cellulose sheets and mass transfer limitations due to soluble enzymes acting on insoluble substrates (Himmel et al, 2007; Chundawat et al, 2011).

1.3 Factors Affecting Biomass Recalcitrance

As mentioned above, the macro- and microscopic properties of lignocellulosic biomass (including its recalcitrance) are partly dependent on the type, abundance and interactions of chemicals within their cell walls. Although cellulose, hemicelluloses and lignin are the common cell wall constituents of all lignocellulosic biomass, their proportion, type and arrangement differ between the types of biomass (herbaceous and woody). For example, woody biomass is more recalcitrant than herbaceous biomass due in part to its higher lignin content (Zhu et al, 2010). Furthermore, woody biomass can be subdivided into
hardwoods (angiosperm) and softwoods (gymnosperm), which have different chemical compositions and degrees of recalcitrance. In general, hardwoods such as poplar (*Populus*) species are less recalcitrant and have a rapid growth rate, which makes them attractive as a potential energy crop (Sannigrahi et al, 2010a). In contrast, softwoods such as lodgepole pine (*Pinus contorta*) are generally considered to be the most recalcitrant type of biomass. However, due to its abundance in many parts of the world such as Russia, Scandinavia and Canada it is likely that softwood biomass will play a significant role in future bioenergy applications.

While the cellulose content of softwood and hardwood biomass is very similar (40-50 % of the total dry matter), there are significant differences in the content and composition of hemicelluloses and lignin. In general, softwoods have higher lignin (26-40 %), and lower hemicelluloses (20-32 %) content than do hardwoods (23-30 % lignin, and 15-35 % hemicelluloses; Fengel and Wegener, 1989; Rowell et al, 2000). These differences have an effect on the physical and chemical properties of the different woody substrates and therefore play a significant role in determining their recalcitrance (Jørgensen et al, 2007).

1.3.1 **Lignin**

Lignin is a highly branched three-dimensional polymer and is largely responsible for the recalcitrance of the plant cell wall. It is composed of the phenylpropanoid monomers guaiacyl (G), syringyl (S), and p-hydroxyphenyl (H), collectively known as monolignols (Figure 1) and is formed by the enzymatic dehydrogenation of the monolignols followed by radical coupling. As a result of this dehydrogenative coupling, the monolignols can be linked through a variety of carbon-carbon bonds or carbon-oxygen bonds, which also vary depending on the source of the lignocellulosic material (Table 1, Figure 2). The structure and
monomer composition of lignin varies depending on the type and source of lignocellulosic material. For example softwood lignin is primarily composed of G units (98 %), whereas hardwood lignin is composed of both G and S units and herbaceous biomass lignin is composed of all three monolignols (Baucher et al, 1998). Additionally, lignin content also varies depending on the source of the lignocellulosic biomass. In general softwoods contain 25-35 % lignin, hardwoods contain 18-25 % lignin and herbaceous biomass contains 10-30 % lignin (Kelley et al, 2004).

![Chemical structures of the phenylpropanoid lignin precursors](image)

**Figure 1.** Chemical structures of the phenylpropanoid lignin precursors a) guaiacyl (coniferyl alcohol, G), b) syringyl (sinapyl alcohol, S) c) para-hydroxyphenyl (p-coumaryl alcohol, H) adapted from Adler (1977).

The main role of lignin is to confer mechanical strength and structural support to the cell wall to prevent compression and bending. This structural support is achieved by forming covalent linkages with hemicellulose, known as the lignin carbohydrate complexes (LCC) (Erickson and Lindgren, 1977; Chabannes et al, 2001). The other roles of lignin are to decrease the permeability of water across the cell wall and protect the cell wall from
microorganisms by providing resistance against the penetration of destructive enzymes into the cell wall (Sarkanen and Ludwig, 1971).

**Table 1.** Summary of linkages found in lignin (adapted from Addler, 1977).

<table>
<thead>
<tr>
<th>Linkage type</th>
<th>Dimer structure</th>
<th>Percent of total linkages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Softwood</td>
</tr>
<tr>
<td>β-O-4</td>
<td>Arylglycerol-β-aryl ether</td>
<td>50</td>
</tr>
<tr>
<td>α-O-4</td>
<td>Noncyclic benzyl aryl ether</td>
<td>2-8</td>
</tr>
<tr>
<td>β-5</td>
<td>Phenylcoumaran</td>
<td>9-12</td>
</tr>
<tr>
<td>5-5</td>
<td>Biphenyl</td>
<td>10-11</td>
</tr>
<tr>
<td>4-O-5</td>
<td>Diaryl ether</td>
<td>4</td>
</tr>
<tr>
<td>β-1</td>
<td>1,2-Diaryl propane</td>
<td>7</td>
</tr>
<tr>
<td>β-β</td>
<td>Linked through side chain</td>
<td>2</td>
</tr>
</tbody>
</table>

Whereas the glycosidic bonds between monomer units in cellulose and hemicellulose can be hydrolyzed under relatively mild conditions, the ether linkages between lignin monomers require either oxidative cleavage or high temperature and low pH for their hydrolysis to occur (El Hage et al, 2010; Sannigrahi et al, 2008). Moreover, the glass transition temperature \( T_g \) of native lignin varies between 100-160 °C (Arora et al, 2010; Selig et al, 2007) above which, it is possible for lignin to soften and become redistributed on the biomass surface (Selig et al, 2007). The \( T_g \) of lignin is largely affected by the type and proportion of linkages, which are in turn affected by the monolignol composition. For example methoxylation at positions 3 and 5 of the aromatic ring prevents the formation of certain linkages (mainly carbon-carbon i.e. condensed bonds), which have a direct effect on the \( T_g \) of lignin.

**1.3.2 Hemicelluloses**

Unlike cellulose, hemicelluloses are heteropolysaccharides with D-xylose, D-mannose, D-galactose, D-glucose, L-arabinose, L-rhamnose (some hardwoods only), 4-O-
Figure 2. Common inter-unit linkages found in lignin. Adapted from Adler (1977) and Pu et al, (2007a).
methyl-D-glucuronic acid and galacturonic acid as components. Hemicelluloses are branched, have a significantly lower degree of polymerization than cellulose (typically below 200) and can be acetylated (Kuhad et al, 1997). The principal hemicelluloses of softwoods such as lodgepole pine are galactoglucomannans and arabinoglucuronoxylans, which represent approximately 20% and 5-10% of the total dry matter respectively (Willför et al, 2005a). In contrast, the hemicellulosic constituent of hardwoods such as poplar is primarily composed of 4-O-methylglucuronoxylans (15-30% of the total dry matter) with only a minor amount (2-5% of the total dry matter) of glucomannans (Willför et al, 2005b). Due to their relatively low degree of polymerization and branched structure, hemicelluloses are easily dissolved in alkaline solutions and are more susceptible to acid-catalyzed hydrolysis of their glycosidic bonds than cellulose (Sjöström, 1993; Timell, 1967).

### 1.3.3 Cellulose

Cellulose is a homopolysaccharide composed of D-glucopyranose units linked via repeating β-(1→4)-glycosidic bonds (Figure 3). While the degree of polymerization and polydispersity of native cellulose is unknown, it has been suggested that wood cellulose is composed of approximately 10,000 glucose residues (Sjöström, 1993). Cellulose chains are linear and have a tendency to aggregate via the formation of inter- and intramolecular hydrogen bonds. The aggregation of 30-36 cellulose chains, which interact with hemicellulose particles at their surfaces leads to the formation of ribbon-like structures known as microfibrils in which, highly ordered (crystalline) regions alternate with less ordered (amorphous) regions (Nishiyama, 2009; Ding and Himmel, 2006). The propensity of cellulose chains to form microfibrils, which undergo further aggregation into fibrils and finally into fibres is responsible for its high tensile strength, makes it insoluble in most
solvents and is partly responsible for the resistance of cellulose to microbial degradation (Ward and Moo-Young, 1989; Sjöström, 1993).

Figure 3. The chemical structure of cellulose.

1.3.4 Plant Cell Wall Structure

The lignocellulosic cell wall is a complex and dynamic structure composed of three regions: the middle lamella, the primary cell wall (P), and the secondary cell wall (Figure 4). The amount, distribution and proportion of lignin, hemicellulose and cellulose in these layers are largely responsible for many of the properties of lignocellulosic biomass including its recalcitrance. The middle lamella is the layer between two neighbouring cells, it is 0.2-1.0 μm thick and contains a high proportion of amorphous material (lignin and hemicellulose), which joins neighboring cells together. The primary cell wall is approximately 0.03-1.0 μm thick, contains a mixture of amorphous and crystalline material and can be thought of as a transition between the middle lamella and the secondary wall (Rowell et al, 2000; Sjöström, 1993). The secondary cell wall contributes up to 89 % of total dry weight in biomass and is subdivided into three layers based on their microfibrillar orientation; these layers are labeled S₁ through S₃ from the outer to the inner layer. The S₁ and S₃ layers are thin at 0.1-0.3 μm, while the S₂ layer is thick at 1-5 μm and is responsible for the strength properties of woody
biomass (Rowell et al, 2000). The $S_1$ layer contains fibrils that are randomly oriented; the $S_2$ layer is composed of parallel fibrillar units oriented at a slight angle to the cell axis and the $S_3$ layer contains parallel fibrillar units forming a flat helix in the transverse direction (Shafizadeh and McGinnis, 1971). Cell wall biosynthesis occurs one layer at a time with the primary cell wall deposited during cell growth and the secondary cell wall deposited after cell growth has ceased (Mohnen et al, 2008). Finally, carbohydrate deposition is followed by lignin biosynthesis and deposition from the middle lamella towards the secondary cell wall (Terashima et al, 1986; 1993).

![Figure 4. Stylized drawing of a typical plant cell wall adapted from Clark (1978).](image)
1.3.5 Distribution of Chemical Components Within the Plant Cell Wall

The distribution of lignin and polysaccharides across the cell wall follow opposite concentration gradients (Janes, 1969). In the case of lignin, its concentration is the highest in the middle lamella (90%) and decreases across the cell wall from the primary cell wall (70%), across the three layers of the secondary cell wall $S_1$ (40%), $S_2$ (15%), and $S_3$ (5% or less). In contrast, the polysaccharide distribution is significantly lower in the middle lamella (10% hemicelluloses and no cellulose) and increases along the primary cell wall (20% hemicelluloses, and 10% cellulose), $S_1$ (25% hemicelluloses, and 35% cellulose), $S_2$ (30% hemicelluloses, and 55% cellulose), and $S_3$ (40% hemicelluloses, and 55% cellulose) (Sjöström, 1993; Table 2). However, because it is only 0.2-1.0 μm thick and accounts for only 2-4% of the total woody substrate, only a small portion (20-25%) of the total lignin is found in the middle lamella. In contrast, the majority of the lignin (70%) is found in the $S_2$ layer of the secondary cell wall though at a significantly lower concentration (Hafrán et al, 2000).

Table 2. Distribution of lignin and polysaccharides (%. w/w) across the cell wall layers (adapted from Sjöström, 1993).

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Middle lamella</th>
<th>Primary cell wall</th>
<th>Secondary cell wall</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>$S_1$</td>
</tr>
<tr>
<td>Cellulose</td>
<td>0</td>
<td>10</td>
<td>35</td>
</tr>
<tr>
<td>Hemicelluloses</td>
<td>10</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>Lignin</td>
<td>90</td>
<td>70</td>
<td>40</td>
</tr>
</tbody>
</table>

1.3.6 Cell Types

In addition to the complex structure of their cell wall, woody biomass is composed of many types of cells which perform a variety of functions such as mechanical support, protection, storage and conduction. Softwood biomass is primarily composed of very long cells (3-4 mm) called longitudinal tracheids, which account for 90-95% of the total wood.
structure. Besides longitudinal tracheids, softwoods contain a small amount (1-2 %) of longitudinal parenchyma cells, which are used for storage as well as resin canals, which secrete resins to assist in wound healing and prevent attack by pests. Hardwoods on the other hand, contain a large variety of cells including vessel elements, which conduct fluid throughout the xylem and form end to end connections via structures called perforation plates. Another hardwood-specific cell type is the fibre tracheid, which unlike the longitudinal tracheids of softwoods are less than 1 mm long and have very thick cell walls. The main role of the fibre tracheids is to provide structural support to the vessel elements. The increased permeability of hardwoods due to the presence of vessel elements is also contributes to their relative ease of delignification (i.e. lower recalcitrance) when compared to softwoods (Pokki et al, 2010; Tesoro et al, 1974). Despite the observed differences in recalcitrance between hardwoods and softwoods, efficient deconstruction of woody biomass to fermentable sugars requires a pretreatment step to modify the physical and chemical properties of the plant cell wall and increase the accessibility of cellulose to cellulolytic enzymes (Chandra et al, 2007).

1.4 Biomass Pretreatment

As mentioned in section 1.2, the recalcitrance of (lignocellulosic) biomass is a major technical challenge to the cost-competitive production of advanced or so called “second generation” biofuels. As a result, a pretreatment step is required to modify the physical and chemical properties of the plant cell wall and increase the accessibility of its cellulose component to cellulolytic enzymes (Mosier et al, 2005a). The pretreatment step is the most crucial stage in the bioconversion process as it has a large impact on the recovery of all the components of the original feedstock and influences the accessibility cellulose to cellulolytic enzymes.
enzymes (and therefore the efficiency of the overall process) (Chandra et al, 2007). While the beneficial effects of pretreatment on the overall bioconversion process have long been recognized (McMillan, 1994), it has also been identified as one of the main costs in producing liquid fuels from biomass and is a significant area of research and development (Merino and Cherry, 2007; Langan et al, 2011).

Ideally the pretreatment step should: be cost effective, require minimal preparation/handling, ensure recovery of all of the biomass components in a useable form, be effective on a wide range of lignocellulosic materials and provide a cellulose-rich fraction that can be efficiently hydrolyzed with low concentrations of enzymes. With regard to the latter requirement, it would be advantageous if the pretreatment process could degrade the cell-wall structure by reducing cellulose crystallinity, degree of polymerization and particle size, while removing hemicellulose and lignin and increasing the enzyme-accessible surface area. However, this type of fractionation/cell wall degradation is challenging for a variety of technical and economic reasons. Therefore, pretreated biomass usually contains some hemicellulose and (especially) residual lignin that is associated with the cellulose-rich material obtained after pretreatment, the amount of which varies depending on the type of pretreatment and its severity (Shevchenko et al, 2001; Chandra et al, 2007).

Several pretreatment methods have been developed to increase the efficiency of enzymatic hydrolysis. These include steam, organosolv, ammonia fibre expansion/explosion (AFEX), lime and dilute acid pretreatments (Agbor et al, 2011; Hendriks and Zeeman, 2009; Taherzadeh and Karimi, 2008). However, as will be discussed in more detail, all pretreatments have certain advantages and disadvantages and therefore it is unlikely that a single pretreatment will provide all the desired outcomes on all potential feedstocks (Langan
et al, 2011). In fact, it is more likely that the type of biomass will dictate the choice of pretreatment. Therefore, effective pretreatment research requires a fundamental understanding of the mechanism of action and subsequent effects of the chosen pretreatment on the target biomass. With regards to their mechanism of action, pretreatment methods can be divided into the following categories: biological, mechanical (milling and grinding), and chemical/physicochemical (steam pretreatment, hydrothermolysis, alkaline, acidic, oxidative, organosolv) and will be discussed in the succeeding sections.

1.4.1 Biological Pretreatment

Biological pretreatment involves the inoculation of lignocellulosic biomass with wood degrading microorganisms such as white rot fungi to modify the chemical composition of lignocellulosic materials. White rot fungi secrete extracellular enzymes such as lignin peroxidases and laccases, which degrade a large amount of lignin and some hemicellulose while leaving the cellulose mostly intact (Lee et al, 2007). Although the majority of the work on biological pretreatment has focused on its applications in the pulp and paper industry, there have been a few studies that evaluate its effectiveness as a pretreatment for bioenergy applications. For example, Taniguchi et al, (2005) showed that pretreatment of rice straw with the white rot fungus Pleurotus ostreatus resulted in the selective degradation of lignin leaving behind a substrate that was more susceptible to enzymatic hydrolysis. A more recent study by Muñoz et al, (2007) showed that incubation of Pinus radiata (a softwood) chips with the white rot fungus Ceriporiopsis subvermispora prior to organosolv pretreatment resulted in improved delignification and susceptibility to enzymatic hydrolysis (from 13 % to 6 % residual lignin and from 55 % to 100 % glucose conversion). The main advantages of biological pretreatment are the low energy requirements and benign environmental
conditions. However, the slow rate of delignification (taking days to weeks) and the large amount of space required to perform the pretreatment limits its application in large scale operations (Sun and Cheng, 2002).

1.4.2 Physical Pretreatments

Physical pretreatments typically rely on the principle of increasing surface area as a result of particle size reduction of the biomass (Zhu et al, 2010). This reduction in particle size is often achieved through a combination of chipping, grinding, and/or milling. Besides increased surface area, mechanical size reduction can improve a substrate’s susceptibility to enzymatic hydrolysis by reducing cellulose crystallinity and degree of polymerization (DP) (Fan et al, 1980a). The main advantage of physical pretreatments is that they are relatively insensitive to the physical and chemical characteristics of the biomass employed. However, size reduction processes are energetically demanding and often consume more energy than is available in the biomass and as a result are generally not economically attractive (Fan et al, 1987; Kumar et al, 2009). It should also be pointed out that physical pretreatments do not (usually) result in significant improvements in cellulose hydrolysis unless accompanied by other pretreatments likely due to the fact that they do not remove a significant amount of lignin and hemicellulose. For example, Zeng and coworkers (2007) showed that reducing the size of corn stover from 425-710 μm to 53-75 μm without further pretreatment resulted in only slight improvements in cellulose hydrolysis yields (from 14 % to 21 %). On the other hand, Ford (1983) showed that reducing the size of Pangola grass stems from 1.0 mm to 0.1 mm had a greater impact on its susceptibility towards cellulytic hydrolysis (from 40 % to 90 % cellulose conversion respectively) than did complete delignification. However, it should be noted that the Pangola grass stems contained half the amount of lignin than did the
corn stover (10 % and % respectively), which is likely to have contributed to the differences in cellulose hydrolysis yields.

1.4.3 Physicochemical/Chemical Pretreatments

Physicochemical/chemical pretreatments can be classified as alkaline, neutral, acidic, oxidative as well as solvent. While this classification is based on the nature of the pretreatments’ mechanism and chemical additives, it is somewhat simple as there can be significant overlap between the different types of pretreatments. For example, as will be discussed in more detail in section 1.5, although organosolv pretreatment is a solvent-based pretreatment, it can be performed under either acidic or alkaline conditions (McDonough, 1993).

Pretreatment with alkaline additives such as sodium hydroxide (NaOH), calcium hydroxide (Ca(OH)$_2$ or lime) and ammonia (NH$_3$) specifically target the cleavage of ether bonds in lignin as well as the hydrolysis of ester bonds in lignin-carbohydrate linkages resulting in the solubilization and partial extraction of lignin (Gupta and Lee, 2010). This produces a highly swollen substrate that has increased accessibility to hydrolytic enzymes (Laureano-Perez et al, 2005; Kim and Lee, 2005). Strong bases such as NaOH can remove a significant portion of the hemicelluloses as soluble oligosaccharides. For example, Varga et al, (2002) showed that pretreatment of corn stover with 10 % (w/w) NaOH resulted in the removal of 95 % of the lignin and 88 % of the hemicellulose. However, because the use of NaOH requires costly chemical recovery systems, Ca(OH)$_2$ though somewhat less effective is the preferred alkaline additive as it is less expensive and easier to recover with well-established lime kiln technologies (Chang et al, 1997; Sierra et al, 2009; Zhao et al, 2008).
As an alternative to the use of more traditional bases such as NaOH and Ca(OH)$_2$, Jin et al, (2010) proposed the use of a novel alkaline pretreatment process using green liquor (GL), which is a mixture of Na$_2$CO$_3$ and Na$_2$S that is part of the chemical recovery process during kraft pulping. The main advantages of GL pretreatment over the more traditional alkaline pretreatments are efficient chemical recovery using proven technologies and ease of incorporation into an existing infrastructure (kraft pulp mills, Jin et al, 2010). However, because GL pretreatment is a mildly alkaline process, only partial delignification (approximately 32 %) can be obtained. As a result, further lignin removal via a post-treatment step is required to lower the enzyme loadings required for efficient enzymatic hydrolysis (Koo et al, 2011).

The addition of oxidizing agents such as hydrogen peroxide or high pressure air/O$_2$ to enhance delignification during alkaline pretreatment is known as alkaline wet oxidation and results in the generation of a cellulose-rich substrate that is highly susceptible to enzymatic hydrolysis. For example, Saha and Cotta (2006, 2007), were able to obtain over 95 % cellulose conversion from alkaline H$_2$O$_2$-pretreated wheat straw and rice hulls. The advantages of alkaline pretreatments (oxidative and non-oxidative) include decreased sugar degradation, recovery of hemicellulosic sugars in oligomeric form and the potential to recover/regenerate the alkaline additives. A further advantage of alkaline pretreatment is that they can be performed over a wide range of temperatures (from 20-170 °C). However, alkali pretreatments have traditionally been considered unsuitable for the production of ethanol from woody biomass due to the requirement of high amounts of alkali, and the loss of alkali via its conversion to irrecoverable salts and/or incorporation into the biomass (Zhu et al, 2010; Gosset et al, 1982). Regarding alkaline wet oxidation processes, although they can
effectively produce a substrate that is readily hydrolyzed by cellulases, they are only suitable for biomass feedstocks containing low levels of lignin as the oxidized lignin has no commercial value and cannot be used as boiler fuel (Galbe and Zacchi, 2002). Further disadvantages of alkaline wet oxidation include the formation of lignin and (to a lower extent carbohydrate) degradation products such as soluble aromatic compounds and organic acids that may inhibit downstream processes as well as the potential to oxidize cellulose, which is detrimental to enzymatic hydrolysis (Xu et al, 2009).

In contrast to alkaline pretreatments, which target the ether bonds in lignin and ester bonds in lignin-carbohydrate linkages, pretreatments under neutral and acidic conditions increase the accessibility of cellulose to cellulases via the removal of hemicellulose and partial depolymerization of cellulose. Neutral pretreatments include hydrothermolysis and uncatalyzed steam pretreatment. Hydrothermolysis is also known as liquid hot water (LHW) pretreatment is one of the oldest methods used in the pretreatment of lignocellulosic biomass (Taherzadeh and Karimi, 2008). Briefly, this process involves the exposure of biomass to water at high temperature and pressure (Weil et al, 1998). The high temperature water acts as an acid and solubilizes the hemicellulose (and some lignin) while leaving most of the cellulose intact (Kumar et al, 2009). The hemicellulose removal in turn increases the substrate’s porosity, which increases its accessibility to cellulases (Jeoh et al, 2007). Mosier et al, (2005b) found that it is possible to obtain over 90 % cellulose conversion after subjecting corn stover to pH-controlled hydrothermolysis for 15 minutes at 190 °C. The advantages of hydrothermolysis include high hemicellulose recovery, short pretreatment times and the ability to pretreat substrates without prior size reduction. However, the solution pH must be constantly monitored and maintained between 4 and 7 to avoid the formation of
inhibitory compounds (Mosier et al, 2005b). Also, as it does not remove a significant amount of lignin, hydrothermolysis cannot be used to process (highly lignified) woody biomass.

Uncatalyzed steam pretreatment of biomass has its origins in the Masonite process, which was developed in the 1920’s for the production of wood particle board (Boehm, 1930). During steam pretreatment, biomass is exposed to steam at high temperature and pressure before the process is terminated by exposing the biomass to atmospheric pressure. The release of pressure increases the biomass’ surface area and promotes fibre separation due to fragmentation by mechanical shear. During uncatalyzed steam pretreatment of herbaceous and hardwood biomass, acetyl groups from hemicellulose are cleaved off and catalyze the partial hydrolysis of hemicellulose to mono- and oligosaccharides (known as autohydrolysis). Lignin on the other hand is redistributed on the fibre’s surface as a result of softening and depolymerization/repolymerization reactions (Shevchenko et al, 2001). The removal of hemicellulose and redistribution of lignin are believed to expose the cellulosic fibres and increase the accessibility of cellulose to cellulolytic enzymes (Kabel et al, 2007). For instance, Grous et al, (1986) reported that 90% of the cellulose present in steam pretreated poplar could be hydrolyzed in 24 h compared to only 15% glucan conversion for untreated chips.

Although autohydrolysis allows the processing of herbaceous and hardwood biomass without the addition of external chemicals, the addition of acidic catalysts prior to pretreatment can improve hemicellulose recovery and decrease the formation of sugar degradation products by allowing the use of lower temperatures and shorter reaction times (Bura et al, 2003). In contrast, steam pretreatment of softwoods requires the addition of an acidic catalyst such as sulfur dioxide (SO₂) or sulfuric acid (H₂SO₄) (Mais et al, 2002;
Shevchenko et al, 2001). However while effective on herbaceous and hardwood biomass, the application of SO$_2$-catalyzed steam pretreatment to softwoods such as Douglas-fir and lodgepole pine has proven considerably more challenging. The remaining recalcitrance of steam pretreated softwoods is largely due to the greater content and increased hydrophobicity of the lignin component of the pretreated softwood substrates (Boussaid et al, 2000; Ewanick et al, 2007; Kumar et al, 2010; Kumar et al, 2012).

The advantages of steam pretreatment include low energy requirement, low water usage, favourable economics (Holtzapple et al, 1989; Duff and Murray 1996) recovery of the majority of the original cellulose and hemicellulose-derived carbohydrates in a fermentable form and the ability to pretreat biomass without prior size reduction (Bura et al, 2003; Clark and Mackie 1987). The major disadvantage of steam pretreatment is the potential formation of sugar degradation products such as 5-hydroxymethylfurfural, furfural and acetic acid, which are inhibitory to subsequent fermentation of the hydrolyzed saccharides (Ewanick et al, 2007; Kabel et al, 2007). In addition, pretreatment under conditions that result in complete hemicellulose removal (high severity) can lead to lignin condensation, which besides increasing the recalcitrance of the pretreated substrate, limits the development of value-added lignin derived co-products and it can usually only be used as a boiler fuel (Shevchenko et al, 2001; Sun et al, 2004). At low pretreatment severities, the residual hemicelluloses and lignin tend to hinder the accessibility of cellulose to cellulases (Ewanick et al, 2007) and as a result of this limitation, steam pretreatment conditions are often a compromise between maximizing sugar recovery, while providing an effective separation of hemicellulose from the lignin-carbohydrate matrix (Mackie et al, 1985). Due to their high (approximately 50 %) lignin content, steam-pretreated softwood substrates are highly recalcitrant and typically require an
additional post-treatment to achieve significant cellulolytic hydrolysis yields (Kumar et al, 2012). Nonetheless, because of its low energy input and the fact that it does not require particle size reduction, acid-catalyzed steam pretreatment is the process that has been suggested as being closest to commercialization (Jørgensen et al, 2007).

Dilute acid pretreatment is similar in concept to acid-catalyzed steam pretreatment as they are both based on the removal of hemicellulose from biomass to increase its porosity resulting in a substrate that is easier to saccharify compared to the initial biomass (Ishizawa et al, 2007). Besides enhancing cellulolytic hydrolysis via hemicellulose removal a further similarity between dilute acid and steam pretreatment is that lignin has been shown to soften, coalesce and migrate to the surface of the biomass matrix where it is redeposited in the form of droplets during cooling. These lignin droplets were found to inhibit the enzymatic hydrolysis of dilute acid pretreated maize stems and steam pretreated Pinus radiata and are believed to contribute to the residual recalcitrance of dilute acid and steam pretreated biomass (Selig et al, 2007; Wong et al, 1988). While the mechanism of action of dilute acid and steam pretreatment is conceptually similar, there are some key differences. For example, the biomass is usually subjected to mechanical comminution prior to dilute acid pretreatment whereas as previously mentioned, steam pretreatment (acid-catalyzed and uncatalyzed) does not require particle size reduction. Another difference between steam pretreatment and dilute acid hydrolysis, is that during dilute acid hydrolysis, the biomass is usually pretreated as a low (5 % w/v) solids slurry, while with steam pretreatment, the moisture content of the biomass is usually 5-10 % (w/v) (Jørgensen et al, 2007). The advantages and disadvantages of dilute acid pretreatment are similar to those of steam pretreatment with the addition that the process and energy costs are higher as a result of the mechanical comminution step, the
lower solids content and the need to neutralize and detoxify the product streams prior to enzymatic hydrolysis (Klinke et al., 2004; Buhner and Agblevor, 2004).

Although the pretreatments mentioned above have shown varying degrees of success with herbaceous and hardwood biomass, reducing the recalcitrance of softwoods is considerably more challenging (Várnai et al., 2010). As a result of the greater recalcitrance of pretreated softwood substrates, higher enzyme loadings (20-40 FPU g\(^{-1}\) cellulose) and longer incubation times are required to achieve effective saccharification (Pan et al., 2007). However, due to the abundance of softwoods in many parts of the world, such as Canada, Russia and Scandinavia, it is likely that they will play a significant role in future biorefinery applications.

The primary options that are currently being assessed for pretreatment of softwoods include processes that modify lignin and/or fractionate biomass, such as cellulose-solvent, SPORL (sulphite pretreatment to overcome recalcitrance of lignocellulose) and organosolv. These processes can effectively pretreat softwoods to generate substrates that are more amenable to cellulolytic hydrolysis (Pan et al., 2008; Zhu et al., 2009; Del Rio et al., 2010). Cellulose-solvent pretreatments such as COSLIF (cellulose solvent and organic solvent lignocellulose fractionation) and ionic liquid (IL) pretreatments are also based on the principle of solubilizing and/or partitioning the various components of the plant cell wall and have received considerable interest over the last decade (da Costa Souza et al., 2009). The COSLIF process is based on the complete fractionation of biomass as a result of the preferential solubility of the various cell wall components in different solvents. Briefly this process involves the dissolution of cellulose and hemicellulose in concentrated H\(_3\)PO\(_4\) followed by their precipitation via the addition of acetone (which dissolves lignin). Washing
the precipitated carbohydrate fraction with water removes most of the hemicellulose leaving behind a pure cellulosic substrate that is highly amenable to cellulolytic hydrolysis (Zhang et al, 2007a).

Ionic liquid (IL) pretreatment is similar in concept to the COSLIF process as it is based on the dissolution of biomass in a suitable solvent (the ionic liquid, salts with a melting point < 100 °C) followed by the addition of an antisolvent (such as water, ethanol or acetone) to precipitate cellulose in a form that is readily hydrolyzed by cellulolytic enzymes (Blanch et al, 2011; Mora-Pale et al, 2011). However, because of the large number of IL’s that are potentially available (up to a trillion), their solvent properties are highly “tunable” thus offering greater selectivity in their ability to fractionate biomass, from complete cell wall dissolution/fractionation to just the selective removal of cellulose or lignin (Mora-Pale et al, 2011; Pu et al 2007; Vitz et al, 2009).

Advantages of cellulose-solvent pretreatments include the ability to pretreat all biomass, the low vapour pressure of the cellulose solvents, which minimizes their losses/environmental impact (Mora-Pale et al, 2011) and in the case of the COSLIF process, moderate temperature (50 °C), which minimizes degradation reactions (Sathitsuksanoh et al, 2010; Zhang et al, 2007a). There are however several challenges preventing the implementation of cellulose-solvent based pretreatments beyond the laboratory. These include high solvent costs, the need for high liquid to solid ratios (> 10:1), the need to dry the biomass prior to pretreatment (to prevent the inhibition of cellulose dissolution by water), the need for extensive particle size reduction prior to pretreatment and the inhibition of enzymatic hydrolysis by trace amounts of ionic liquids, (Banarjee et al 2011; da Costa Sousa et al, 2009; Mora-Pale et al, 2011; Zhang et al, 2007a).
The SPORL process is a two stage pretreatment that combines a mild thermochemical treatment with a mechanical size reduction step. During the first stage, wood chips are impregnated with a solution of sodium bisulphite in H$_2$SO$_4$ at 160-190 °C. The addition of bisulphite increases the solution pH, which results in the removal of hemicellulose as a mixture of oligo- and monomeric sugars and reduces the formation of sugar degradation products (Shuai et al, 2010, Wang et al, 2009). In addition to hemicellulose removal, the first stage results in the sulphonation and partial removal of lignin, which softens the wood chips, and decreases the energy required for mechanical size reduction (Zhu et al, 2010).

Besides energy savings, sulphonation increases the hydrophilicity of lignin, which reduces the hydrophobic interactions with cellulases and improves the enzymatic hydrolysis of cellulose. Recent studies, have shown that it is possible to achieve > 90 % cellulose conversion from SPORL-pretreated spruce and red pine at cellulase loadings of 15 FPU g$^{-1}$ cellulose without further delignification despite removal of only 30 % of the lignin present in the initial feedstock (Zhu et al, 2009; Shuai et al, 2010). In contrast and as previously discussed, green liquor pretreatment results in comparable lignin removal, yet requires further delignification and higher enzyme loadings to achieve comparable cellulose conversion yields. (Yu et al, 2011). Advantages of the SPORL process include relative ease of incorporation into existing infrastructure (mechanical pulp mills) and the generation of lignosulphonates, which have several commercial applications such as dyes, emulsifiers, asphalt and binding agents for animal feed pellets amongst others (Fengel and Wegener, 1989). However, while the SPORL process generates a substrate that is readily hydrolyzed at moderate cellulase loadings (15-20 FPU g$^{-1}$ cellulase), further decreases in cellulase loadings
to 2.5 FPU g\(^{-1}\) cellulose and lower are required to reduce the costs associated with the bioconversion process (Merino and Cherry, 2007).

Although capable of pretreating a variety of lignocellulosic feedstocks (including softwoods), the implementation of cellulose-solvent and SPORL pretreatments into future biorefinery applications will likely be limited by several factors. These include the requirement for cost-effective solvent recovery systems and the need for relatively high cellulase loadings respectively. However, the organosolv process is a promising pretreatment that can be readily scaled up and is capable of fractionating softwoods into a cellulose-rich solid fraction, an aqueous hemicellulose stream and a highly pure sulphur-free dry lignin fraction (Duff and Murray, 1996).

1.5 Organosolv Pretreatment

The organosolv process was originally developed as a pulping method for the chemical fractionation of lignocellulosic biomass using organic solvents (Kleinert and Tayenthal, 1931). Briefly, this process involves treating wood chips with a mixture of water, an organic solvent such as ethanol, methanol or acetone and a (usually acidic) catalyst at 150-200 °C and 400 PSI. This results in the removal of the majority of the hemicelluloses and lignin through the cleavage of glycoside linkages, lignin-carbohydrate bonds, lignin-lignin bonds and solubilization of the lignin in the organic solvent leaving behind a cellulose-rich solid (McDonough, 1993; Sarkanen, 1990). However, high solvent costs and inevitable solvent losses associated with the process have prevented it from being used in industrial (pulping) applications (Rydholm, 1965). Stringent environmental regulations, which imposed the need for reduced emissions and toxic effluents on the pulp and paper industry led to renewed interest in the organosolv process as an environmentally friendly alternative to kraft
pulping of hardwoods and softwoods during the 1980’s (Stockburger, 1993; Azis and Sarkanen, 1989). This resulted in the development of various organosolv pulping processes that were subjected to commercial scale up including the ALCELL™ (alcohol cellulose), Organocell and ASAM (alkali sulphite anthraquinone methanol) processes (Stockburger, 1993; Hergert, 1998). Several factors including high energy requirements, solvent costs and challenges with solvent recovery conspired to prevent the various organosolv processes from becoming commercially viable alternatives to kraft pulping. However, because of its potential for the development of co-products from lignin and its capacity for scale up, the organosolv process is an attractive option for the pretreatment of woody biomass and is the basis of the process used by the Canadian company “Lignol Energy Corporation” (Pan et al, 2005; 2006a).

1.5.1 Alcohol-Based Organosolv Pretreatment

Despite the fact that organosolv pretreatment can be carried out with a variety of organic solvents, low molecular weight (C1-C4) primary alcohols are the solvent of choice due to their effectiveness as delignification agents and relative ease of recovery (Zhao et al, 2009a). A study by Ghose and coworkers (1983) evaluated the efficiency of various aliphatic alcohols on the delignification of agricultural residues and found that n-butanol was the most selective delignification agent due to its increased hydrophobicity. However, due to their lower cost methanol and ethanol are the most widely used solvents for organosolv pretreatment (Zhao et al, 2009). Further advantages to the use of methanol and ethanol as pretreatment solvents include their low boiling point (65 °C and 78 °C respectively), which allows their recovery via simple distillation and complete miscibility with water (Sarkanen, 1990).
The major advantage to the use of methanol as a pretreatment solvent is the lower energy requirements during solvent recovery (due to its lower boiling point) and ease of penetration into the lignocellulosic material (Chum et al, 1988). However, because of its lower toxicity and reduced tendency to form highly flammable vapours at low temperatures, ethanol (EtOH) is the preferred solvent for organosolv pretreatment. A further advantage to the use of ethanol is that losses during pretreatment can be readily replenished as it is the final product of the bioconversion process. Other than ethanol and methanol, higher boiling point alcohols such as ethylene glycol and glycerol have been successfully applied as pretreatment solvents (Gharpuwy et al, 1983; Sun and Chen, 2007). However, their high cost and energy consumption during recovery decreases their viability as pretreatment solvents (Zhao et al, 2009a).

Besides the ability to use various solvents, organosolv pretreatment can be carried out under acidic and alkaline conditions although acid-catalyzed organosolv processes are more commonly employed. Similar to steam pretreatment, hardwoods and agricultural residues can be subjected to organosolv pretreatment with or without a catalyst. During uncatalyzed organosolv pretreatment, delignification is promoted by the release of acetic acid from hemicelluloses (Sarkanen, 1990). However without the addition of a catalyst, higher temperatures (< 190 °C) are required for effective delignification to occur (Pye and Lora, 1991). In contrast (and also similar to steam pretreatment), organosolv pretreatment of softwoods requires the addition of a catalyst due to the fact that softwood hemicelluloses contain significantly less acetyl groups (1-2 % w/w) than do hardwood hemicelluloses (3-5 % w/w) (Pye and Lora, 1991; Sjöström, 1993).
1.5.2 Organic Acid and Peracid-Based Organosolv Pretreatment

Although low boiling point alcohols such as methanol and ethanol are effective lignin solvents, low molecular weight organic acids such as acetic and formic acid and their corresponding peracids can also be used as solvents for organosolv pretreatment (Baeza et al., 1991; Vazquez et al., 2000; Pan et al., 2006c). As will be discussed in section 1.6.3, an effective solvent for a particular solute should have a Hildebrand solubility parameter ($\delta$) that is close to that of the solute, which in the case of lignin is approximately $11$ (cal/cm$^3$)$^{1/2}$ (Pan and Sano, 1999). Formic and acetic acid have $\delta$ values of $12.1$ and $10.1$ (cal/cm$^3$)$^{1/2}$ respectively and are therefore effective lignin solvents that have been used with relative success in the pulping of hardwoods and softwoods (Nimz and Casten, 1986; Erismann et al., 1994).

With regards to the use of organic acid-based organosolv pretreatment for bioconversion processes, the results have been considerably less successful despite their excellent lignin solvent properties. For example, Baeza et al. (1991) found that although formic acid pretreatment of Pinus radiata D. Don sawdust resulted in significant delignification, the overall cellulose hydrolysis yields were only $56\%$. Similarly, Vazquez et al. (2000) found that acetic acid pretreatment of Eucalyptus did not result in significant improvements on the rate or extent of cellulose conversion despite extensive hemicellulose removal and delignification. Those results were attributed to the residual lignin being found on the fibres’ surface and preventing the accessibility of cellulose to cellulolytic enzymes.

Similar results were obtained in a more recent study by Pan and coworkers (2006c), who compared the susceptibility of acetic acid and ethanol-organosolv pretreated Douglas-fir to enzymatic hydrolysis. However rather than the residual lignin preventing cellulose
accessibility, the negative effects of acetic acid pretreatment on cellulose hydrolysis were attributed to decreased interactions between the cellulose and cellulases as a result of cellulose acetylation. This hypothesis was further supported by the fact that deacetylation of the acetic-acid pretreated substrate resulted in improved hydrolysis yields (from 10% to 60% respectively; Pan et al, 2006c). Further disadvantages to the use of organic acids as pretreatment solvents include their highly corrosive nature and the fact that they do not swell carbohydrates (Young and Davis, 1986; Young, 1998). As a result, organic acids though effective as lignin solvents are not generally used for the pretreatment of lignocellulosic biomass.

In contrast to organic acids, organic peracids such as peracetic acid (PAA) are powerful oxidizing agents that are highly selective towards lignin and are readily prepared by reacting the corresponding organic acid with hydrogen peroxide and catalytic amounts of sulphuric acid (Zhao et al, 2009b). Advantages of organic peracid-based organosolv pretreatment include mild pretreatment conditions (20-80 °C and atmospheric pressure) and high carbohydrate yields due to the fact that organic peracids do not react with carbohydrates (Teixeira et al, 1999a; 1999b). However, there are some significant disadvantages such as chemical costs, risk of explosion and equipment corrosion (Zhao et al, 2009a). Also, unlike other organosolv processes, the oxidized lignin produced during organic peracid-based organosolv pretreatment cannot be used for co-product applications. Therefore, it is unlikely that organosolv pretreatments based on organic peracids will ever be economically feasible.

1.5.3 Acetone-Based Organosolv Pretreatment

Besides low molecular weight alcohols and organic acids, ketones can be used as solvents for the organosolv pretreatment of lignocellulosic materials. Acetone is the most
widely used ketone as it has a δ value of 10 (cal/cm$^3$)$^{1/2}$, which is comparable to that of acetic acid (10.1 (cal/cm$^3$)$^{1/2}$) and is therefore an effective lignin solvent and is fully miscible with water (Zhao et al, 2009a). Further advantages to the use of acetone are that it does not get incorporated into the biomass or form an azeotrope with water and has a low boiling point (56 °C), all of which facilitate its recovery. Paszner and Chang (1983), developed a process called acid-catalyzed organosolv saccharification (ACOS), which uses aqueous acetone (50-90 % v/v), and catalytic amounts of mineral acids to separate all lignocellulosic materials into a high-purity lignin and fermentable sugars. The ACOS process was further improved to include an aqueous extraction step to fractionate the biomass into a hemicellulose-derived oligosaccharide stream, high purity lignin and glucose (Paszner et al, 1985).

The major advantage of the ACOS process is that it produces pure glucose, which can be readily fermented, thus eliminating the need of an enzymatic hydrolysis step (Jeong, 1994). However, there are some challenges that need to be addressed before the process can be implemented at an industrial scale. For example, ACOS pretreatment is energy intensive as it requires the continuous flow of solvent at very high temperature (230 °C) and pressure (1500 PSI) (Paszner and Chang, 1987). Furthermore, because the cost of acetone is higher than that of methanol and ethanol, efficient solvent recovery is required.

Overall, the organosolv process has the potential to become a viable pretreatment for the production of ethanol from lignocellulosic biomass especially softwoods as it can generate a high-purity sulphur-free lignin fraction that can provide additional revenue in the form of co-products. Of the various solvents that are available, ethanol is the most likely to be used in future applications due to its lower toxicity, ease of recovery via simple distillation
and because losses during pretreatment can be readily replenished as it is the final product of
the bioconversion process.

1.6 Factors Affecting Delignification

The rate and extent of delignification during organosolv pretreatment is a complicated
process that is partly governed by the chemical environment that is provided by the various
pretreatment solvents and catalysts and the physicochemical properties of the biomass itself.
These properties include density, permeability to pretreatment liquors, lignin content and
chemistry (i.e. the type and amount of linkages). Other factors that influence the rate and
extent of delignification are the process variables such as cooking time and temperature. For
example, Tirtowidjojo et al, (1988) showed that the rate of delignification of acid-catalyzed
organosolv pulping doubled with every 10 °C increase in cooking temperature over the range
of 130-170 °C. Therefore, a thorough understanding of these factors is required for the
development of viable biorefineries based on the organosolv process.

1.6.1 Effect of Permeability on Delignification

For lignin to be effectively solubilized during chemical pulping (including organosolv
pretreatment), it must maintain constant contact with the pretreatment liquor. This contact is
established when the pretreatment chemicals enter the wood via vessels (in hardwoods) and
tracheids (in softwoods) and allows the chemicals to diffuse and maintain contact with lignin.
As mentioned earlier in section 1.3.6, hardwoods are generally more permeable to
pretreatment liquors due to the presence of vessel elements, which contributes to their ease of
delignification compared to softwoods. Besides penetration of the pretreatment liquor via
vessels and tracheids, effective contact between lignin and the pretreatment liquor is partially
governed by the thickness of the wood chip. Generally, wood chips that are too thick will
have untreated centres due to incomplete penetration of the pretreatment liquor (Adams et al, 1989). Additionally, swelling of the cell wall as a result of exposure to pretreatment liquor enhances delignification by increasing pore size, which allows the diffusion of lignin from the cell wall (Marton, 1971; Chum et al, 1988). Finally, fibre separation during pulping enhances further delignification by allowing lignin fragments to diffuse out of the cell wall (Marton, 1971).

1.6.2 Lignin Composition and Structure

Besides the permeability of biomass to the pretreatment liquor, lignin content and structure play significant roles in the overall selectivity of delignification and efficiency of the organosolv process. One of the most well documented relationships between lignin and ease of delignification is the relative amount of syringyl and guaiacyl units (known as the S:G ratio). In guaiacyl units, only the third position of the aromatic ring is methoxylated, whereas in syringyl units the third and fifth positions are methoxylated (Figure 1). Therefore, guaiacyl units are able to form linkages at position 5 with other monolignols, while syringyl units are only able to form linkages at position 4 of the aromatic ring and the propane side chain. The formation of 5-5’ carbon-carbon bonds between two guaiacyl units is one of the reasons softwood lignin (which is mostly composed of guaiacyl units) is more recalcitrant than hardwood lignin (Chang and Sarkanen, 1973).

Besides the proportion of syringyl and guaiacyl units, the linkages between individual monolignols have a significant effect on the recalcitrance of lignin. While there are several types of bonds in the lignin macromolecule, ether bonds (specifically β-O-4 ethers) are the predominant linkage (Table 1). Therefore the effectiveness of the organosolv process (which is based on the breakdown of lignin into soluble fragments) is largely dependent on the
extent of ether bond cleavage (Sarkanen, 1990). Interestingly, Tsutsumi et al. (1995) showed that β-aryl ether lignin model compounds that contained syringyl units were more reactive (cleaved faster and to a greater extent) than those that only contained guaiacyl units. These differences in the rate and extent of β-aryl ether cleavage were attributed to differences in reactivity as a result of the chemical structures of the model compounds. However, it is possible that the formation of 5-5’ carbon-carbon bonds in the model compound that contained only guaiacyl units contributed to the lower extent of ether cleavage.

1.6.3 Effects of the Chemical Environment

The choice of solvent and chemical additives is of the utmost importance as they are responsible for the chemical environment in which lignin degradation and solubilization takes place. The ability of a solvent to dissolve lignin depends on its ability to form hydrogen bonds and its Hildebrand solubility parameter ($\delta$), which is calculated by the following equation (Hildebrand and Scott, 1950):

$$\delta = (-E/V_l)^{1/2}$$

(1)

where $-E$ is the energy of vaporization to a gas at zero pressure, and $V_l$ is the molal volume of the liquid. As mentioned in section 1.5.2, an effective solvent for a given solute should have a $\delta$ value that is close to that of the solute, which in the case of lignin ranges from ~11-14 (cal/cm$^3$)$^{1/2}$ (Schuerch, 1952, Pan and Sano, 1999). Therefore solvents ranging in $\delta$ values from ~10-14 (cal/cm$^3$)$^{1/2}$ such as acetone, butanol, dioxane, methanol, ethanol, formic acid and acetic acid, ($\delta$ values of 10, 11.4, 10, 14.5, 12.9, 12.1 and 10.1 (cal/cm$^3$)$^{1/2}$ respectively) are effective lignin solvents (Schuerch, 1952, Wang et al, 2011).

It should be noted that, for effective liquor penetration and fibre swelling, solvents need to be mixed with water (Yawalata, 2001; Ni and van Heiningen, 1997). However,
because water has a δ value of ~22.5 (cal/cm$^3$)$^{1/2}$ its addition increases the δ value of the pretreatment liquor, which decreases the effectiveness of delignification and promotes condensation and precipitation of the dissolved lignin fragments (Bose and Francis, 1999; Pan et al, 2006b; 2008). Therefore, besides the type of solvent, the ratio of solvent to water in the pretreatment liquor is a parameter that needs to be taken into consideration as it has a significant effect on delignification. Previously, Goyal et al, (1992) showed that during organosolv pulping of hardwoods, the maximum degree of delignification was achieved when the ethanol concentration was 70 % (w/v). These results agreed with a later study by Bose and Francis (1999), which showed that, in the case of Norway spruce, the maximum degree of delignification was obtained at an ethanol concentration of 76 %. That study also showed that ethanol concentrations higher than 90 % had a negative effect on delignification, which was attributed to the contraction of the cell wall in the organic solvent, which prevented the diffusion of the pretreatment liquor.

Although the solvent to water ratio in the pretreatment liquor (and corresponding δ value) plays a significant role in governing the effectiveness of the pretreatment, the solubility of lignin is inversely proportional to its molecular weight (Schuerch, 1952; Yawalata, 2001). Therefore, effective delignification requires that the lignin macromolecule be broken down into smaller fragments that are soluble in the pretreatment liquor. This cleavage of the lignin macromolecule can be achieved by the use of chemical additives to change the chemical environment of the pretreatment liquor. As mentioned in section 1.5.1, organosolv pretreatment can be carried out under either acidic or alkaline conditions. Under acidic conditions, organosolv delignification is due to the solvolysis of α- and β-O-4 aryl ether linkages in the lignin macromolecule (Meshgini and Sarkanen, 1989; Sarkanen, 1990;
McDonough, 1993). Previous studies using model compounds showed that, under the same acidity and temperature, the cleavage of α-O-4 aryl ether linkages requires significantly lower activation energy than does the cleavage of β-O-4 aryl ethers (80-118 vs. 148-151 kJ mol⁻¹ respectively, Meshgini and Sarkanen, 1989). Therefore, it is likely that in the early stages, delignification is primarily due to the solvolytic cleavage of α-O-4 aryl ether bonds.

The acid-catalyzed mechanism of α-O-4 aryl ether solvolysis is believed to occur via the protonation of the oxygen bound the α-carbon to generate an alcohol leaving group and a benzyl carbonium ion intermediate. This intermediate can then react with either water or the alcohol solvent to generate a benzyl alcohol or ether respectively (Figure 5) and with other nucleophiles, which can significantly alter the properties of the lignin. For example sulphurous acid can be used to generate lignosulphonates, which are significantly more hydrophilic than native lignin (Sjöström, 1993) and as discussed in section 1.4.3, have significant industrial applications. However, an undesirable (though minor) competing reaction is the condensation of the carbonium intermediate with an aromatic nucleus (Sarkanen, 1990).

![Figure 5](image-url)

**Figure 5.** Acid-catalyzed solvolytic cleavage of α-O-4 aryl ether linkages via the formation of a benzyl carbonium ion intermediate. N = ᵃ-OH, ᶊOCH₃, ᶇO(CH₂)ₙCH₃, etc. Adapted from Sarkanen (1990) and McDonough (1993).
Another major lignin reaction during acid-catalyzed organosolv pretreatment is the solvolytic cleavage of β-aryl ether bonds (Bose and Francis, 1999; Kishimoto and Sano, 2002). Although their cleavage requires higher activation energy and acidity, β-aryl ethers account for 50 to 60% of the interunit linkages in lignin and therefore their solvolytic cleavage plays a significant role in determining the extent of lignin degradation to soluble fragments (Bose and Francis, 1999). The solvolytic cleavage of β-aryl ether bonds is believed to occur via two reaction mechanisms. The first (and faster) mechanism involves the protonation of the oxygen at the α-carbon and the formation of a benzyl carbonium ion. The benzyl carbonium ion is stabilized by the release of a proton from the β-carbon and subsequent formation of a carbon-carbon double bond, followed by the rapid cleavage the β-aryl ether (Figure 6a).

Similar to the reaction mechanism described above, the second (and slower) mechanism of β-aryl ether solvolysis involves the formation of a benzyl carbonium intermediate. However, the benzyl carbonium ion is stabilized by the release of formaldehyde from the γ-carbon, followed by the formation of a double bond between the α- and β-carbons and the cleavage of the β-aryl ether (Figure 6b; Sarkanen 1990; McDonough, 1993). As a result of the acidic environment, the formaldehyde released during β-aryl ether cleavage, can undergo (undesirable) condensation reactions with aromatic moieties (Figure 7; Sarkanen, 1990; Chum et al, 1999). Finally, β-aryl ether structures containing free phenolic groups may undergo homolytic cleavage and rearrangements to form highly condensed structures (Sarkanen, 1990; Kishimoto and Sano, 2002).

It should be noted that besides enhancing delignification, acidic catalysts such as HCl and H$_2$SO$_4$ can decrease the strength properties of the cellulose fibres as a result of acid catalyzed
Figure 6. Acid-catalyzed solvolytic cleavage of β-O-4 aryl ether linkages. (a) Reaction involving the formation of a benzyl carbocation at the α-carbon followed by the formation of a ketone at the β-carbon or (b) reaction involving the release of formaldehyde from the γ-carbon. Adapted from Sarkanen (1990) and McDonough (1993).
cellulose and hemicellulose hydrolysis. However, while this reduction in strength properties is not desirable if the fibres are to be used in papermaking, from a bioconversion perspective it may be advantageous due to increased surface area and improved mixing during subsequent enzymatic hydrolysis (Pan et al, 2006b).

Similar to acid-catalyzed processes, organosolv delignification under alkaline conditions is due to the cleavage of α- and β-aryl ether linkages. However, there are significant differences in their reaction mechanisms. For example the cleavage of α-aryl ethers is restricted to free phenolic groups and proceeds via the formation of a quinone methide intermediate followed by the elimination of the substituent at the α-carbon and subsequent incorporation of a nucleophile such as the hydroxide ion (Figure 8a). In the case of β-aryl ethers, an ionized hydroxyl group at the α-carbon can act as a nucleophile and displace the neighbouring β-aryl ether forming an oxirane ring, which can be opened by the addition of a nucleophile (Figure 8b; McDonough, 1993). Besides α- and β-aryl ether cleavage, lignin condensation can occur via the addition of carbanions (formed by the resonance-related delocalization of the negative charge of the phenolate ions) to the α-carbon of a quinone methide intermediate (McDonough, 1993).

In contrast to acid-catalyzed organosolv processes, organosolv pretreatment under alkaline conditions can be thought of as a modification of conventional alkaline pulping processes such as kraft and soda pulping. In general, it is believed that by introducing an organic solvent into the process, it is possible to obtain higher delignification rates and selectivity compared to the parent process (Norman et al, 1993). This enhanced delignification is possibly due to the solubilization of lignin at pH > 10 as a result of the ionization of phenolic groups. However, organosolv pretreatment under alkaline conditions is limited by the same
factors as conventional alkaline pretreatments i.e. larger than catalytic amounts of alkali (15-25 % w/w on oven-dried wood), incorporation of the alkali into the biomass and costly chemical recovery systems (Black, 1991; Stockburger, 1993; Zhu et al, 2010).

Figure 7. Examples of acid-catalyzed lignin condensation reactions as a result of (a) the release of formaldehyde during solvolytic cleavage of β-O-4 aryl ether bonds or (b) the homolytic cleavage of β-O-4 aryl ether bonds containing free phenolic groups. Adapted from Chum et al, (1999) and Sarkanen (1990) respectively.

1.7 Topochemistry of Delignification

Despite being susceptible to cellulolytic hydrolysis, acid-catalyzed organosolv-pretreated substrates contain relatively high amounts (10-20 %) of residual lignin (Pan et al, 2008; Hallac et al, 2010a; Koo et al, 2011). To understand this apparent contradiction, the topochemistry of delignification of the organosolv process compared to other pulping
processes needs to be considered. In the majority of the pulping processes including kraft and acid sulphite, delignification proceeds in two stages. In the first stage, lignin is primarily removed from the secondary wall, while in the second stage lignin is removed from the secondary wall and the middle lamella (Procter et al, 1967; Saka et al, 1982). In the case of organosolv pretreatment the topochemistry of delignification appears to be the opposite to

**Figure 8.** Solvolytic cleavage of lignin under alkaline conditions. Example of α-ether cleavage on lignin units containing free phenolic groups (a) and β-O-4 ether cleavage (b). B− = −OH, −OCH₂CH₃, −OCH₃ etc. Adapted from McDonough (1993).
that of the other processes. During the first (fast) stage, lignin is completely removed from the middle lamella and cell corners, resulting in complete and early fibre separation while in the second stage, delignification is limited to the secondary wall and proceeds at a much lower rate (Paszner and Behera, 1989; Hallac et al, 2010a). It is believed that the selective removal of lignin from the middle lamella allows cellulolytic hydrolysis to occur from the lumen and the middle lamella (Hallac et al, 2010a).

1.8 Enzymatic Hydrolysis of Lignocellulosic Substrates

In addition to pretreatment, enzymatic hydrolysis of the (pretreated) lignocellulosic substrates is a significant barrier to the cost effective commercialization of cellulosic ethanol technologies due to their high price and large amount of protein/enzyme required (Merino and Cherry, 2007; Yang et al, 2011). Projects carried out by companies such as Novozymes and Genencor (DuPont) as well as research institutions such the National Renewable Energy Laboratory (NREL) have resulted in 20-30 fold reductions in the cost of cellulase production (American Chemical Society, 2005; Merino and Cherry, 2007; Aden and Foust 2009; Teter, 2012). However, it is estimated that a further 3-6 fold reduction in enzyme costs (from the current $ 0.68-0.32 gallon⁻¹ ethanol to $ 0.10 gallon⁻¹ ethanol or less) is necessary to meet the US Department of Energy Biomass Program’s enzyme cost goal (Aden, 2008; Klein-Marcushamer et al, 2012). This reduction in enzyme costs can be achieved via further decreases in production costs, development of strategies to reduce the enzyme loadings required for effective cellulose conversion or a combination of both (Wingren et al, 2005; Wyman, 2007).

Overall, enzymatic hydrolysis of lignocellulosic substrates is a slow and complex process that is influenced by the physicochemical properties of the substrate and the mode of
action of the cellulase system (Mansfield et al, 1999; Chandra et al, 2007; Yang et al, 2011). This process is further complicated by the presence of residual lignin and hemicelluloses, which contribute to the residual recalcitrance of the pretreated substrates. As a result, efforts to decrease the cellulase loadings needed to achieve significant cellulose conversion require a better understanding of the cellulase enzyme system as well as the interactions between cellulolytic enzymes and the pretreated lignocellulosic substrates.

1.8.1 The Cellulase System

Enzymatic hydrolysis of cellulose to glucose is a multistage process that is carried out by a number of bacterial and fungal enzymes that are collectively known as the cellulase system. Briefly this process involves the adsorption of the soluble enzymes onto the insoluble cellulose substrate, hydrolysis to fermentable sugars, and desorption of the enzymes. The major components of the cellulase system are the cellobiohydrolases (CBH, EC: 3.2.1.91, also known as exoglucanases), endoglucanases (EG, EC: 3.2.1.4) and β-glucosidases (EC 3.2.1.21). The cellobiohydrolases are subdivided into CBH I and CBH II, which release cellobiose in a processive manner from the reducing and non-reducing ends of the cellulose chain respectively. On the other hand, the endoglucanases hydrolyze the internal β-1,4-glycosidic bonds within the cellulose chain rapidly reducing its degree of polymerization. Filamentous fungi such as *Trichoderma reesei* produce five kinds of EG (EG I-V). Finally, β-glucosidases hydrolyze cellobiose and other soluble oligoglucans to glucose (Warren 1996; Teeri 1997; Lynd et al, 2002; Zhang and Lynd 2004). The three components of the cellulase system act in synergism by creating accessible sites for each other and removing end-product inhibition (Eriksson et al, 2002; Väljamäe et al, 2003) (Figure 9). *T. reesei* has been studied for over
Figure 9. Simplified representation of the enzymatic hydrolysis of cellulose showing the mode of action of the various components of the cellulase system. Modified from Arantes and Saddler (2010).
sixty years and is the most well characterized cellulase producing microorganism (Reese et al, 1950; 1956; Zhang et al, 2006a). The major components of the T. reesei cellulase system are CBH I and CBH II, which comprise approximately 60% and 20% of the total protein content respectively (Goyal et al, 1991; Teeri, 1997).

Most glycoside hydrolyases (including cellulases) are modular proteins composed of a catalytic domain (CD) and a carbohydrate binding module (CBM) (Palonen et al, 2004; Jorgensen et al, 2007). The role of the CBM is to bring the catalytic domain into contact with the substrates’ surface, which is essential for effective cellulolytic hydrolysis. In fact, the removal of the CBM has been shown to result in significant decreases in the rate of cellulose hydrolysis (Suurnäkki et al, 2000). Adsorption of cellulases on pure cellulose has been extensively studied and is thought to involve a combination of hydrogen bonding and hydrophobic interactions (Baker et al, 2001; Reinikainen et al, 1995). Specifically, site directed mutagenesis studies have shown that conserved aromatic amino acids are essential for the function of fungal CBM’s (Reinikainen et al, 1992). It is also believed that CBM’s can enhance cellulosic hydrolysis by disrupting hydrogen bonds between adjacent cellulose chains (Wang et al, 2003; Boraston et al, 2004).

1.8.2 Factors Affecting Enzymatic Hydrolysis of Lignocellulosic Substrates

As previously mentioned, enzymatic hydrolysis of cellulose is a heterogeneous process that involves soluble enzymes acting on an insoluble substrate. This process is influenced by several factors that are associated with the nature of the cellulase system (enzyme-related factors) as well as the substrate (substrate-related factors). The major enzyme-related factors affecting the rate and yield of cellulose conversion include inhibition
of cellobiohydrolases by cellobiose (end product inhibition) and the need to optimize the cellulase system.

End product inhibition of cellobiohydrolases by cellobiose is directly proportional to the initial substrate concentration during enzymatic hydrolysis (Xiao et al, 2004; Bezerra and Dias, 2005). High substrate concentration also results in decreased cellulose hydrolysis yields as a result of incomplete mixing and mass transfer (Taherzadeh and Karimi, 2007). However, despite its inhibitory effect towards cellulolytic hydrolysis and mass transfer limitations, it is generally accepted that future biorefinery applications will require high substrate concentrations to decrease the costs associated with fermentation and distillation (Wingren et al, 2003; Kristensen et al, 2009). Fortunately, there are several suitable strategies that can be employed to reduce end product inhibition. These include supplementation with excess β-glucosidase to ensure complete cellobiose hydrolysis, removal of fermentable sugars by membrane filtration and simultaneous saccharification and fermentation (Breuil et al, 1992; Wingren et al, 2003; Gan et al, 2005).

Improving the performance of the cellulase system is a significant area of research and development with efforts focusing on screening for new cellulose-degrading microorganisms and improving existing fungal strains via genetic modification and random mutagenesis (Jørgensen et al, 2007). While these efforts have resulted in significant progress towards the development of cheaper and more efficient enzymes, these are optimized for one specific substrate and therefore the same degree of efficiency may not be encountered when applied to other substrates (Zhang et al, 2006a). In fact, several studies have shown that production of cellulases on the target substrates results in enzyme preparations that exhibit greater performance than commercially available cellulase formulations (Baker et al, 1997;
McMillan et al, 2001; Jørgensen et al, 2006). This improvement in enzyme performance is likely due to optimized ratios of individual components of the cellulase system (which as mentioned earlier act in synergism) as a result of production on the target substrate. Therefore, it may be advantageous for future biorefineries to have on-site enzyme production facilities that could be fine-tuned to produce cellulase preparations that are optimized for their substrates.

Besides optimizing the cellulase system for the target substrate, supplementing the cellulase system with auxiliary enzymes and proteins that are not directly involved in the hydrolysis of cellulose is a possible approach to improve the enzymatic hydrolysis of lignocellulosic substrates (Berlin et al, 2005a). For example previous studies (Berlin et al, 2005a; 2006a; 2007; Hu et al, 2011) have shown that removal of residual hemicelluloses, improves the ease of hydrolysis of pretreated corn stover, hardwood and softwood substrates. This increase in ease of hydrolysis is likely due to increased accessibility of cellulose to cellulases and to a lesser extent synergistic effects between cellulases and hemicellulases especially xylanases (Hu et al, 2011). Other recent studies have focused on supplementing the cellulase system with non-catalytic proteins such as expansins and swollenins. These proteins do not catalyze the hydrolysis of cellulose but have been shown to disrupt the crystalline structure of cellulose and increase its accessibility to cellulases (Cosgrove, 2000; Saloheimo et al, 2002). However, the feasibility of using these proteins beyond the laboratory remains to be demonstrated.

As mentioned earlier, in addition to these enzyme-related factors, the enzymatic hydrolysis of lignocellulosic materials is influenced by the physicochemical properties of the substrate. These include the physicochemical properties of cellulose such as crystallinity,
degree of polymerization (cellulose DP) and cellulase-accessible surface area as well as the content, physical properties and distribution of the residual lignin and hemicelluloses (Grethlein 1985; Grous et al, 1986; Mooney et al, 1999; Shevchenko et al, 2000; Chandra et al, 2008a).

Previous studies (Wood et al, 1975; Fan et al, 1980b) have suggested that cellulose crystallinity can be used as an indicator of its reactivity towards cellulases and subsequent studies have proposed that the enzymatic hydrolysis of amorphous cellulose is 3 to 30 times faster than that of crystalline cellulose (Ooshima et al, 1983; Zhang and Lynd 2004). However, it should be noted that those studies were performed on pure cellulosic substrates and therefore only provide a simplified interpretation of the role of cellulose crystallinity as an indicator of its susceptibility towards enzymatic hydrolysis. Indeed, subsequent work with more heterogeneous lignocellulosic substrates such as steam pretreated biomass has shown that, when other substrate characteristics are considered, cellulose crystallinity cannot be used alone to predict the rate or extent of cellulose hydrolysis. As a result it was concluded that when studying lignocellulosic substrates, other substrate characteristics such cellulose degree of polymerization, accessibility to cellulases and chemical composition are just as important as cellulose crystallinity (Puri 1984; Ramos et al, 1993 Chandra et al, 2007).

As noted earlier, the degree of polymerization of cellulose (cellulose DP, chain length) is another substrate property that has been implicated as having an effect in governing a substrate’s susceptibility to enzymatic hydrolysis (Mansfield et al, 1999; Chandra et al, 2007). Since CBH I is the major component of the cellulase system (60-65 % w/w; Teeri, 1997), it would be intuitive to expect that reductions in cellulose DP would improve hydrolysis yields by generating more reducing ends for CBH I to act on. It would also be
intuitive to expect that substrate’s with high cellulose DP would benefit from the supplementation of endoglucanases to reduce cellulose DP and generate more cellulose chains for CBH I and CBH II to act on. Nonetheless, our understanding of the impact of cellulose chain length on its susceptibility to enzymatic hydrolysis is still limited as some studies failed to find relationships between enzymatic hydrolysis and cellulose DP (Sinistyn et al, 1991; Zhang and Lynd, 2006a), while other studies have shown that substrates with low cellulose DP hydrolyze more quickly and to a greater extent than did those with high cellulose DP (Puri, 1984; Martinez et al, 1997; Pan et al, 2008). However, it should be noted that generating substrates with reduced cellulose DP often requires more stringent conditions, which undoubtedly affect other substrate properties such as accessibility of cellulose to cellulases (Chandra et al, 2007). As a result, further studies are necessary to elucidate the impact of cellulose DP on cellulosytic hydrolysis.

Although cellulose crystallinity and DP are believed to influence the susceptibility of cellulose to enzymatic hydrolysis, cellulose accessibility to cellulases has been proposed as the key property governing a lignocellulosic substrate’s susceptibility toward cellulosytic hydrolysis (Chandra et al, 2008b; Arantes and Saddler, 2010 and 2011; Rollin et al, 2011). However, while crystallinity and degree of polymerization are properties of cellulose; accessibility of cellulose to cellulases is governed by a variety of factors including particle size (exterior surface area), pore volume (interior surface area) and chemical composition (i.e. the presence and distribution of hemicelluloses and lignin). With regards to the effects of exterior surface area, previous work (Mooney et al, 1999) showed that when substrates containing equal amounts of lignin were subjected to enzymatic hydrolysis, the fines (fibres < 0.2 mm) were hydrolyzed considerably faster than were the longer fibres. The internal
surface area is related to the substrates porosity and is 1-2 orders of magnitude larger than the external surface area (Chang et al, 1981). Several studies have found direct correlations between the substrate’s porosity and its reactivity towards cellulases with the rate-limiting pore volume being 5.1 nm (Grethlein, 1985; Grous et al, 1986). In the same vein, subsequent work by Tanaka et al, (1988) proposed that the enzymatic hydrolysis of lignocellulosic substrates is enhanced when the pore volume is large enough to accommodate the large and small components of the cellulase system thus maintaining its synergistic action.

Several methods have been developed to determine the overall surface area of lignocellulosic materials and can be classified by the type of surface area that they measure. For example, particle size analyzers such as the fibre quality analyzer (FQA) measure the exterior surface area, while solute exclusion and mercury porosimetry measure the interior surface area. Techniques such as Simons’ stain (SS) and Nitrogen (N\textsubscript{2}) adsorption measure a combination of the interior and exterior surface area. One of the most widely used procedures in determining the surface area of lignocellulosic materials is the nitrogen (N\textsubscript{2}) adsorption technique using the Brunauer-Emmett-Teller (BET) method (Brunauer et al, 1938; Haselton, 1954) as it measures both the internal and external surface area (Chandra et al, 2008a).

Briefly, this technique involves exposing previously dried lignocellulosic material to N\textsubscript{2} at a series of increasing pressures and low temperatures. The total surface area that is accessible to the N\textsubscript{2} probe can be determined by measuring the number of N\textsubscript{2} molecules adsorbed on the lignocellulosic material the size of the N\textsubscript{2} molecule and the mass of the lignocellulosic material. The major advantage of the N\textsubscript{2} adsorption technique is that it can provide a quantitative measurement of cellulose accessibility. However, the potential collapse of
substrate pores during the drying process can underestimate the actual surface area (Laivins and Scallan, 1993; Esteghlalian et al, 2001).

To circumvent the potential closure of substrate pores during the drying process, researchers have used the solvent drying technique proposed by Thode et al, (1958). This technique involves exposing the lignocellulosic material to a series of solvents of increasing hydrophobicity followed by an air drying step to ensure the absence of water. The solvent drying process followed by the N\textsubscript{2} adsorption technique was successfully used by Fan and coworkers (1981) to determine the influence of cellulose accessibility on the susceptibility of pure cellulosic substrates to enzymatic hydrolysis. However, it should be pointed out that the solvent drying technique is a labour-intensive process that requires several days per sample and is therefore may not be suitable for the routine determination of cellulose accessibility. Furthermore, N\textsubscript{2} molecules are approximately 3200 smaller than cellulases and can access many pores and cavities on the fiber surface that are inaccessible to cellulases thus overestimating the total accessibility of cellulose to cellulases (Neuman and Walker, 1992).

Similar to the N\textsubscript{2} adsorption technique, Simon’s stain (SS) can be used to measure the interior and exterior surface area of lignocellulosic substrates (Chandra et al, 2008a). This technique was originally developed for the microscopic evaluation of mechanical damage undergone by pulp fibres during refining and is based on the competitive adsorption of two dyes (direct blue and direct orange) in an aqueous environment (Simons, 1950). The direct blue dye has a smaller molecular size and weaker affinity for cellulose compared with the direct orange dye (Yu et al, 1995; Chandra et al, 2008a). As a result of its greater affinity for cellulose, the direct orange dye can penetrate the larger pores within the lignocellulosic substrate and displace the direct blue dye (Yu and Atalla, 1998). Moreover, Yu et al, (1995)
showed that the orange dye is composed of two subfractions with hydrodynamic radii of 5-7 nm and 12-36 nm, which are equal or larger than the rate limiting pore size for cellulose hydrolysis by cellulases (5.1 nm, Grethlein, 1985). Due to their differences in size and affinities for cellulose, the ratio of adsorbed orange to blue dye can be used to estimate the proportion of large and small pores while the maximum adsorption of the orange dye can estimate the overall accessibility of cellulose to cellulases (Chandra et al, 2008a).

Esteghlalian et al, (2001) used the Simon Stain (SS) method to correlate the extent of hydrolysis of Kraft pulp samples dried to different degrees (never dried, oven dried, air dried and freeze dried). The ratios of adsorbed orange to blue dye were directly proportional to the extent of cellulolytic hydrolysis of the substrates. More recently, Chandra and coworkers (2008b) modified this technique to increase throughput, decrease time required for analysis and improve reproducibility. This allowed them to show a direct correlation between the results of this technique and the ease of hydrolysis of lignocellulosic substrates pretreated by a variety of methods (Chandra et al, 2008b; 2009). The major advantages of the SS technique include, unlike N₂ adsorption, the samples are not dried prior to analysis and it also measures the portion of cellulose component that is accessible to molecules that are similar in size or larger than cellulases. Additional advantages include the fact that the SS method is simple to perform, is relatively quick and is highly reproducible. The major disadvantage of the SS technique is that the results are only semiquantitative and therefore only provide an estimate of the substrate’s surface area.

Besides the interior and exterior surface area, the structure, location and content of residual hemicelluloses and (especially) lignin are known to influence the accessibility of cellulose to cellulases and therefore a substrate’s susceptibility to hydrolysis (Chandra et al,
Hemicelluloses are closely associated with cellulose and lignin and can cover the fibre surface, preventing the accessibility of cellulose to cellulases. However, due to their sensitivity to acid-catalyzed hydrolysis (Bura et al, 2002), the removal of hemicelluloses during pretreatment has not been a major issue. Rather, studies have focused in minimizing the degradation of hemicellulosic sugars so that they can be recovered in a fermentable manner (Chandra et al, 2007). Unfortunately, efforts to recover hemicellulosic sugars often require less severe pretreatment conditions, which can lead to increases in the lignin content of the solid fraction and decreased susceptibility to enzymatic hydrolysis (Chandra et al, 2007).

As mentioned earlier, residual lignin in the pretreated biomass is one of the major obstacles preventing rapid and complete enzymatic hydrolysis of pretreated lignocellulosic substrates at low enzyme loadings (Chandra et al 2007). Similar to hemicelluloses, residual lignin is thought to cover the fibre’s surface and inhibit cellulolytic hydrolysis by preventing the accessibility of cellulose to cellulases (Mooney et al, 1998). However unlike hemicelluloses, residual lignin can also inhibit cellulolytic hydrolysis via the (mostly) irreversible adsorption of cellulases on lignin rather than cellulose (Sewalt et al, 1997; Palonen et al, 2004; Berlin et al, 2006b, Nakagame et al, 2010). The adsorption of cellulases on lignin is thought to be mediated by a variety of factors including hydrophobic interactions, hydrogen bonding and ionic interactions (Ooshima et al, 1986; Sewalt et al, 1997; Eriksson et al, 2002; Berlin et al, 2006b; Pan et al, 2008).

Although there have been several studies regarding the effect of lignin on cellulolytic hydrolysis, the exact nature by which lignin inhibits cellulases has not been resolved. For example, Mooney et al, (1998) used four softwood pulps with different lignin contents
(ranging from 4% to 31%) to study the effect of lignin on cellulase adsorption. Interestingly, it was found that lignin content did not have an effect on cellulase adsorption. As a result the authors attributed the inhibitory effect of lignin on cellulolytic hydrolysis to be mostly caused by decreased accessibility of cellulose to cellulases. In contrast, subsequent studies (Berlin et al., 2006b; Nakagame et al., 2010 and 2011a; Rahikainen et al., 2011) have shown that the addition of lignin isolated from steam- and organosolv-pretreated softwoods to Avicel (a commercially available microcrystalline cellulose preparation) had a negative effect on its susceptibility to enzymatic hydrolysis. Those studies attributed the inhibitory effect of lignin on cellulolytic hydrolysis to be caused by non-productive binding of cellulases to lignin. However, it should be noted that the study by Mooney and coworkers used Douglas-fir refiner mechanical pulp in which the chemical structure of lignin is not modified to a great extent. In contrast, the subsequent studies used lignin isolated from pretreated (steam and organosolv) substrates which had undergone significant changes in their chemical structure including condensation reactions that increased its hydrophobicity (Shevchenko et al., 2001; Berlin et al., 2006b; Nakagame et al., 2011a). Thus it is possible that in the case of native or near-native lignins their main mechanism of inhibition is by restricting the accessibility of cellulose to cellulases, while in the case of residual lignins, their mechanism of inhibition is though a combination of restricted accessibility to cellulases and non-productive binding. This hypothesis is supported by the fact that it has been estimated that 60-70% of the added enzyme remains bound to lignin after complete cellulose hydrolysis of steam pretreated substrates (Lu et al., 2002; Berlin et al., 2005b). Therefore, pretreatments that remove lignin (such as the organosolv process) may be advantageous to decrease the costs associated with enzyme losses (especially when using recalcitrant feedstocks such as softwoods).
1.9 Thesis Overview and Objectives

1.9.1 Background

The conversion of lignocellulosic biomass to ethanol (i.e. second generation ethanol) is a promising approach to reduce global dependency on fossil fuel monopolies held by politically volatile countries. Moreover, second generation ethanol is considered carbon neutral and therefore its consumption has the potential to reduce the atmospheric carbon dioxide burden (Lynd et al, 1991). However, considerably more fundamental work is needed to address the technical and logistic challenges associated with the commercialization of second generation ethanol. These include the high cost of cellulolytic enzymes, their comparatively low specific activity and the need for a pretreatment to modify the cell wall properties and increase the accessibility of cellulose to cellulases.

Although several pretreatment processes have been developed to improve the overall efficiency of cellulose hydrolysis, it is unlikely that a single pretreatment will provide the desired outcomes on all potential feedstocks (Langan et al, 2011). For example, while steam-pretreated hardwoods and agricultural residues are readily hydrolyzed at moderate (10-15 FPU g\(^{-1}\) cellulose) cellulase loadings, (steam-pretreated) softwoods are considerably more recalcitrant and require very high (> 40 FPU g-1 cellulose) cellulase loadings to achieve effective (> 70 %) cellulose conversion (Bura et al, 2009; Kumar et al, 2012). The difference in the recalcitrance of softwoods compared to agricultural residues and hardwoods is due in part to their higher lignin content and the potential of the guaiacyl-rich softwood lignin to undergo condensation reactions during pretreatment. These reactions increase the hydrophobicity of the lignin, which results in non-productive interactions between lignin and cellulases (Kumar et al 2010 and 2012).
However, while lignin removal results in great improvements in a substrate’s susceptibility to enzymatic hydrolysis, near-complete delignification (though technically possible with current pulping and bleaching technologies) is not economically feasible for biorefinery applications. This is largely due to the difference in price between bleached pulp and glucose/ethanol. For example, the current (March 2012) price of northern bleached softwood kraft pulp (NBSK) according to the PIX Pulp Benchmark Index is US $ 870 ton\(^{-1}\). In contrast, the price of glucose is US $ 423 ton\(^{-1}\) and the benchmark price for the cost-competitive production of second generation ethanol has been calculated at US $ 1.33 gallon\(^{-1}\) (Aden, 2008). Therefore, it is probable that regardless of the type of biomass or pretreatment, the resulting lignocellulosic substrate will always contain some lignin. Nonetheless, the effective processing of softwoods requires pretreatments that can either modify lignin or fractionate biomass such as the organosolv process.

As previously mentioned, the organosolv process is one of the few pretreatments that can effectively process softwoods to generate cellulose-rich (\(>\) 80%) substrates that are amenable to enzymatic hydrolysis (Pan et al, 2005, 2007 and 2008). Further advantages of the organosolv process include the generation of a high-purity sulphur-free lignin fraction that has potential for co-product applications (and can improve the overall process economics) and the proven capability to be scaled up (Stockburger, 1993; Hergert, 1998, Pan et al, 2005 and 2007). Nonetheless, there are several challenges that need to be addressed before the organosolv process can be used as a commercially viable pretreatment. These include the time and energy required for pretreatment, recovery of hemicellululosic sugars in a fermentable form, the formation of sugar degradation products that may inhibit fermentation, as well as solvent recovery, which is not only necessary to reduce process economics but also
to prevent the inhibition of hydrolysis, and fermentation (Sun and Cheng, 2002). Moreover, although organosolv-pretreated substrates are readily hydrolyzed at moderate to high enzyme loadings and low solids content (10-40 FPU g\(^{-1}\) cellulose and 2% w/v), obtaining significant cellulose conversion at low enzyme loadings (2.5-5 FPU g\(^{-1}\) cellulose) and high solids content (10% w/v and higher) remains a significant challenge.

However, comparatively little work has looked at the mechanisms governing the susceptibility of organosolv-pretreated substrates to cellulolytic hydrolysis. This lack of a mechanistic understanding of the enzymatic hydrolysis of organosolv-pretreated substrates has undoubtedly arisen from the fact that the majority of the mechanistic studies related to the organosolv process thus far have been performed from a papermaking perspective (Diaz et al, 2004). Furthermore, the majority of the studies that have used the organosolv process as a pretreatment for bioconversion have either focused on the optimization of process parameters and hydrolysis yields (Pan et al, 2005, 2006a, 2007, Huijgen et al, 2011) or on lignin characterization (El Hage et al, 2009 and 2010; Hallac et al, 2010b; Sannigrahi et al, 2010b). Although some studies (Pan et al, 2008; Hallac et al, 2010a and 2010c; Cateto et al, 2011; Koo et al, 2011b) have mentioned the possible contribution of substrate-related factors such as cellulose crystallinity, degree of polymerization and cellulose-accessible surface area on the ease of enzymatic hydrolysis of organosolv-pretreated substrates, their exact role is still unknown. In addition, those studies were performed at high (20-40 FPU g\(^{-1}\) cellulose) enzyme loadings, which may conceal the effects of the above mentioned substrate properties by saturating the substrate with enzyme.

The primary goal of this thesis is to further understand the influence of substrate-related factors on the susceptibility of organosolv-pretreated softwoods to enzymatic
hydrolysis. It is hoped that besides contributing to the elucidation of the more fundamental
enzyme-substrate interactions, the work described in this thesis will contribute to the
improvement of the organosolv process to produce substrates that exhibit enhanced cellulose
hydrolysis yields at lower enzyme loadings.

1.9.2 Research Approach

When this research was initiated, we hypothesized that the physical and chemical
inhibition of enzymatic hydrolysis by the residual lignin were the key substrate-related
factors that influenced the susceptibility of organosolv-pretreated softwood to enzymatic
hydrolysis. We also hypothesized that as an alternative to complete delignification, it may be
possible to identify (and determine the exact contribution of) other substrate-related factors
that influence an organosolv-pretreated substrate’s susceptibility to enzymatic hydrolysis.
Moreover, it was anticipated that once identified, these factors could be modified to produce
a substrate exhibiting superior cellulose conversion yields at low enzyme loadings.

The overall goal of this research is to increase our understanding of the interactions
between cellulolytic enzymes and lignocellulosic substrates generated by the organosolv
process. To achieve this goal, the initial work focused on confirming the nature (i.e. physical,
chemical or both) of the inhibitory role of the residual lignin on the enzymatic hydrolysis of
organosolv-pretreated mountain pine beetle-killed lodgepole pine (Pinus contorta, a
softwood) and hybrid poplar (Populus tricocarpa x P. deltoides, P. deltoides x P. nigra, and
P. nigra x P. maximowzii a hardwood). Mountain pine beetle-killed lodgepole pine was
chosen as a representative softwood because of its abundance in British Columbia (BC) and
much of the Pacific Northwest. On the other hand, hybrid poplar was chosen as a
representative hardwood because it is likely to be used as an energy crop due to its low recalcitrance and rapid growth rate (Sannigrahi et al, 2010c).

Organosolv pretreatment was performed under conditions that were previously optimized (in our laboratory) for the individual feedstocks as a compromise between carbohydrate recovery and ease of enzymatic hydrolysis (Pan et al, 2006a and 2007). However, the previous work (unexpectedly) found that hybrid poplar was more recalcitrant to organosolv-pretreatment and required more severe conditions than did mountain pine beetle-killed lodgepole pine. As a result, a secondary goal of the initial work was to confirm these previous findings.

Upon determining the nature of the inhibitory role of lignin, we next focused on elucidating the substrate properties that play the most important role in influencing the susceptibility of organosolv-pretreated softwoods to enzymatic hydrolysis. To achieve this goal, a set of substrates with varying physicochemical properties were generated by altering the chemical additives (neutral acidic and alkaline) and solvent (ethanol and butanol) used during pretreatment. The substrate properties that were examined in this study included cellulose DP, fibre length, surface chemistry, chemical composition and the accessibility of cellulose to cellulases. It was hypothesized that in addition to differences in physicochemical properties these substrates would exhibit different degrees of susceptibility toward cellulolytic hydrolysis. Consequently it was thought likely that correlations between the different substrate properties (especially cellulose accessibility to cellulases) and susceptibility to enzymatic hydrolysis could be determined.

After identifying the substrate properties that appeared to influence enzymatic hydrolysis we next focused our efforts on attempting to individually alter these properties to
determine their role in the enzymatic hydrolysis of organosolv-pretreated softwoods. To confirm the role of fibre length on enzymatic hydrolysis, organosolv-pretreated lodgepole pine was size fractionated and the susceptibility of the size-fractionated substrates to enzymatic hydrolysis was assessed. As surface area is inversely proportional to particle size it was anticipated that the smaller sized substrates would exhibit greater susceptibility towards cellulolytic hydrolysis.

The role of cellulose DP on enzymatic hydrolysis was determined by preparing a set of substrates with varying cellulose DP’s followed by complete delignification and cellulose dissolution and regeneration (to remove the influence of the residual lignin and cellulose crystallinity respectively). The regenerated substrates were then subjected to a secondary hydrolysis. Similar to the effects of fibre length on enzymatic hydrolysis it was anticipated that the substrates exhibiting lower cellulose DP would be more amenable to enzymatic hydrolysis.

Finally, the role of cellulose accessibility to cellulases on cellulolytic hydrolysis was investigated by subjecting the organosolv pretreated substrate to chemical and mechanical treatments in the form of neutral sulphonation and refining. It was anticipated that both treatments would increase cellulose conversion albeit by two different mechanisms. It was thought that refining would increase fibre swelling, as well as cellulose accessibility due to fibrillation. On the other hand, it was believed that neutral sulphonation would decrease the non-specifically interactions between lignin and cellulases (due to the increased hydrophilicity) of the sulphonated lignin as well as increased accessibility of cellulose to cellulases (due to increased fibre swelling).
In summary, it is hoped that the information generated from this thesis will improve our understanding of the mechanisms that govern the effectiveness of the cellulase system during the enzymatic hydrolysis of lignocellulosic substrates generated by the organosolv process and contribute to the improvement of the overall biomass-to-ethanol-process.
Chapter 2: Materials and Methods

2.1 Feedstock Preparation

Hybrid poplar (POP) wood chips were an equal blend of three hybrid poplar species (*Populus tricocarpa* x *P. deltoides*, *P. deltoides* x *P. nigra*, *P. nigra* x *P. maximowzii*) and were provided by the British Columbia Forest service. Two sets of gray phase mountain pine beetle killed lodgepole pine (*Pinus contorta*, LPP) wood chips were used in this study. The first set (LPP-A), was used in sections 3.1, 3.3 and 3.4 was harvested in Prince George, British Columbia, Canada and generously provided by FPinnovations-PAPRICAN division. The second set (LPP-B) was used in section 3.2 and was generously provided by Dr. Colette Breuil in the form of fourteen one meter long bolts of wood from seven trees located in central British Columbia. The bolts were debarked, air-dried, split by axe and chipped using a custom designed chipper. The wood chips were screened on a plate screen and the fraction larger than 2.5 cm x 2.5 cm and smaller than 5.0 x 5.0 cm (and approximately 0.5 cm thick) was collected as the feedstock for organosolv pretreatment. Samples of the POP and LPP feedstocks were ground using a Wiley mill and the fraction passing through a 40-mesh (0.42 mm) opening was collected for compositional analysis as will be described in section 2.5 (Table A1).

2.2 Organosolv Pretreatment

The laboratory scale organosolv pretreatment was performed on a custom-built four-vessel (2L each) rotating digester (Aurora products Ltd. Savona, BC, Canada) as described by Pan et al, (2005). Briefly, 200 g (oven-dried weight equivalent, ODW) batches of never-dried chips were incubated in a mixture containing water, alcohol solvent (either ethanol or butanol) and a chemical additive. The vessels containing the biomass and pretreatment liquor
were heated at a rate of 3 °C min^{-1} and held at the target temperature for a specified time. For the initial work (Section 3.1), pretreatment was performed according to the conditions described by Pan et al (2006a, 2007) for POP (high severity) and LPP (low severity) substrates respectively (Table 3). For the second study (section 3.2), the pretreatment conditions and chemical additives are shown on Table 4. For the third and fourth studies (sections 3.3 and 3.4 respectively), pretreatment was carried out at the “low severity” conditions used in the first study.

Table 3. Summary of low and high severity pretreatment conditions used to generate the organosolv-pretreated LPP and POP substrates studied in section 3.1.

<table>
<thead>
<tr>
<th>Condition</th>
<th>T (°C)</th>
<th>t (min)</th>
<th>[EtOH]</th>
<th>[H₂SO₄]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low severity (LS)</td>
<td>170</td>
<td>60</td>
<td>65</td>
<td>1.10</td>
</tr>
<tr>
<td>High severity (HS)</td>
<td>180</td>
<td>60</td>
<td>50</td>
<td>1.25</td>
</tr>
</tbody>
</table>

T, temperature (°C); t, time at temperature; [H₂SO₄], sulfuric acid charge (% w/w on oven-dried wood); [EtOH], ethanol concentration (% v/v).

After pretreatment, the digester vessels were cooled to room temperature in a water bath, opened and the (pretreated) substrates were separated from the spent liquor using a nylon cloth. The substrates were washed (3 x 300 mL) with a warm (60 °C) solution of solvent and water at the same concentration as the pretreatment liquor. The washes were combined with the spent liquor and sampled immediately for the determination of furfural and 5-hydroxymethylfurfural (HMF). The substrates were then washed with water (3 x 300 mL) at 60 °C and the washes were discarded. The washed substrates were homogenized in a standard British disintegrator for 5 minutes and passed through a laboratory flat screen with 0.203 mm slits (Voith, Inc., Appleton, WI) to remove rejects (unfiberized wood). The screened substrates were transferred to a Buchner funnel, filtered, and stored at 4 °C for the determination of screened yield, physicochemical characterization and enzymatic hydrolysis.
Table 4. Summary of pretreatment conditions and chemical additives used to generate the organosolv-pretreated LPP substrates studied in section 3.2.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Pretreatment conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAEM60 EtOH</td>
<td>200 °C, 60 min, 78 % EtOH, 0.025 M MgCl₂</td>
</tr>
<tr>
<td>NAEM30 EtOH</td>
<td>205 °C, 60 min, 78 % EtOH, 0.025 M MgCl₂</td>
</tr>
<tr>
<td>H₂SO₄ EtOH</td>
<td>170 °C, 60 min, 65 % EtOH, 1.1 % H₂SO₄</td>
</tr>
<tr>
<td>SO₂ EtOH</td>
<td>170 °C, 60 min, 65 % EtOH, 1.1 % SO₂</td>
</tr>
<tr>
<td>NaOH EtOH</td>
<td>170 °C, 60 min, 65 % EtOH, 20 % NaOH</td>
</tr>
<tr>
<td>NAEM60 BuOH</td>
<td>200 °C, 60 min, 78 % BuOH, 0.025 M MgCl₂</td>
</tr>
<tr>
<td>NAEM30 BuOH</td>
<td>205 °C, 60 min, 78 % BuOH, 0.025 M MgCl₂</td>
</tr>
<tr>
<td>H₂SO₄ BuOH</td>
<td>170 °C, 60 min, 65 % BuOH, 1.1 % H₂SO₄</td>
</tr>
<tr>
<td>SO₂ BuOH</td>
<td>170 °C, 60 min, 65 % BuOH, 1.1 % SO₂</td>
</tr>
<tr>
<td>NaOH BuOH</td>
<td>170 °C, 60 min, 65 % BuOH, 20 % NaOH</td>
</tr>
</tbody>
</table>

NAEM = neutral alkaline earth metal, EtOH = ethanol, BuOH = butanol, a Solvent concentration as % (v/v), b Acid and alkali charges as % (w/w) on oven-dried wood.

2.3 Preparation of Size-Fractionated Organosolv-Pretreated Lodgepole Pine

Substrates

Fibre size fractionation was carried out in a Bauer-McNett Fibre classifier fitted with 14-, 28-, 48-, 100-, and 200-mesh screens (corresponding to sieve openings of 1.19, 0.60, 0.30, 0.15, and 0.074 mm, respectively). The fraction retained by a given screen was termed RX where X refers to the mesh size (i.e. the fraction retained by the 14-mesh screen is R14, by the 28-mesh screen R28, etc.). The fraction that passed through all the screens was termed the P200 fraction and was obtained by collecting the water/P200 mixture that is usually sent to waste into a large container, allowed to settle for 7 days, decanted and filtered through a 400-mesh screen. The filtrate was recirculated three times to minimize the loss of material. The fractionated substrates were stored at 4 °C for further characterization and enzymatic hydrolysis.

2.4 Chemical and Mechanical Treatments on Organosolv-Pretreated Lodgepole Pine

Neutral sulphonation of the residual lignin was performed in a Parr reactor by a modification of the procedure described by Mooney et al (1998). Briefly, 10 g ODW of never
dried organosolv-pretreated substrate was suspended in an aqueous solution containing sodium sulfite (pH 7) to a final solids concentration of 10 %. The final concentration of sodium sulfite was 0.25 M. The pulp slurries were heated to 140 °C and incubated for 1 h after which the sulphonated substrates were cooled down to room temperature in a water bath, filtered under suction, and washed with tap water (5 x 1 L) followed by deionized water (2 x 1 L). The initial water wash was recirculated three times to avoid the loss of fines. A control experiment was performed at the same conditions as the sulphonation experiments but without sodium sulfite.

Lignin extraction of the organosolv-pretreated substrate was achieved according to the following procedure: approximately 10 g ODW of never dried substrate was resuspended in an aqueous ethanol solution (final ethanol concentration 65 %, v/v and liquid to solid ratio 7:1). The resulting pulp slurry was transferred to a round bottom flask and extracted under reflux for 3 h changing the ethanol solution every hour. The extracted substrate was filtered under suction while warm, washed (3 x 50 mL) with warm aqueous ethanol (60 °C, 65 % v/v) to remove any lignin that may have reprecipitated on the fibres’ surface followed by deionized water (3 x 500 mL). The washes were combined with the spent liquor and discarded and the partially delignified substrate was resuspended overnight in 250 mL deionized water at 4°C and filtered under suction.

Partial oxidative delignification of the organosolv-pretreated substrate was achieved with sodium chlorite in 1 % (v/v) acetic acid as will be described in section 2.8 with the exception that rather than overnight, the substrates were incubated for 5 min. Incorporation of carboxyl and sulphonate groups into the residual lignin as a result of the chemical treatments was determined by conductometric titration as described by Katz et al, (1984).
Mechanical treatments in the form of PFI-mill refining were carried out as follows: approximately 20 g ODW of never dried substrate was resuspended in water to a final solids concentration of 10 % (w/v). The resuspended substrate was then transferred to a PFI-mill with a plate gap of 0.2 mm and refined for 2,500, 10,000, and 20,000 revolutions according to the Pulp and Paper Technical Association of Canada's (PAPTAC) standard method C7. The refined substrates were transferred to a Buchner funnel and filtered. The filtrate was recirculated three times to prevent the loss of material. All post-treated substrates were stored at 4°C for further characterization and enzymatic hydrolysis. Post-treatment conditions are summarized on Table 5.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Post-treatment conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>OSLPP</td>
<td>NA</td>
</tr>
<tr>
<td>Sulph-control</td>
<td>H₂O, 140 °C, 60 min</td>
</tr>
<tr>
<td>Sulph</td>
<td>0.25 M Na₂SO₃, pH 7, 140 °C, 60 min</td>
</tr>
<tr>
<td>OS-LPP EX</td>
<td>65 % (v/v) EtOH, 80 °C, 180 min</td>
</tr>
<tr>
<td>OS-LPP DL</td>
<td>50 %² (w/w) NaClO₂, 1 % (v/v) acetic acid, 20 °C, 5 min</td>
</tr>
<tr>
<td>Ref-1</td>
<td>2500 revolutions, plate gap 0.2mm</td>
</tr>
<tr>
<td>Ref-2</td>
<td>10000 revolutions, plate gap 0.2mm</td>
</tr>
<tr>
<td>Ref-3</td>
<td>20000 revolutions, plate gap 0.2mm</td>
</tr>
</tbody>
</table>

² NaClO₂ concentration on oven-dried pulp.

### 2.5 Compositional Analysis of Initial Feedstocks and Pretreated Substrates

The (wet-basis) moisture content of the initial feedstocks and the pretreated substrates were determined from the mass loss after drying to constant weight at 105 °C in a convection oven. The Klason lignin content of the initial feedstocks and the pretreated substrates was determined according to TAPPI standard method T-222 om-98. The hydrolysate was retained for determination of monosaccharide composition and acid-soluble lignin. Acid soluble lignin was determined on a Cary 50 UV-Vis spectrometer at 205 nm as described by Dence (1992). Monosaccharides in the filtrate were determined with a DX-3000 HPLC system.
(Dionex, Sunnyvale, CA), equipped with an anion exchange column (Dionex CarboPac PA1) and an ED40 electrochemical detector, with fucose (0.25 mg mL$^{-1}$) as the internal standard. The column was eluted with deionized water at a flow rate of 1 mL min$^{-1}$. Aliquots (20 μl) were injected after being passed through a 0.45 μm nylon syringe filter (Chromatographic Specialties Inc., Brockville, ON, Canada). Baseline stability and detector sensitivity were optimized by post column addition of 0.2 M NaOH at a flow rate of 0.5 mL min$^{-1}$ using a Dionex AXP pump. The column was reconditioned using 1 M NaOH after each analysis. Monosaccharides (arabinose, galactose, glucose, xylose and mannose) in the substrates were quantified with reference to standards.

2.6 Chemical Analysis of Spent Pretreatment Liquors

Monosaccharides in the liquid samples were determined by dilution of the pretreatment liquor in deionized water followed by HPLC analysis as described in section 2.5. For substrates produced under acidic and neutral conditions, total oligomeric sugars in the liquid fraction were determined by the addition of 0.7 mL of 72 % (w/w) H$_2$SO$_4$ to 15 mL of the liquid sample and the volume made up to 20 mL with deionized water. In the case of substrates produced under alkaline conditions, oligomeric sugars were determined by the addition of enough 72 % (w/w) H$_2$SO$_4$ to decrease the pH of 10 mL of the liquid sample to 2 and the exact volume was recorded. Samples and standards were autoclaved at 121 °C for 1 h to ensure the complete hydrolysis of oligosaccharides and analyzed by HPLC.

Sugar degradation products (furfural, HMF and acetic acid) were determined on a Dionex 500 HPLC system equipped with a GP40 gradient pump, an AS3500 autosampler and an AD20 absorbance detector. A BioRad Aminex HPX-87H column (BioRad, Hercules, CA) was used at 50 °C and eluted with 5 mM H$_2$SO$_4$ at flow rate of 0.6 mL min$^{-1}$. Diluted
aliquots (20 μl) were injected after being passed through a 0.45-μm PTFE filter (Chromatographic Specialties Inc.). Furfural, HMF and acetic acid were determined by absorbance at 280 nm.

Ethanol soluble lignin (ethanol organosolv lignin, EOL) in the spent pretreatment liquor was determined by diluting a 10 mL aliquot of the pretreatment liquor in 40 mL of deionized water at pH 2 (in pre-weighed 50 mL polypropylene centrifuge tubes). The EOL was allowed to precipitate overnight and centrifuged at 5000 rpm for 30 min, washed with deionized water (3 x 30 mL) and lyophilized. Acid soluble lignin in the spent pretreatment liquor was determined as described in section 2.5 after precipitation of the EOL.

2.7 Enzymatic Hydrolysis of Pretreated Substrates

Commercial cellulases (Spezyme CP) and β-glucosidase (Novozym 188) were provided by Genencor International Inc. (a Danisco division, Rochester, NY) and Novozymes (Franklington, NC), respectively. Cellulase and β-glucosidase activities were determined according to established protocols (Ghose, 1987; Wood and Bhat, 1988) and are expressed in terms of filter paper units (FPU, 1 FPU = 2.08 mg) and international units (IU) respectively.

Batch hydrolysis of the untreated feedstocks and the pretreated substrates was performed at 2 % and 10 % (w/v) solids content in 50 mM acetate buffer, pH 4.8, with 0.004 % tetracycline and 0.003 % cycloheximide, to prevent microbial contamination. Cellulase was used at loadings ranging from 2.5, to 20 FPU g⁻¹ of cellulose in the substrate with supplementation of β-glucosidase at a ratio of 1:2 FPU: IU to avoid inhibition by cellobiose accumulation. The reaction mixture (50 mL) was incubated at 150 rpm, 50 °C, in a rotary
shaker and sampled periodically for glucose determination by HPLC as described in section 2.5. All hydrolysis experiments were conducted in triplicate.

2.8 Adsorption of Bovine Serum Albumin on Organosolv Pretreated Substrates

The pretreated lodgepole pine and poplar substrates were incubated at 2% (w/v) solids content in 50 mM sodium acetate buffer (pH 4.8) at 50 °C with bovine serum albumin (BSA) at varying concentrations (0.10-1.20 mg mL⁻¹). After 3 h, the entire flask contents were transferred to 50 mL polypropylene centrifuge tubes and centrifuged for 10 minutes at 4000 rpm. The free BSA content in the supernatant was determined using ninhydrin (Sigma, St. Louis, MO, USA) as described by Starcher (2001) with BSA as the protein standard. The adsorbed BSA was calculated from the difference between the initial BSA concentration and the free BSA content in the supernatants. The Langmuir theory of adsorption was applied and the concentration of adsorbed BSA (\(A_{\text{BSA}}\)) was given by the following equation:

\[
A_{\text{BSA}} = \frac{\sigma_{\text{BSA}} [S][\text{BSA}]}{1+K[\text{BSA}]}
\]

where \(\sigma_{\text{BSA}}\) is the maximum BSA adsorption capacity for the substrate (mg g⁻¹) \(K\) is the adsorption equilibrium constant, [S] and [BSA] are the concentration of substrate and BSA at equilibrium (mg mL⁻¹) respectively. Values for \(\sigma_{\text{BSA}}\) were calculated from the linear transformation of the adsorption isotherm and are the average of triplicate experiments (Appendix A, Figure A1).

To determine whether BSA adsorbed solely to lignin or to lignin and carbohydrates, organosolv-pretreated lodgepole pine (low severity) was delignified with sodium chlorite in 1 % (v/v) according to the guidelines found in the Pulp and Paper Technical Association of Canada’s (PAPTAC) Useful method G10.U. Briefly, 3 g (ODW) of never-dried substrate, were resuspended in 30 mL of a solution composed of 5 % (w/v) NaClO₂ dissolved in 1%
(v/v) acetic acid and incubated overnight at room temperature and in the dark. The
delignified pulp slurry was thoroughly washed with deionized water until the filtrate was
colourless and the delignification and washing procedures were repeated until all traces of
colour were removed from the pulp. The delignified substrate was incubated with BSA as
described above with the exception that BSA was used at a single concentration (0.5 mg mL\(^{-1}\)). The free BSA was also determined as described above with the exception that the
delignified substrate was incubated for 3, 6, 12, 24, 48 and 72 h prior to determining protein
centration.

2.9 The Effect of Lignin on the Enzymatic Hydrolysis of Organosolv-Pretreated
Substrates

Enzymatic hydrolysis of the pretreated substrates before and after NaClO\(_2\)-mediated
delignification or lignin blocking with BSA were performed as described in section 2.7 with
the exception that cellulase was added at a single dose of 5 FPU g\(^{-1}\). For the effects of lignin
blocking, amounts of BSA corresponding to maximum adsorption capacity of the substrate
(\(\sigma_{\text{BSA}}\)) were added (and incubated at 50 °C with shaking at 150 rpm) 24 h prior to the
addition of cellulases.

2.10 Adsorption of Cellulases During and After Complete Cellulose Conversion

Batch hydrolysis experiments were conducted at varying cellulase loadings as
described above with the exception that the reaction mixtures were not sampled for glucose
determination and each flask corresponded to a single time point. At the appropriate
sampling time, the entire flask contents were transferred to 50 mL polypropylene centrifuge
tubes and centrifuged for 10 min at 4,000 rpm. A 40 \(\mu\)L sample of the supernatant was then
analyzed for protein content as described in section 2.8. Control flasks without enzyme were
analyzed as described above to account for the degradation of tetracycline and cycloheximide to detectable amines and for the possibility of endogenous protein in the substrate. All cellulase adsorption experiments were performed in triplicate.

2.11 Determination of Contact Angle

The static contact angle technique was used to determine the surface hydrophobicity of LPP and POP subjected to organosolv-pretreatment at low and high severity. Briefly, pulp handsheets (60 g m\(^{-2}\)) from the pretreated substrates were prepared using distilled water according to TAPPI test method T-205 sp-06. A 15 mm by 10 cm handsheet strip was attached (smooth side up) onto a KSV contact angle measurement system (KSV Instruments Ltd., Helsinki, Finland) consisting of a CCD video camera, a frame grabber, an adjustable sample stage and a LED light source. A water drop (14 μl) was allowed to fall onto the solid from a syringe tip to produce a sessile drop and an image was recorded every 0.067 s. For contact angle measurements, the frames were analyzed for the point at which the drop stabilized and remained symmetrical on the sheet surface. The drop usually stabilized 2-3 frames (0.134-0.201 s) after contact with the sheet. The initial equilibrium contact angle was measured in the frame with image analysis software and is the average of 10 measurements.

2.12 Determination of Lignin Composition by Thioacidolysis

The chemical composition of lignin in the (extractive-free) untreated and pretreated hybrid poplar substrates was determined by thioacidolysis as described by Robinson et al, 2009. Specifically, the samples were dried to constant weight in a convection oven at 105 °C and passed through a Wiley mill equipped with a 40-mesh and stored in a desicator until used. The samples (10 mg wood flour or 40 mg pretreated substrate) were then weighed into a 5 mL glass vials with teflon-lined screw-caps (Wheaton). One mL of freshly made reaction
mixture (10 % (v/v) boron trifluoride etherate and 2.5 % (v/v) ethanethiol, in freshly distilled dioxane 96 % (v/v)) was then added to each vial containing the samples and blanketed with nitrogen gas prior to sealing. The reaction mixture was heated at 100 °C with hourly manual agitation. After 4 h, the reaction was quenched by placing the vials at -20 °C for 10 min followed by the addition of 0.2 mL of the internal standard (5 mg mL⁻¹ tetracosane in dichloromethane), and 0.3 mL of a 0.4 M aqueous sodium bicarbonate solution. The reaction products were then extracted from the aqueous solution by the addition of 2 mL deionized water and 1 mL dichloromethane followed by vortexing for 30 s and phase separation. A 1 mL aliquot of the lower organic phase was removed and cleared of residual water by passing the sample through a Pasteur pipette packed with an inch of granular anhydrous sodium sulphate into a 2 mL (polypropylene) microcentrifuge tube. The samples were then collectively evaporated to dryness in a Vacufuge (Eppendorf) (approximately 1.5 hr at 45°C), and resuspended in overnight 1 mL dichloromethane at -20 °C.

The resuspended samples were then derivatized by combining a 20 μl aliquot with 20 μl pyridine and 100 μl N,O-bis(trimethylsilyl) acetamide (Sigma). After incubation for at least 2 h at 25°C a 1 μl aliquot of the reaction products was analyzed (splitless injection) on a Hewlett Packard 5890 series II gas chromatograph (GC), fitted with an autosampler, a flame ionization detector (FID), a 30 m RTX5ms 0.25 mm ID capillary column and helium as the carrier gas at a flow rate of 1 mL min⁻¹. The inlet and detector temperatures were set to 250 °C and the oven temperature profile was set as follows: the initial temperature 130 °C was held for 3 min and increased at 3 °C min⁻¹ for 40 min to a final temperature of 250 °C where it was held for 5 minutes before being cooled to 130°C. Identification and quantification of
the guaiacyl- and syringyl-derived monolignol moieties was performed as described by Rolando et al., (1992).

2.13 Generation of Substrates Enriched in Small Fibres

To obtain a set of substrate enriched in small fibres or “fines”, 10 g batches of organosolv-pretreated lodgepole pine were subjected to a primary enzymatic hydrolysis at cellulase loadings of 5, 10, 20, and 25 FPU g$^{-1}$ cellulose and 2 % (w/v) solids content as described in section 2.7. After the primary hydrolysis (4 h), the substrates were cooled down to 4 °C, filtered under suction, and the filtrate recirculated to minimize the loss of small fibres. Cellulases adsorbed to the prehydrolyzed substrates were removed via the addition of proteases as described by Nakagame et al, (2010) followed by re-suspending and washing the substrates under suction three times with 100 mL distilled H$_2$O. After the primary hydrolysis, the prehydrolyzed substrates were delignified with sodium chlorite as described in section 2.8 and their chemical composition was determined as described in section 2.5. Further characterization of the prehydrolyzed substrates included the determination of fibre length distribution, viscosity-average cellulose degree of polymerization and cellulose crystallinity as will be described in sections 2.16, 2.19 and 2.20 respectively. Finally, the prehydrolyzed substrates were subjected to a secondary hydrolysis at a cellulase loading of 2 FPU g$^{-1}$ cellulose. A control substrate was generated as described above but without the addition of cellulases during the primary hydrolysis.

2.14 Preparation and Enzymatic Hydrolysis of Phosphoric Acid Swollen Cellulose

Selected substrates (organosolv-pretreated lodgepole pretreated under acidic and alkaline conditions as well as the small fibre-enriched prehydrolyzed with 25 FPU g$^{-1}$) were delignified as described in section 2.8 followed by the removal of residual hemicelluloses as
described in the TAPPI standard method T203 cm-99. Briefly, 1.5 g (ODW) of never-dried delignified substrate was resuspended in enough deionized water and 50 % (w/v) NaOH to give a final volume of 75 mL and a final NaOH concentration of 17.5 % (w/v). The pulp slurry was stirred for 30 min at which time 100 mL of deionized water was added to dilute the NaOH concentration to 9.5 % (w/v) and the pulp slurry was stirred for a further 30 min. The pulp slurry was then filtered in vacuo and rinsed (3 x 30 mL) with 9.5 % (w/v) NaOH followed by deionized water until the pH of the filtrate was neutral.

Phosphoric acid swollen cellulose (PASC) substrates were then prepared as described by Zhang et al., (2006b). Approximately 0.2 g (ODW of never dried substrate) was added to a 50 mL polypropylene centrifuge tube followed by the slow addition of ice-cold 86.2 % (w/v) \( \text{H}_3\text{PO}_4 \) (with vigorous stirring) so that the final phosphoric acid concentration was 83.2 % (w/v). The cellulose-\( \text{H}_3\text{PO}_4 \) mixture was incubated in ice-cold water with stirring every 15 min to ensure complete cellulose dissolution. After 1 h, the dissolved cellulose was precipitated by the (slow) addition of ice-cold deionized water (with stirring) until a final volume of 50 mL was achieved. The precipitated cellulose was recovered by centrifugation (5000 g, 20 min). The pellet was resuspended in ice-cold deionized water and centrifuged four times to remove excess phosphoric acid. Approximately 0.5 ml of 2 M \( \text{Na}_2\text{CO}_3 \) was then added to the pellet to neutralize the residual phosphoric acid. The neutralized pellet was resuspended in 45 mL ice-cold deionized water and centrifuged twice more to remove excess \( \text{Na}_2\text{CO}_3 \). The phosphoric acid swollen cellulose was resuspended on more time in 45 mL of ice-cold deionized water, filtered under suction through a glass–fibre filter to remove excess water and subjected to enzymatic hydrolysis at 2 FPU g\(^{-1}\) cellulose as described in section 2.7.
2.15 Surface Analysis by X-Ray Photoelectron Spectroscopy (XPS)

Pulp handsheets (60 g m\(^{-2}\)) from the pretreated substrates were prepared using distilled water according to TAPPI test method T-205 sp-06. Surface chemical characterization was carried out with a Leybold Max 200 X-ray photoelectron spectrometer (Cologne, Germany) with a monochromated Al-K\(\alpha\) X-ray source. The detector position was at an angle of 90\(^\circ\) relative to the sample surface. The samples for analysis (1 cm\(^2\)) were cut from the handsheets, attached to the sample holder and evacuated in a prechamber for 12 h. The high resolution spectra were charge corrected using the C-C component of the C\(_{1s}\) signal at 285 eV as an internal standard. A Gaussian curve fitting program (XPSPEAK 4.1) with the Shirley background was used to deconvolute the C\(_{1s}\) signal. The following binding energies relative to the C-C position were used: 1.7 ± 0.2 eV for C-O, 3.1± 0.2 for C=O, or O-C-O, and 4.2 ± 0.3 for O=C-O (Laine et al, 1994). The theoretical surface lignin coverage (TSLC, \(\Phi_{\text{lig}}\)) was calculated from the O/C ratios as described by Laine et al, (1994) according to the following equation:

\[
\Phi_{\text{lig}} = (O/C_{\text{sample}}) - O/C_{\text{(cellulose)}})/(O/C_{\text{(lignin)}} - O/C_{\text{(cellulose)}}) \tag{3}
\]

where \(O/C_{\text{sample}}\) is the O/C ratio of the analyzed sample, and \(O/C_{\text{(cellulose)}}\) and \(O/C_{\text{(lignin)}}\) are the theoretical O/C ratios of pure cellulose and lignin (0.83 and 0.33 respectively, Laine et al, 1994).

2.16 Fibre Analysis

Fiber length, width and size distribution of the substrates was measured using a Fiber Quality Analyzer (LDA02, OpTest Equipment, Inc., Hawkesbury, ON, Canada). Briefly, a dilute suspension of fibers with a fiber frequency of 25–40 events per second was transported through a sheath flow cell where the fibers are oriented and positioned. The images of the
fibers were detected by a built-in CCD camera, and the length of the fibers was measured by circular polarized light. The experiments were conducted according to the procedure described by Robertson et al, (1999). All samples were run in triplicate. Individual fibres from the untreated poplar and LPP feedstocks were obtained by cutting the wood chips longitudinally into a few slices of less than 0.5 mm thickness. The wood slices were macerated in a 1:1 mixture of glacial acetic acid:hydrogen peroxide (30 % from stock bottle) at 70 °C for 48 h to remove lignin and hemicellulose. The macerated wood chips were washed extensively with distilled water and the fibres disintegrated in a small Hamilton Beech mixer. The fibre size and distribution was determined as described above.

2.17 Water Retention Value

Fibre swelling was estimated by the water retention value (WRV), which was performed according to the Technical Association of the Pulp and Paper Industry's, (TAPPI) useful method UM 256. Briefly, 0.5-1.0 g (ODW) of never-dried substrate was resuspended in 50 mL deionized water and shaken vigorously to break the pulp apart. The pulp slurry was allowed to soak overnight at room temperature and filtered through a 200-mesh screen in a centrifuge cup. The filtrates were recirculated three times to prevent the loss of fines and the resulting pulp pads were centrifuged (900 g, 25 °C) for 30 min. The wet (centrifuged) pulp pads were weighed, dried overnight at 105°C and reweighed. The WRVs were calculated as the weight of water retained in the pulp pad after centrifugation divided by the dry weight of the fibers according to the following equation:

$$\text{WRV} = \frac{W_w - W_d}{W_d}$$  (4)

where $W_w$ is the weight of the wet sample after centrifuging, and $W_d$ is that of the dried sample. To determine the effect of residual lignin on the (fibre) swelling of hybrid poplar and
lodgepole pine pretreated at varying severities, the substrates were subjected to sodium chlorite-mediated delignification as described in section 2.8 followed by determination of the WRV. All WRV measurements were performed in triplicate.

2.18 Simons’ Stain

Cellulose accessibility to cellulases was estimated by Simons’ stain according to the procedure by developed by Chandra et al, (2008b). Briefly, direct orange (DO, Pontamine Fast Orange 6RN) and direct blue (DB, Pontamine Fast Sky Blue 6BX) dyes were obtained from Pylam Products Co. Inc. (Garden City, NY). Fractionation of the orange dye was performed according to Esteghlalian et al, (2001). For each substrate, approximately 100 mg (ODW) of never-dried pulp samples were weighed into six 15 mL polypropylene centrifuge tubes. Each tube received 1.0 mL of phosphate-buffered saline solution at pH 6. The DO solution (10 mg mL\(^{-1}\)) was added in a series of increasing volumes (0.25, 0.50, 0.75, 1.0, 1.5, and 2.0 mL) to the six tubes containing pulp sample and PBS. The DB solution (10 mg mL\(^{-1}\)) was also added to each tube using the same series of increasing volumes, resulting in each set of tubes containing a 1:1 mixture of DO and DB dyes at increasing concentrations. Distilled water was added to increase the final volume of the samples to 10.0 mL. The tubes were then incubated overnight at 70 °C with shaking at 200 rpm. After the incubation period, the tubes were centrifuged at 5,000 rpm for 5 min, and a sample of the supernatant was placed in a cuvette and the absorbance read on a Cary 50 UV-Vis spectrophotometer at 624 and 455 nm. The amount of dye adsorbed onto the fiber was determined using the difference in the concentration of the initial added dye and the concentration of the dye in the supernatant according to the Beer–Lambert law. The extinction coefficients were calculated by preparing standard curves of each dye and measuring the slope of their absorbance at 455 and 624 nm.
The values calculated and used in this thesis were $\varepsilon_{O_{455}} = 35.62$, $\varepsilon_{B_{455}} = 2.59$, $\varepsilon_{O_{624}} = 0.19$, $\varepsilon_{B_{624}} = 15.62$ (L g$^{-1}$ cm$^{-1}$).

### 2.19 Determination of Cellulose Degree of Polymerization

To determine the viscosity average degree of polymerization, the pretreated substrates were carefully delignified as described on section 2.8 followed by the removal of residual hemicelluloses as described in the TAPPI standard method T203 cm-99. Briefly, 1.5 g (ODW) of never-dried delignified substrate was resuspended in enough deionized water and 50% (w/v) NaOH to give a final volume of 75 mL and a final NaOH concentration of 17.5% (w/v). The pulp slurry was stirred for 30 min at which time 100 mL of deionized water was added to dilute the NaOH concentration to 9.5% (w/v) and the pulp slurry was stirred for a further 30 min. The pulp slurry was then filtered in vacuo and rinsed (3 x 30 mL) with 9.5% (w/v) NaOH followed by deionized water until the pH of the filtrate was neutral.

The viscosity of substrate solutions containing 0.06%, 0.1%, 0.125%, and 0.5% (w/v) delignified and hemicellulose-free substrates in 0.5 M cupriethylenediamine was measured on a capillary viscometer (Cannon Ubbelohde Viscometer, Cannon Instrument Co., State College, PA) according to the guidelines found in TAPPI standard method T230 om-99. The specific viscosity of the substrate solutions was determined according to the following equation:

$$\eta_{sp} = \frac{\eta_c - \eta_o}{\eta_o}$$

where $\eta_c$ is the viscosity of the sample at concentration $c$ and $\eta_o$ is the viscosity of the solvent. The intrinsic viscosity ($\eta_{int}$) of each substrate was calculated by extrapolating a plot of $\eta_{sp}/c$ as a function of $c$ to $c = 0$ as described by Lapierre et al, (2009) (Figure A2). The
viscosity average cellulose degree of polymerization (DP\textsubscript{v}) was calculated from the intrinsic viscosity by the following equation (Van Heiningen et al, 2004):

\begin{equation}
DP_v = (1.65\eta_{\text{int}})^{1.11}
\end{equation}

where \eta_{\text{int}} is the intrinsic viscosity of the substrate. All viscosity measurements were performed in triplicate.

As an alternative to the viscosity average cellulose degree of polymerization, the molecular weight distribution of the organosolv substrates studied in section 3.2 was determined by gel permeation chromatography (GPC) analysis of their tricarbanyl derivatives. The delignified and hemicellulose-free were subjected to tricarbanylation as described by Schroeder and Haigh, (1979). Briefly, approximately 20 mg of oven-dried substrate was placed in glass tubes. The samples were then resuspended in 7 mL anhydrous pyridine followed by the addition of 3 mL phenylisocyanate. The flasks were then sealed with Teflon stoppers and incubated at 80 °C with periodic vortexing for 48 h at which time the reaction was stopped by the addition of 1 mL methanol. The tricarbanylated cellulose was recovered by precipitation in 4 vol. of methanol followed by centrifugation at 5,000 rpm. The precipitated cellulose tricarbanylate was washed three times with methanol to remove traces of pyridine and air-dried. The samples were resuspended overnight in tetrahydrofuran (THF, final concentration 2 mg mL\textsuperscript{-1}) at 4 °C and filtered through a 0.45 μm filter. The GPC analysis of the tricarbanylated samples (20 μl injection volume) was carried out in an Agilent 1100 HPLC system (Palo Alto, Ca) equipped with two styragel columns (HR5E and HR1 purchased from Waters, Milford, MA) in tandem. THF was used as the mobile phase at a flow rate of 1 mL min\textsuperscript{-1}. The GPC calibration curve was generated from the elution profile of polystyrene standards. The tricarbanylated samples and polystyrene standards were detected
by a refractive index detector. The degree of polymerization (DP) of cellulose was obtained by dividing the molecular weight of the tricarbanylated polymer by the molecular weight of tricarbanylated anhydroglucose (DP = MW/519) as described by Mansfield et al., (1997).
Both the number average (DP_N) and molecular weight average (DP_W) were determined.

2.20 Determination of Cellulose Lateral Order Index (Crystallinity)

Cellulose crystallinity was determined on a Spectrum One Fourier-transform infrared spectrometer (FT-IR) with a PIKE MIRacle™ fitted with single bounce attenuated total reflectance (ATR) accessory (Perkin Elmer, Wellesley, MA). Briefly, approximately 0.1 g ODW of never-dried delignified substrate was resuspended in 50 mL of deionized water and shaken vigorously to break the pulp apart. The resuspended substrate was then transferred to a Buchner funnel, filtered to create a pulp pad and air-dried overnight. The samples were pressed uniformly against the diamond surface, and mid-IR spectra were obtained by averaging 64 scans from 4,000 to 600 cm\(^{-1}\) at a spectral resolution of 4 cm\(^{-1}\) resolution. The spectra were normalized using the Spectrum One software supplied with the equipment.

Cellulose crystallinity was calculated as the ratio of the maximum absorption at 1425 cm\(^{-1}\) and 898 cm\(^{-1}\), which correspond to CH\(_2\) scissoring and the vibrational mode of C1 with four atoms attached to it and are characteristic of crystalline and amorphous \(\beta\)-glycosidic linkages respectively (Jeihanipour et al, 2010). Cellulose crystallinity of the untreated feedstocks was determined as described above with the exception that the wood chips were partially homogenized by grinding on a Wiley mill fitted with a 40-mesh screen prior to delignification and analysis. Reported values are the average of 10 measurements at random positions in the pulp/wood flour pad.
Chapter 3: Results and Discussion

3.1 Characterization and Enzymatic Hydrolysis of Organosolv-Pretreated Hybrid Poplar and Lodgepole Pine

3.1.1 Background

While there have been significant improvements in the bioconversion of less recalcitrant lignocellulosic feedstocks such as agricultural residues and hardwoods, increasing the bioconversion of softwoods such as lodgepole pine has been considerably more challenging. As a result, more severe pretreatment conditions, higher enzyme loadings and longer incubation times are often required to achieve effective saccharification (Várnai et al, 2010). Among the many options that are available for the effective pretreatment of lignocellulosic substrates, only those processes that solubilize or otherwise modify lignin have effectively processed softwoods to generate substrates that are readily hydrolyzed by cellulolytic enzymes (Agbor et al, 2011). One pretreatment that satisfies this criterion is the ethanol organosolv (OS) process.

The principal advantage of the OS process is its ability to generate a high-purity sulfur-free lignin fraction that can be used to develop value added co-products such as antioxidants, adhesives, polyurethane foams and carbon fibres (Pan et al, 2005; 2006a). In contrast, other leading pretreatment technologies such as dilute acid hydrolysis and steam pretreatment produce a low quality lignin that tend can only used as a boiler fuel (Sannigrahi et al, 2010). Other advantages of OS pretreatment include the relative ease of solvent recovery, generation of a cellulose-rich solid substrate that is readily hydrolyzed by cellulolytic enzymes and a (hemicellulose derived) sugar-rich liquid fraction. Finally, the OS
process is capable of effectively pretreating a variety of lignocellulosic feedstocks such as, hardwoods, softwoods and grasses (Pan et al, 2006a; 2007; Hallac et al, 2010a).

Although OS pretreatment can reduce the recalcitrance of woody biomass, moderate to high enzyme loadings (10-40 FPU g^{-1} cellulose) are generally required for its efficient and rapid hydrolysis. Possible reasons for the remaining recalcitrance of the pretreated substrates include restricted accessibility of cellulose to cellulases by residual lignin and hemicelluloses and various properties of cellulose itself such as crystallinity and degree of polymerization (Jeoh et al, 2007; Pan et al, 2008). In addition, lignin can affect the enzymatic hydrolysis of cellulose by irreversibly binding to cellulases reducing their effective concentration (Nakagame et al, 2010).

In our initial work, we studied the changes in the physicochemical properties of softwood and hardwood biomass during organosolv pretreatment at two different conditions (low and high severity, LS and HS respectively, Table 3) to try to determine whether those properties correlated with the resulting substrates’ susceptibility towards enzymatic hydrolysis. Mountain pine beetle-killed lodgepole pine (LPP) and hybrid poplar were used as representatives of softwoods and hardwoods respectively. The possible inhibitory role of the residual lignin on the enzymatic hydrolysis of organosolv-pretreated lignocellulosic substrates at moderate to low (5-10 FPU g^{-1} cellulose) enzyme loadings was also investigated.

3.1.2 Mass Balance and Chemical Composition of the Pretreated Substrates

As mentioned previously, the OS process generally fractionates lignocellulosic substrates into its three major component streams. The cellulose-rich solid substrate, a high-purity sulphur-free lignin fraction (ethanol organosolv lignin, EOL) and an aqueous fraction
containing mono- and oligomeric saccharides, soluble lignin fragments and sugar-degradation products such as furfural, 5-hydroxymethylfurfural and acetic acid. The chemical composition of the initial feedstocks is shown in Appendix A, Table A1 and the mass balances of the four substrates used in this study are summarized on Table 6.

Table 6. Mass balance of organosolv-pretreated lodgepole pine and hybrid poplar

<table>
<thead>
<tr>
<th>Yield (%)</th>
<th>Lodgepole pine</th>
<th>Hybrid poplar</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LS b HS c</td>
<td>LS HS</td>
</tr>
<tr>
<td>Solid Fraction</td>
<td>48.5 47.2</td>
<td>53 54.1</td>
</tr>
<tr>
<td>Rejects</td>
<td>1.4 0.7</td>
<td>1.9 2.4</td>
</tr>
<tr>
<td>Ethanol organosolv lignin</td>
<td>11.7 11.2</td>
<td>10.2 10.9</td>
</tr>
<tr>
<td>Water soluble fraction:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acid soluble lignin</td>
<td>4.2 4.1</td>
<td>4.8 4.4</td>
</tr>
<tr>
<td>Arabinan</td>
<td>0.5 0.4</td>
<td>0.2 0.2</td>
</tr>
<tr>
<td>Galactan</td>
<td>1.2 1.1</td>
<td>0.4 0.3</td>
</tr>
<tr>
<td>Glucan</td>
<td>2.2 2.4</td>
<td>0.5 0.3</td>
</tr>
<tr>
<td>Xylan</td>
<td>1.8 1.7</td>
<td>7.7 6.0</td>
</tr>
<tr>
<td>Mannan</td>
<td>3.4 3.2</td>
<td>0.7 0.5</td>
</tr>
<tr>
<td>Furfural</td>
<td>3.1 3.6</td>
<td>1.8 2</td>
</tr>
<tr>
<td>HMF</td>
<td>3.1 3.5</td>
<td>0.1 1.0</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>10.3 11.1</td>
<td>11.1 10.7</td>
</tr>
<tr>
<td>Losses d</td>
<td>8.6 9.2</td>
<td>7.6 7.2</td>
</tr>
</tbody>
</table>

a % of extractive-free dry wood, b LS = low severity, c HS = high severity, d losses = 100 % - total yield.

Our results show that at the conditions used in this study, increasing pretreatment severity did not appear to have a significant effect on delignification or carbohydrate degradation. This was especially true in the case of the lodgepole pine (LPP), which exhibited nearly identical mass balances. However, slightly different results were obtained in the case of hybrid poplar. For example, increasing pretreatment severity resulted in a small decrease in the amount of xylan in the aqueous fraction (from 7.7 % to 6.0 %) and a ten-fold increase in the formation of Hydroxymethylfurfural (HMF) (from 0.1 % to 1.0 %). These results suggested that hybrid poplar was somewhat more sensitive to changes in pretreatment conditions than was LPP.
Compositional analysis of the pretreated substrates confirmed that the different pretreatment conditions used in this study did not have a significant effect on delignification or carbohydrate degradation (Table 7). Interestingly, both the OS-pretreated lodgepole pine and hybrid poplar (OSLPP and OSPOP respectively) displayed similar cellulose contents (ranging from 77 to 81 % w/w). However, while the cellulose contents of the pretreated substrates were similar, the OSPOP substrates exhibited significantly lower residual lignin than did the OSLPP substrates (approximately 8 % vs. 18-20 % respectively, Table 7). These results suggest that hybrid poplar was more readily delignified than was the LPP. Indeed, the OSPOP substrates exhibited a significantly higher delignification selectivity (defined as the solids yield divided by the % percentage of the initial lignin remaining in the solid substrate) than did the OSLPP substrates (2.9 and 3.0 vs. 1.5 and 1.6 for the OSPOP and OSLPP substrates respectively, Table 8). These results were expected due to the differences in lignin content and structure between softwoods and hardwoods. However, they contradict previous work by Pan et al. (2006b; 2007), which suggested that hybrid poplar was more recalcitrant than was LPP and therefore required more severe pretreatment conditions for effective delignification to occur.

**Table 7.** Chemical composition (% w/w) of organosolv pretreated lodgepole pine and hybrid poplar, numbers in parentheses indicate the standard deviation (n = 3).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Arabinan</th>
<th>Galactan</th>
<th>Glucan</th>
<th>Xylan</th>
<th>Mannan</th>
<th>Lignin&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>OSLPP-LS</td>
<td>0.2 (0.1)</td>
<td>bdl&lt;sup&gt;b&lt;/sup&gt;</td>
<td>79.2 (1.4)</td>
<td>bdl</td>
<td>2.0 (0.2)</td>
<td>19.8 (0.3)</td>
</tr>
<tr>
<td>OSLPP-HS</td>
<td>0.2 (0.0)</td>
<td>bdl</td>
<td>77.5 (1.2)</td>
<td>bdl</td>
<td>2.2 (0.0)</td>
<td>18.3 (0.2)</td>
</tr>
<tr>
<td>OSPOP-LS</td>
<td>0.2 (0.0)</td>
<td>bdl</td>
<td>78.9 (1.6)</td>
<td>5.7 (0.0)</td>
<td>2.1 (0.2)</td>
<td>8.2 (0.7)</td>
</tr>
<tr>
<td>OSPOP-HS</td>
<td>0.2 (0.1)</td>
<td>bdl</td>
<td>81.0 (1.2)</td>
<td>6.9 (0.3)</td>
<td>2.6 (0.6)</td>
<td>7.8 (0.4)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Total lignin = AIL + ASL  <sup>b</sup>bdl = below detection limit.

Pan et al. (2007, 2008) attributed the unexpected ease of delignification of LPP to possible compositional and morphological changes (such as increased permeability to pretreatment chemicals) caused by the mountain pine beetle infestation. However a more
likely explanation is that the different size of the untreated feedstocks (< 0.6 x 0.2 cm and 2.5 x 2.5 to 5 x 5 cm for the hybrid poplar and LPP respectively) had an effect on the ease of delignification due to the type equipment used during pretreatment. While some pretreatments such as ammonia fibre explosion (AFEX) and dilute acid hydrolysis benefit from particle size reduction prior to pretreatment (Chundawat et al, 2007), organosolv pretreatment requires thorough mixing for effective liquor penetration and delignification to occur. In our laboratory this mixing is achieved in a rotating digester. Because of the nature of the rotating digester, it is possible that the smaller hybrid poplar chips used by Pan et al, (2006b) were packed at the bottom of the digester vessel. This could have resulted in improper mixing thus requiring more severe conditions to achieve effective delignification when compared with this study (in which the initial feedstock particle size was similar).

**Table 8.** Influence of pretreatment severity on delignification selectivity and gross fiber characteristics.

| Substrate | Delignification selectivity
deg | Average fiber length (mm) | Fines content (%) |
<table>
<thead>
<tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>numerical</td>
<td>Weighted</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Length</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>weighted</td>
<td></td>
</tr>
<tr>
<td>Un-LPP</td>
<td>NA</td>
<td>1.79</td>
<td>3.19</td>
</tr>
<tr>
<td>LPP-LS</td>
<td>1.5</td>
<td>0.62</td>
<td>1.95</td>
</tr>
<tr>
<td>LPP-HS</td>
<td>1.6</td>
<td>0.60</td>
<td>2.07</td>
</tr>
<tr>
<td>Un-POP</td>
<td>NA</td>
<td>0.73</td>
<td>1.08</td>
</tr>
<tr>
<td>POP-LS</td>
<td>2.9</td>
<td>0.43</td>
<td>0.70</td>
</tr>
<tr>
<td>POP-HS</td>
<td>3.0</td>
<td>0.38</td>
<td>0.65</td>
</tr>
</tbody>
</table>

*Delignification selectivity = solids yield/remaining lignin (as % of initial lignin)*

However, while the mass balance and compositional analysis of the pretreated substrates’ can provide valuable information about the feedstocks’ reactivity towards pretreatment, it is difficult to ascertain the substrates’ susceptibility to enzymatic hydrolysis based on chemical composition alone. For example, Rollin et al, (2011) showed that switchgrass that had been pretreated with the cellulose solvent and organic solvent
lignocellulose fractionation (COSLIF) process was more susceptible to enzymatic hydrolysis than was aqueous ammonia-pretreated switchgrass (90 % vs. 60 % cellulose conversion after 72 h at 15 FPU g\(^{-1}\) cellulose respectively) despite having a significantly higher lignin content (24.6 % vs. 10 % respectively). Therefore, it was apparent that it would be advantageous to determine other substrate properties such as particle size, cellulose crystallinity, cellulose DP and accessibility of cellulose to cellulases.

3.1.3 Substrate Characterization

In addition to the role that chemical composition might have on the substrate’s susceptibility to cellulytic enzymes, gross fibre characteristics such as length, width and size distribution (i.e. external surface area) have been implicated as having an influence on the enzymatic hydrolysis of lignocellulosic substrates (Mooney et al, 1999; Pan et al, 2008; Yeh et al, 2010). It is generally believed that small fibres (especially the fines i.e. fibres < 0.2 mm) are more susceptible to cellulytic hydrolysis due to their larger surface area (Mansfield et al, 1999; Pan et al, 2008).

The fibre size and distribution of the untreated feedstocks and the OSLPP substrates were measured with a fibre quality analyzer (FQA). The fibre length distribution curve showed a marked shift in fibre frequency towards smaller fibres (< 0.5 mm) as a result of pretreatment. This increase in the frequency of small fibres was accompanied by the virtual elimination of the proportion of large (> 1.5 mm) fibres (Figure 10A) which, as expected (based on the fibre length distribution curves), resulted in a decrease in the numerical and length-weighted average fibre length (from 1.79 mm to approximately 0.6 mm and from 3.19 mm to approximately 2 mm respectively, Table 8). As was also expected, the decrease in fibre length was accompanied by a large increase in the fines content (from 31 % to 55 %,
corresponding to an 80 % increase, Table 8). Interestingly, pretreatment severity did not influence the changes in fibre length distribution, average fibre length and fines content. This suggested that, at the conditions used in this study, the gross fibre characteristics of the OSLPP substrates were not affected by changes in the pretreatment severity.

Similar results were observed with the OSPOP substrates (Figure 10B). However, while the shift in fibre length distribution was not as marked as in the case of the LPP substrate, the relative decrease in the length-weighted average fibre length (from 1.08 mm to 0.70 mm and 0.65 mm) was similar (approximately 30 %). It was also apparent that unlike the LPP, the hybrid poplar was more susceptible to changes in pretreatment severity as evidenced by the fact that at high severity, the proportion of fines increased to a greater extent than at low severity (from 28.9 % to 43.9 % and 36.4 % respectively Table 8).

Overall, it was evident that for both the LPP and hybrid poplar, OS pretreatment resulted in significant decreases in fibre length accompanied by large increases in the fines content. This was likely due to the fact that the OS pretreatment is typically carried out under acidic conditions, which results in acid-catalyzed fibre damage and increased surface area (Pan et al, 2008). Although this type of “fibre cutting” is undesirable during the papermaking process (due to decreased paper strength), the increased surface area available to cellulases as a result of acid-catalyzed fibre damage is thought to be one the reasons why the organosolv process is effective in reducing the recalcitrance of lignocellulosic substrates (Pan et al, 2008). In contrast, the kraft process (the predominant pulping process used in the paper industry) is
Figure 10. Fibre length distribution of mountain pine beetle-killed lodgepole pine (LPP) (A) and hybrid poplar (POP) (B) before and after organosolv (OS) pretreatment.
carried out under alkaline conditions and results in the production of pulps that contain large fibres and a smaller proportion of fines (Pan et al., 2008; Lapierre et al., 2009).

Besides the chemical composition and fibre length of the substrate other characteristics such as the degree of polymerization (DP) and crystallinity of the cellulose are properties that are likely to influence the ease of enzymatic hydrolysis of a substrate. With regards to cellulose DP, it is has been proposed that longer cellulose chains can form more inter- and intramolecular hydrogen bonds, which limit the accessibility of the cellulose to cellulases, decreasing the susceptibility to cellulolytic hydrolysis (Puri, 1984). In addition, lower cellulose DP results in increased cellulose chain ends that are available for cellubiohydrolases (especially Cel 7a) to act on (Väjljämä et al., 2001; Zhang and Lynd, 2004). However, the extent of the influence of cellulose DP on enzymatic hydrolysis is still unclear and has been the focus of several studies. Some of those studies suggested that a substrate’s susceptibility towards enzymatic hydrolysis is at least partially governed by the degree of polymerization of its cellulose component (Martinez et al., 1997; Pan et al., 2008; Cateto et al., 2011) while others (Sinistyn et al., 1991; Zhang and Lynd, 2006a) have suggested that cellulose DP does not affect its susceptibility towards enzymatic saccharification.

In the work reported here (using the intrinsic viscosity technique), it was evident that the cellulose DP’s of the OSLPP substrates were approximately 3 times lower that than those of the OSPOP substrates (605 and 531 vs. 1886 and 1523 respectively, Table 9). These results suggest that the cellulose in LPP was more extensively depolymerized during pretreatment than was the cellulose in hybrid poplar. A possible explanation for this observation comes from the fact that, as previously mentioned, the delignification selectivity
of the pretreatment towards hybrid poplar was twice as high as that for the LPP substrate (Table 8). It is thus conceivable that in the case of hybrid poplar, a greater proportion of the carbohydrate components would be preserved during the pretreatment. In contrast, (and because of its lower delignification selectivity) pretreatment of LPP would not only remove the majority of the hemicelluloses but also cause more extensive cellulose depolymerization while retaining a greater proportion of the lignin in the solid fraction (Tables 8 and 9).

Table 9. Effect of pretreatment severity on the cellulose crystallinity, cellulose accessibility (Simons’ stain), fibre swelling (water retention value, WRV) and cellulose DP of organosolv-pretreated lodgepole pine and hybrid poplar, numbers in parentheses indicate the standard deviation.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Crystallinity ( \frac{A_{1425}}{A_{898}} )</th>
<th>( \frac{A_{\text{max}} \text{ DO}}{A_{\text{max}} \text{ DB}} )</th>
<th>( A_{\text{max}} \text{ DO} ) (mg g(^{-1}))</th>
<th>WRV</th>
<th>( \text{DP}_v )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Un-LPP</td>
<td>0.71 (0.07)</td>
<td>0</td>
<td>0</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>OSLPP-LS</td>
<td>0.89 (0.01)</td>
<td>2.0 (0.1)</td>
<td>83.1 (1.7)</td>
<td>2.4 (0)</td>
<td>605 (56)</td>
</tr>
<tr>
<td>OSLPP-HS</td>
<td>0.82 (0.02)</td>
<td>2.0 (0.1)</td>
<td>81.2 (3.4)</td>
<td>2.2 (0.1)</td>
<td>531 (33)</td>
</tr>
<tr>
<td>Un-POP</td>
<td>0.83 (0.05)</td>
<td>0</td>
<td>0</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>OPOP-LS</td>
<td>0.88 (0.01)</td>
<td>1.7 (0.1)</td>
<td>109.9 (2.8)</td>
<td>3.2 (0.1)</td>
<td>1886 (38)</td>
</tr>
<tr>
<td>OPOP-HS</td>
<td>0.88 (0.01)</td>
<td>1.7 (0.1)</td>
<td>102.5 (3.0)</td>
<td>3.4 (0)</td>
<td>1523 (34)</td>
</tr>
</tbody>
</table>

\( ^a \)Crystallinity measurements are the average of 10 separate measurements, all others are the average of 3 separate experiments. \( ^b \) N.D.= not determined.

Interestingly, our results show that at the conditions used in this study, the LPP was less affected by changes in pretreatment conditions as the high severity pretreatment resulted in a small (approximately 12 %) decrease in DP compared to the low severity conditions. In contrast (and similar to the effects of pretreatment severity on fibre length), hybrid poplar appeared to be more sensitive to changes in pretreatment conditions as evidenced by the greater (approximately 20 %) reduction in cellulose DP. Analogous to the effects of pretreatment on fibre length, the acid-catalyzed depolymerization of cellulose during organosolv pretreatment is thought to be one of the reasons behind the effectiveness of the organosolv process as a pretreatment for woody biomass (Pan et al, 2008).
Similar to the effect of cellulose DP on the enzymatic hydrolysis of cellulose, there has been considerable debate on the influence of cellulose crystallinity on cellulolytic hydrolysis. For example, several studies (Fan et al, 1991; Sinitsyn et al, 1991; Zhang and Lynd, 2004) have found that the amorphous regions of cellulose are more susceptible to enzymatic hydrolysis than are the crystalline regions. However, those studies used relatively pure model cellulose substrates rather than lignocellulosic biomass (i.e. without the influence of residual lignin and hemicelluloses) and therefore provide a somewhat simple interpretation of the role of cellulose crystallinity as an indicator of its susceptibility towards enzymatic hydrolysis. In fact, subsequent work with lignocellulosic substrates such as steam-pretreated biomass failed to find a correlation between cellulose crystallinity and its susceptibility towards enzymatic hydrolysis (Tanahashi et al, 1989; Ramos et al, 1993). As a result of this lack of resolution of influence, it is likely that, when studying real lignocellulosic substrates, the effect of other substrate properties (such as chemical composition and accessibility of cellulose to cellulases) on the enzymatic hydrolysis of cellulose are just as important as the properties of cellulose itself (Puri, 1984; Tanahashi et al, 1989).

In the current study, it seemed that besides reducing cellulose DP, the acidic conditions employed during pretreatment increased the cellulose crystallinity (measured as the lateral order index, LOI) of the OSLPP and OSPOP substrates (from 0.71 to 0.89 and 0.82 and from 0.83 to 0.88 respectively, Table 9). This increase in cellulose crystallinity is likely due to hydrolysis of some of the amorphous cellulose (and hemicellulose) during pretreatment resulting in an enrichment of crystalline cellulose in the pretreated substrates (alongside a reduction in cellulose DP and fibre length). Interestingly, the changes in pretreatment severity did not appear to have a significant effect on cellulose crystallinity.
suggesting that the low severity conditions were sufficient to selectively remove the majority of the amorphous material.

Although cellulose DP and crystallinity are believed to influence the effectiveness of enzymatic hydrolysis, it has been proposed that the overall accessibility of cellulose to cellulases is the key substrate characteristic that limits its hydrolysis (Chandra et al 2008b; Rollin et al, 2011). However unlike crystallinity and degree of polymerization, which are properties of cellulose, the accessibility of cellulose to cellulases is influenced by a variety of factors including particle size (exterior surface area), pore volume (interior surface area), the content/distribution of residual lignin and hemicelluloses as well as cellulose crystallinity and DP. In the work reported here, the accessibility of cellulose to cellulases was estimated by the Simon’s stain technique. Briefly, this technique is based on the competitive adsorption of two direct dyes (blue and orange) where the orange dye has a higher molecular weight and affinity for cellulose and can penetrate and displace the blue dye from the larger pores within the cellulose structure. Yu et al, (1995) showed that the orange dye is composed of two subfractions with hydrodynamic radii of 5-7 nm and 12-36 nm, which are equal or larger than the rate limiting pore size for cellulose hydrolysis by cellulases (5.1 nm, Grethlein, 1985). Due to their differences in size and affinity for cellulose, the ratio of adsorbed orange to blue dye can be used to estimate the relative proportion of large and small pores while the maximum adsorption capacity for the orange dye can estimate the overall accessibility of cellulose to cellulases (Chandra et al, 2008b).

Similar to the effects on chemical composition, fibre length and cellulose crystallinity, changes in pretreatment severity did not appear to affect the ratio of orange to blue dye adsorption. However, the OSPOP substrates exhibited a somewhat lower ratio of
adsorbed dyes than did the OSLPP substrates (1.7 and 2.0 respectively, Table 9) suggesting a higher amount of blue dye-accessible pores in the OSPOP substrates. In contrast, the OSPOP substrates exhibited a significantly higher adsorption capacity for the orange dye than did the OSLPP substrates (109.9 and 102.5 mg g\(^{-1}\) vs. 83.1 and 81.2 mg g\(^{-1}\) for OSPOP and OSLPP substrates respectively, Table 9). These results suggested that the cellulose component of the OSPOP substrates was more accessible to cellulases than was that of the OSLPP substrates. These differences in cellulose accessibility were likely due to the higher lignin content of the OSLPP substrates when compared to the OSPOP substrates and may be responsible for possible differences in susceptibility towards enzymatic saccharification.

Fibre swelling is a key aspect of increasing the accessibility of cellulose to enzymes and it has also been proposed to play a major role in determining a substrate’s susceptibility to cellulosolytic hydrolysis (Chandra et al, 2007). Fibre swelling is known to be influenced by several factors including the presence and nature of charged groups in the interior and exterior of the fibres, the pH of the medium, substrate hydrophilicity and the presence of electrolytes (Lindstrøm and Carlsson, 1982). The extent of fibre swelling can be estimated by calculating the centrifugal Water Retention Value (WRV) developed by Jayme (1944). As previous work (Ogiwara and Arai, 1968; Bendzalova et al, 1996; Luo and Zhu, 2010) showed that the WRV could provide a good indication of a substrates’ susceptibility towards enzymatic hydrolysis, we next assessed the WRV for the OSLPP and OSPOP substrates. It was evident from the WRV’s that like cellulose accessibility to cellulases, the OSPOP substrates exhibited a higher degree of fibre swelling than did the OSLPP substrates (3.2 and 3.4 vs. 2.4 and 2.2 respectively, Table 9). This is probably due to both the lower residual lignin and (especially) the higher hemicellulose content in the OSPOP substrates.
Specifically, lignin restricts fibre swelling while hemicelluloses (particularly xylan) promote fibre swelling by forming a hydrophilic layer on the cellulose surface, which enhances water uptake and prevents interactions between the crystalline regions of adjacent cellulose fibrils (Eriksson et al, 1991; Mooney et al, 1998; Fonseca-Silva et al, 2011).

Thus it appeared that the LPP and hybrid poplar substrates behaved differently during pretreatment. As mentioned earlier, the hybrid poplar was easier to delignify and produced a substrate that exhibited enhanced fibre swelling and increased cellulose accessibility to cellulases. In contrast, the cellulose component of the LPP substrate was more susceptible to acid-catalyzed cellulose damage than that of hybrid poplar, as evidenced by the lower cellulose DP of the OSLPP substrates. However, the OSLPP substrates were quite similar with regards to their chemical composition, fines content, cellulose DP, crystallinity and accessibility to cellulases despite having been prepared under different severities, suggesting that the LPP was relatively insensitive to changes in pretreatment conditions. In contrast, hybrid poplar was more sensitive to changes in pretreatment conditions, as evidenced by the higher fines content and lower cellulose DP of the OSPOP substrate pretreated at high severity (OSPOP-HS).

3.1.4 Susceptibility of Organosolv-Pretreated Lodgepole Pine and Hybrid Poplar towards Enzymatic Hydrolysis

To measure the effect of pretreatment severity on the enzymatic hydrolysis of the pretreated substrates and to establish a target for subsequent reductions in enzyme requirements, the 72 h cellulose hydrolysis yields were measured as a function of cellulase loading. It was evident that both the pretreated lodgepole pine and hybrid poplar substrates were highly susceptible toward enzymatic hydrolysis as near complete (> 96%) cellulose
hydrolysis could be achieved at 7.5 FPU g-1 cellulose (Figure 11A and B). However, reducing the enzyme loadings below 7.5 FPU g-1 cellulose resulted in significant decrease in cellulose hydrolysis. For example, at 5 and 2.5 FPU g⁻¹ cellulose, the cellulose hydrolysis yields of the OSLPP substrates decreased to 70 % and 25 % respectively (Figure 11A). This decrease in cellulose conversion was likely due to the inhibition of cellulolytic hydrolysis by the residual lignin, which corresponded to approximately 20 % of the substrates’ dry weight (Table 7). Although decreasing cellulase loadings resulted in a decrease in the hydrolysis yields of the OSLPP substrates, it was apparent that changes in pretreatment severity did not affect their susceptibility towards enzymatic hydrolysis (Figure 11A). This is likely due to the fact that, as previously mentioned, changes in pretreatment severity did not appear to have a significant effect on the OSLPP substrates’ physicochemical properties including chemical composition and the accessibility of the cellulases to the substrate.

However, different results were observed with the OSPOP substrates. For example, when pretreated at low severity (OSPOP-LS), decreasing the cellulase loadings below 7.5 FPU g⁻¹ cellulose also resulted in a decrease in cellulose hydrolysis. However, the overall decrease was much lower than that observed with the OSLPP substrates (from 98 % at 7.5 FPU g⁻¹ cellulose to 89 % and 47 % at 5 and 2.5 FPU g⁻¹ cellulose respectively, Figure 11B). These results suggest that (as expected due to its higher accessibility of cellulose to cellulases and lower lignin content) the OSPOP-LS substrate was much less recalcitrant than were the OSLPP substrates. On the other hand, decreasing the cellulase loading during the enzymatic hydrolysis of the OSPOP-HS substrate resulted in a substantial decrease in cellulose conversion (from 96 % at 7.5 FPU g⁻¹ cellulose to 62 % and 32 % at 5 and 2.5 FPU g⁻¹ cellulose respectively, Figure 11B) to a level that is comparable to that of the OSLPP
substrates. These results were unexpected as the OSPOP-HS substrate showed similar physicochemical properties and had a similar chemical composition as did the OSPOP-LS substrate. Moreover, the OSPOP-HS substrate also had a higher proportion of fines and lower cellulose DP (Tables 8 and 9), which are thought to influence the substrate’s susceptibility towards cellulosytic hydrolysis (Mooney et al, 1999; Pan et al, 2007; Pan et al, 2008).

A possible explanation for these unexpected results is that the conditions used in the high severity pretreatment resulted in a redistribution of some of the residual lignin towards the substrate’s surface restricting the accessibility of cellulose to cellulases. It is also possible that the redistribution of the lignin resulted in increased non-productive interactions with cellulases. However in the case of the OSLPP substrates it is possible that these phenomena were masked by the larger amounts of residual lignin in the substrates. Supporting evidence for this hypothesis is provided by the fact that the OSPOP-HS substrate exhibited a somewhat lower accessibility of cellulose to cellulases compared to the OSPOP-LS substrate, as evidenced by its lower maximum adsorption capacity for the direct orange dye (102.5 vs. 109.9 mg g\(^{-1}\) respectively, Table 9).

Alternatively, it is possible that the conditions used during the high-severity pretreatment promoted the condensation of the residual lignin, which increased its hydrophobicity. This condensed lignin could in-turn inhibit the enzymatic hydrolysis of the pretreated substrate by non-productively binding with cellulases. It is also possible that, due to the greater degree of condensation of the guaiacyl-rich softwood lignin, changes in the chemical structure of the residual lignin in the OSLPP substrates (as a result of pretreatment
Figure 11. The effects of varying pretreatment severity on the susceptibility of organosolv-pretreated lodgepole pine (A) and hybrid poplar (B) to cellulytic hydrolysis. Substrates were resuspended at 2% (w/v) solids content in 50 mM Na-acetate. Hydrolysis conditions: 50 °C, 150 rpm. Error bars indicate the standard deviation (n = 3).
severity) did not affect the non-productive binding of lignin to cellulases. This possible mechanism is supported by the fact that, as previously mentioned, decreasing the ethanol concentration in the pretreatment liquor (such as in the case of the high-severity pretreatment) is known to promote lignin condensation (Bose and Francis, 1999; Pan et al, 2006b; 2008).

3.1.5 Determination of the Nature of the Residual Recalcitrance of Organosolv-Pretreated Substrates

To try to determine the nature of the residual recalcitrance of both the OSLPP substrates as well as the OSPOP-HS substrate, we next determined the effect of pretreatment severity on the substrates’ hydrophobicity, using the static contact angle technique. Hodgson and Berg (1988) have used this technique to measure the contact angle of α-cellulose (14.0 °) and (lignin-rich) thermomechanical pulps (42.8 ° and 51.2 °) to show that lignin is significantly more hydrophobic than cellulose. Similarly Maximova et al, (2004) found that the adsorption of lignin to either cellulose fibres or mica increased their contact angle. More recently this technique was used by Heiss-Blanquet and co-workers (2011) to show that lignin removal during steam- and organosolv-pretreatment of wheat straw decreased the contact angle (and consequently the hydrophobicity) of the pretreated substrates when compared to untreated wheat straw.

It was anticipated that, because of their higher lignin content, the OSLPP substrates would be more hydrophobic than the OSPOP substrates. It was also anticipated that the OSPOP-HS substrate would be somewhat more hydrophobic as a result of either the redistribution of lignin towards the fibres’ surface or lignin condensation due to the higher severity condition. As expected, our results show that the OSLPP substrates had a greater
contact angle than did the OSPOP substrates (65.8° and 62.2° vs. 52.6° and 58.9° respectively, Table 10) indicating their greater hydrophobicity. As was also expected, an increase in the hydrophobicity of the OSPOP-HS substrate compared to the OSPOP-LS substrate was observed suggesting that the greater recalcitrance of the OSPOP-HS substrate could be due to its increased hydrophobicity.

**Table 10.** Surface characterization and maximum BSA adsorption capacity of organosolv-pretreated lodgepole pine and hybrid poplar, numbers in parentheses indicate the standard deviation.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>C1 (%)</th>
<th>C2 (%)</th>
<th>C3 (%)</th>
<th>C4 (%)</th>
<th>O/C</th>
<th>Contact angle (°)</th>
<th>A$_{max}$ BSA (mg g$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPP-A</td>
<td>35.6</td>
<td>52.7</td>
<td>11.7</td>
<td>bdl</td>
<td>0.49</td>
<td>65.8 (3.5)</td>
<td>84.8 (2.5)</td>
</tr>
<tr>
<td>LPP-B</td>
<td>30.7</td>
<td>56.2</td>
<td>13.2</td>
<td>bdl</td>
<td>0.51</td>
<td>62.2 (2.1)</td>
<td>88.1 (1.6)</td>
</tr>
<tr>
<td>POP-A</td>
<td>22.2</td>
<td>62.4</td>
<td>14.1</td>
<td>1.2</td>
<td>0.57</td>
<td>52.6 (2.0)</td>
<td>36.0 (2.3)</td>
</tr>
<tr>
<td>POP-B</td>
<td>20.8</td>
<td>62.5</td>
<td>15.7</td>
<td>1.0</td>
<td>0.55</td>
<td>58.9 (2.2)</td>
<td>48.7 (3.6)</td>
</tr>
</tbody>
</table>

*Maximum BSA adsorption capacity and contact angles measurements are the average of 3 and 10 separate experiments respectively. bC1= unoxidized carbon (C-C, C-H), C2= carbon with one oxygen bond (C-O), C3= carbon with two oxygen bonds (O-C=O or C=O), C4= carbon with three oxygen bonds (O=C-O).*

Although these results suggested that the greater recalcitrance of the OSLPP and OSPOP-HS substrates was caused by their hydrophobicity. However, it should be noted that contact angle measurements are affected by several factors. These include substrate porosity, chemical composition, distribution of lignin on the fibres’ surface and changes in cellulose crystallinity (Böras and Gatenholm, 1999; Fardim and Duran, 2002; Negro et al, 2003; Kristensen et al, 2008). As a result, the surface chemical composition of the pretreated substrates was analyzed using X-ray photoelectron spectroscopy (XPS), which enabled the analysis of the substrate’s surface to a depth of approximately 5-10 nm (Tshabalala, 2005). The surface analysis by XPS can be expanded into survey and high-resolution spectra, which provide information about the surface elemental composition (which in the case of lignocellulosic materials is mostly carbon and oxygen) and the chemical state of each atom on the samples’ surface respectively.
Earlier work on the high-resolution surface analysis of lignocellulosic materials by XPS showed that the carbon peak could be subdivided into four different peaks representing the four classes of carbon atoms (C$_1$-C$_4$) present in lignocellulosic biomass. The C$_1$ carbons are bound to other carbons or to hydrogen and are found in lignin. The C$_2$ carbons are bound to one oxygen atom (C-O) and are found mostly in carbohydrates. The C$_3$ carbons are bound to two oxygen atoms or a carbonyl group (C=O, O-C=O) and the C$_4$ carbons represent carboxyl groups (O=C-O) (Dorris and Gray, 1978a and b; Gray, 1978; Palonen and Viikari 2004). Subsequent studies on the applicability of XPS analysis to lignocellulosic-derived materials showed that the oxygen to carbon ratio can be used to estimate the relative proportion of lignin and carbohydrates on the substrates’ surface. For example, lignin-rich surfaces possess O/C ratios that are closer to that of pure lignin (0.33) while carbohydrate-rich surfaces show O/C ratios closer to those of hemicelluloses and cellulose (0.81-0.83 respectively) (Mjörberg, 1981; Laine et al, 1994).

The XPS analyses showed that the O/C ratios of the OSLPP substrates were lower than those of the OSPOP substrates (0.49 and 0.51 vs. 0.57 and 0.55 respectively, Table 10) suggesting that (as expected due to their greater overall lignin content and hydrophobicity) the surface of the OSLPP substrates possessed a greater amount of lignin than did the surface of the OSPOP substrates. Moreover, deconvolution of the high resolution carbon spectra indicated that the OSLPP substrates contained a greater proportion of C$_1$ carbons than did the OSPOP substrates (35.6% and 30.7 % vs. 22.2 % and 20.8 %, respectively Table 10), confirming the results obtained from the O/C ratios. In addition to having nearly identical O/C ratios to the OSLPP-LS and OSPOP-LS substrates, the OSLPP-HS and OSPOP-HS substrates showed a slightly lower proportion of C$_1$ carbons (Table 10), suggesting that
increasing pretreatment severity did not promote the redistribution of lignin to the substrate’s surface.

Since XPS analysis suggested that changes in pretreatment conditions did not affect the chemical composition of the substrate’s surface it was hypothesized that the greater recalcitrance of the OSPOP-HS substrate might be due to increased nonspecific interactions between the residual lignin and cellulases, resulting from lignin condensation during pretreatment. Therefore, to determine whether the increase in pretreatment severity might have promoted lignin condensation, we next investigated the chemical structure of the residual lignins using the thioacidolysis technique. This technique is based on the selective cleavage of monolignols involved in uncondensed β-O-4 linkages by the soft nucleophile ethanethiol and the Lewis acid boron trifluoride (Figure 12), and can provide valuable information on the chemical composition of the lignin sample (Lapierre et al, 1984; Rolando et al, 1992). In addition, the total monolignol yield can be used as an indirect measurement of lignin condensation (Baumberger et al, 2002; Pinto et al, 2002; Heiss-Blanquet et al, 2011).

As expected, organosolv pretreatment resulted in a decrease in the monolignol yield (and therefore an increase in lignin condensation) that was proportional to pretreatment severity (from 950 μmol g⁻¹ lignin in the untreated hybrid poplar to 238 and 179 μmol g⁻¹ lignin in the OSPOP-LS and OSPOP-HS substrates respectively, Table 11). Similar results were observed by Moon et al, (2011) who found that increasing pretreatment severity during the hydrothermolysis of poplar resulted in significant decreases in the monolignol yield, despite an enrichment in the lignin content of the pretreated residues. Besides decreases in the monolignol yield, our results showed a marked decrease in the S:G ratio that was proportional to pretreatment severity (from 1.75 in the untreated hybrid poplar to 1.64 and
1.40 for the OSPOP-LS and OSPOP-HS substrates respectively, Table 11). These results agree with a previous work of Tsutsumi et al, (1995), which showed that the syringyl units involved in β-aryl ether linkages were more reactive than were guaiacyl units. Moreover, the enrichment in guaiacyl units could increase the likelihood of lignin condensation via the formation of carbon-carbon linkages at position 5 of the aromatic ring. Overall, the decrease in monomer yield and S:G ratio as a result of pretreatment severity support the suggestion that the recalcitrance of the OSPOP-HS substrate was due to increased nonspecific interactions between the (more condensed) residual lignin and cellulases.

Figure 12. Proposed mechanism of the thioacidolysis reaction with lignin. Adapted from Rolando et al, (1992).

Although it is likely that increased nonspecific interactions between lignin and cellulases were responsible for the greater recalcitrance of the OSPOP-HS substrate, the physical effects of lignin (i.e. restriction of cellulose accessibility to cellulases) could not be
ruled out. In addition, because of their greater amounts of residual lignin, it is likely that the recalcitrance of the OSLPP substrates at low enzyme loadings was due to both nonspecific interactions between lignin and cellulases and the restricted accessibility of cellulose. Therefore we next decided to determine whether the nature of the inhibitory effect of lignin on the enzymatic hydrolysis of the organosolv-pretreated substrates was chemical, physical or a combination of both. To achieve this goal, the enzymatic hydrolysis of the pretreated substrates (at 5 FPU g\(^{-1}\) cellulose) was determined before and after complete delignification or the prior addition of bovine serum albumin (BSA) to remove the chemical effects of lignin. Bovine serum albumin was chosen as a suitable lignin blocking agent as it does not bind to cellulose or influence its susceptibility towards enzymatic hydrolysis (Yang and Wyman, 2006; Zhu et al, 2009b). The amount of BSA added to each substrate corresponded to its maximum adsorption capacity as determined by the linear transformation of their Langmuir adsorption isotherms (Appendix A, Figure A1).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>S:G</th>
<th>Total monomer yield (µmol g(^{-1}) lignin(^a))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>1.75 (0.04)</td>
<td>950 (54)</td>
</tr>
<tr>
<td>OSPOP-LS</td>
<td>1.64 (0.01)</td>
<td>238 (10)</td>
</tr>
<tr>
<td>OSPOP-HS</td>
<td>1.40 (0.06)</td>
<td>179 (14)</td>
</tr>
</tbody>
</table>

It was evident that prior addition of BSA to the OSLPP substrates resulted in significant improvements in the rate and extent of cellulolytic hydrolysis (from 68 % to 92 % and from 72 % to 98 % for the OSLPP-LS and OSLPP-HS substrates respectively, (Figure 13A and B). As expected, these results suggested that lignin-blocking with BSA decreased nonspecific interactions between cellulases and the residual lignin thus increasing their availability to react with cellulose. However, complete lignin removal resulted in even more significant
Figure 13. Effect of delignification and lignin blocking on the enzymatic hydrolysis (5 FPU g\(^{-1}\) cellulose) of lodgepole pine subjected to organosolv pretreatment at low (A) and high severity (B). Substrates were resuspended at 2% (w/v) solids content in 50 mM Na-acetate. Hydrolysis conditions: 50 °C, 150 rpm. Error bars indicate the standard deviation (n = 3).
improvements with over 90% of the cellulose hydrolyzed within 24 h (Figure 13A and B) suggesting that lignin also inhibits cellulosic hydrolysis by preventing the accessibility of cellulose to cellulases. This was supported by the fact that the delignified OSLPP-LS substrate showed a significant increase in cellulose accessibility as indicated by an increase in direct orange dye adsorption (From 83.1 to 102 mg g$^{-1}$, Tables 9 and 12). It should be also be noted that (as expected based on their physicochemical properties and susceptibility to enzymatic hydrolysis) both OSLPP substrates showed nearly identical behaviour towards enzymatic hydrolysis after lignin-blocking and complete delignification. These results further support the suggestion that, unlike hybrid poplar, LPP was more resistant to changes in pretreatment conditions.

**Table 12.** Effects of lignin removal on cellulose accessibility (Simon’s stain), and fibre swelling (water retention value, WRV) of lodgepole pine and hybrid poplar pretreated at low severity, numbers in parenthesis indicate the standard deviation (n = 3).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$A_{\text{max}}$ DO/$A_{\text{max}}$ DB</th>
<th>$A_{\text{max}}$ DO (mg g$^{-1}$)</th>
<th>WRV</th>
</tr>
</thead>
<tbody>
<tr>
<td>OSLPP-LS$_{DL}$</td>
<td>1.8 (0.2)</td>
<td>102.0 (1.0)</td>
<td>2.9 (0.3)</td>
</tr>
<tr>
<td>OSPOP-LS$_{DL}$</td>
<td>1.8 (0.1)</td>
<td>112.7 (2.8)</td>
<td>3.3 (0.1)</td>
</tr>
</tbody>
</table>

While it was apparent that the residual lignin inhibited the enzymatic hydrolysis of the OSLPP substrates through a combination of influences such as the nonspecific binding to cellulases and restricting the accessibility of cellulose to cellulases, the OSPOP substrates behaved differently. In the case of the OSPOP-LS substrate, it was clear that the residual lignin did not affect its susceptibility to enzymatic hydrolysis (at 5 FPU g$^{-1}$ cellulose) as neither lignin-blocking with BSA nor complete delignification had a significant effect on cellulose conversion (approximately 90% after 72 h Figure 14A). The lack of any effect of lignin on the enzymatic hydrolysis of the OSPOP-LS substrate was likely because the cellulose component was already more accessible to cellulases than was the cellulose...
Figure 14. Effect of delignification and lignin blocking on the enzymatic hydrolysis (5 FPU g⁻¹ cellulose) of hybrid poplar subjected to organosolv pretreatment at low (A) and high severities (B). Substrates were resuspended at 2% (w/v) solids content in 50 mM Na-acetate buffer. Hydrolysis conditions: 50 °C, 150 rpm. Error bars indicate the standard deviation (n = 3).
component of the OSLPP substrates as indicated by the maximum adsorption capacity of
direct orange dye and the XPS results (Tables 9 and 10 respectively). In addition, complete
delignification of the OSPOP-LS substrate resulted in a marginal increase on direct orange
dye adsorption (109.9 vs. 112.7 mg g$^{-1}$, Tables 9 and 12). These results suggest that the
relatively small amounts of residual lignin in the OSPOP-LS substrate did not prevent the
accessibility of the enzymes to the cellulose.

Although the residual lignin did not appear to influence the susceptibility of the
OSPOP-LS substrate to enzymatic hydrolysis, it was evident that, in the case of the OSPOP-
HS substrate, lignin blocking and complete delignification resulted in identical improvements
in cellulose hydrolysis (from 62 % to 85 % after 72 h, Figure 14B). This suggested that the
greater recalcitrance the OSPOP-HS substrate was solely due to nonspecific interactions
between the residual lignin and cellulases. As previously discussed, it is likely that these
interactions are a result of structural changes in the residual lignin as a consequence of the
high-severity pretreatment conditions. As was the case for the OSPOP-LS substrate, the lack
of any physical effect of the residual lignin on the hydrolysis of the OSPOP-HS substrate was
likely due the greater enzyme accessibility of the cellulose.

These results suggested that, prior to delignification, the OSPOP substrates were at
least as susceptible to enzymatic hydrolysis as were the OSLPP substrates. However, after
complete delignification the opposite was observed with over 90 % of the cellulose in the
OSLPP substrates hydrolyzed after 24 h compared to only 47 % for the OSPOP substrates
(Figures 13 and 14). These results were unexpected since the delignified OSLPP substrates
displayed lower cellulose accessibility to cellulases (measured by the adsorption of direct
orange dye) than did the OSPOP substrates (102 vs. 112.7 mg g$^{-1}$ respectively, Table 12). A
possible explanation is that, when the inhibitory effect of lignin was removed, the lower hemicellulose content and cellulose DP of the OSLPP substrates contributed to their ease of enzymatic hydrolysis. Previous studies (Jeoh et al., 2007; Berlin et al., 2007) have suggested that (similar to lignin) residual hemicelluloses can prevent the accessibility of cellulose to cellulases. However, since the majority of the hemicelluloses were solubilized during pretreatment it is likely that their inhibitory effect was eliminated. This is supported by the fact that almost complete (89 %) cellulose conversion was observed after 72 h at relatively low (5 FPU g\(^{-1}\) cellulose) cellulase dosages. Alternatively, it is possible that the higher DP of the cellulose in the OSPOP substrates compared to that of the OSLPP substrates (1886 and 1523 vs. 605 and 531 respectively, Table 9) contributed to their initially slow saccharification due to the smaller number of cellulose chain ends for cellobiohydrolases to act on (Väljämae et al., 2001). However, once enough cellulose chains were generated via the action of endoglucanases, cellulolytic hydrolysis proceeded to near completion.

Overall, it was evident that non-productive binding of residual lignin to the cellulases had a significant impact on the enzymatic hydrolysis of three of the four substrates used. To try to further clarify the mechanisms involved we next tried to determine the amount of cellulases that might be bound to the lignin-rich residue obtained after the near-complete hydrolysis of cellulose. The OSPOP and OSLPP substrates were subjected to batch hydrolysis at 7.5 FPU g\(^{-1}\) cellulose as that was the lowest enzyme loading that resulted in near complete (> 95 %) cellulose conversion (Figure 11A and B) and the amount of protein in solution was determined after 72 h. It was apparent that a significant amount (39 %) of the added cellulases remained bound to the lignin rich residue after the complete enzymatic hydrolysis of the OSLPP substrates. In contrast, we were able to recover 93 % of the
enzymes added to the OSPOP-LS substrate and 80 % of those added to the OSPOP-HS substrate (Figure 15).

Overall, these results support the suggestion that, in the case of the OSLPP substrates, the inhibitory effect of the residual lignin was due to the combination of nonspecific interactions between the enzymes and lignin as well as restricting the accessibility of cellulose to the cellulases. Moreover, the fact that virtually all of the added enzymes were recovered after the near complete cellulose hydrolysis of the OSPOP-LS substrate supports the observation that its residual lignin did not have a significant effect on its susceptibility towards cellulolytic hydrolysis. However, in the case of the OSPOP-HS substrate we were only able to recover 80 % of the added enzymes. This supported the suggestion that nonspecific interactions between the residual lignin and cellulases restricted the effective hydrolysis of its cellulose component.

![Figure 15](image)

**Figure 15.** Effect of pretreatment conditions on enzyme recovery after complete cellulose hydrolysis. Substrates were resuspended at 2% (w/v) solids content in 50 mM Na-acetate buffer for 72 hours at a cellulase loading of 7.5 FPU g\(^{-1}\) cellulase. Hydrolysis conditions: 50 °C, 150 rpm. Error bars indicate the standard deviation (n = 3).
3.1.6 Enzymatic Hydrolysis of Organosolv-Pretreated Lodgepole Pine and Hybrid Poplar at Increased Solids Content

Although enzymatic hydrolysis of lignocellulosic substrates at a low (< 5% w/w) solids concentration can provide a great deal of information about the effectiveness of the pretreatment process, (Wyman et al, 2005; Ingram et al, 2011) it is generally accepted that a higher solid concentration will be required to make the overall biomass-to-ethanol process economically viable (Kristensen et al, 2009; Wingren et al, 2003). For example previous studies (Stenberg et al, 2000; Wingren et al, 2003) have suggested that the increased glucose concentration obtained by increasing the solids content from 5% to 8% (w/w) can reduce the total ethanol production costs by as much as 20%. Therefore, the susceptibility of the OSLPP-LS and OSPOP-LS substrates to cellulolytic hydrolysis at higher (10% w/v) solids content was next investigated.

It was evident that at cellulase loadings of 5 FPU g\(^{-1}\) cellulose, increasing the solids content decreased the extent of cellulose conversion for both substrates (Figure 16). These results were expected since it is well know that at low solids content, cellulose fibres are suspended in free water and mix with relative ease, while increasing the solids content above 8% (w/w) results in increased fibre-fibre interactions, which prevent effective mixing and limit the accessibility of cellulose to water and cellulases (Duffy and Titchener, 1975; Hodge et al, 2008; Kristensen et al, 2009; Zhang et al, 2009). However, the OSLPP-LS substrate appeared to be relatively less affected by the increased solids content than was the OSPOP-LS substrate. Specifically, in the case of the OSLPP-LS substrate, increasing the solids content from 2% to 10% decreased the 72 h cellulose conversion yields from 68% to 61% corresponding to an 11% decrease. On the other hand, the 72 h cellulose hydrolysis yield for
the OSPOP-LS substrate decreased from 89 % to 68 % (Figure 16), which corresponded to a decrease in cellulose conversion of approximately 24 %.

![Graph showing cellulose conversion](image_url)

**Figure 16.** Comparison of the 72 h cellulose hydrolysis yields of organosolv-pretreated lodgepole pine and hybrid poplar at 2 and 10 % (w/v) solids content and 5 FPU g\(^{-1}\) cellulose before and after removing the inhibitory effect of the residual lignin. Solid bars: 2 % solids, hatched bars: 10 % solids. Hydrolysis conditions: 50 mM Na-acetate buffer, 50 °C, 150 rpm. Error bars indicate the standard deviation (n = 3).

These results were unexpected as the OSPOP-LS substrate was more susceptible to hydrolysis at 2 % solids content than was the OSLPP-LS substrate. However, the fact that the OSPOP-LS substrate had a greater hemicellulose content and higher cellulose DP than did the OSLPP-LS substrate (Tables 7 and 9) was the likely explanation for these apparently contradictory results. With regards to the residual hemicelluloses, as mentioned in section 3.1.5, it is unlikely that they presented a physical barrier to the accessibility of cellulose to cellulases. However, it is well known that residual hemicelluloses improve paper strength by increasing fibre flexibility and promoting interfibre hydrogen bonds during the dewatering process (Roberts, 1996; Peel, 1999; Helmerius et al, 2010). Therefore it is possible that,
when the solids content was increased, the interfibre hydrogen bonds promoted by the residual hemicelluloses contributed to the decrease in mixing and to the greater decrease in 72 h cellulose conversion.

It is also possible that the higher cellulose DP of the OSPOP-LS substrate contributed to its greater recalcitrance at higher solids content by restricting the accessibility to cellulases via the formation of fibril networks held together through interchain hydrogen bonds (Puri, 1984) while having a relatively minor effect at lower solids content. In contrast, because of its significantly lower cellulose DP and hemicellulose content, it is possible that increasing the solids content from 2 % to 10 % did not have as great an impact on the accessibility to cellulases (and therefore the ease of enzymatic hydrolysis) of the OSLPP-LS substrate. However, as the large differences in the lignin content of the OSLPP-LS and OSPOP-LS substrates complicates this comparison, the substrates were delignified prior to investigating the differences in their susceptibility to cellulosic hydrolysis.

It was apparent that complete delignification improved the 72 h cellulose conversion yields of both substrates. However in the case of the hybrid poplar (OSPOP-LS\textsubscript{DL}) substrate, the improvements were relatively minor (from 68 % to 74 %, Figure 16). These results were in line with the previous observation that at a 2 % substrate concentration, the residual lignin did not appear to influence the susceptibility of the OSPOP-LS substrate to cellulosic hydrolysis (or the accessibility of its cellulose component to cellulases). In contrast, the delignified lodgepole pine substrate (OSLPP-LS\textsubscript{DL}) showed a substantial improvement in its 72 h cellulose hydrolysis yields (from 61 % to 84 %, Figure 16) and (as previously discussed) a significant increase in cellulose accessibility to cellulases. These results support the suggestion that at increased solids content the higher cellulose DP and hemicellulose
content of the OSPOP-LS substrate restrict its effective mixing. This in turn decreased its susceptibility to enzymatic hydrolysis by restricting the accessibility of the cellulose component. On the other hand (and similar to the results observed at low solids content), the main factor limiting the enzymatic hydrolysis of the OSLPP-LS substrate (and possibly other organosolv-pretreated softwoods) at higher solids content appeared to be its residual lignin.

3.1.7 Conclusions

This study compared the effect of pretreatment severity on the physicochemical properties and susceptibility to cellulosic hydrolysis of organosolv-pretreated lodgepole pine and hybrid poplar (as representatives of softwood and hardwood biomass respectively) with a particular focus on the inhibitory effect of the residual lignin. Both feedstocks produced substrates that were readily hydrolyzed at relatively low (7.5 FPU g\(^{-1}\) cellulose) enzyme loadings. However, the hybrid poplar substrate displayed a greater delignification selectivity and lower recalcitrance at low solids content than did the lodgepole pine, likely due to the differences in the structure and content of lignin between softwoods and hardwoods.

Although the changes in pretreatment severity did not affect the physicochemical properties or susceptibility of organosolv pretreated lodgepole pine substrates to enzymatic hydrolysis, the hybrid poplar became more recalcitrant as pretreatment severity increased. This increase in recalcitrance was likely due to non-productive interactions between lignin and cellulases caused by the increased condensation and hydrophobicity of the residual lignin. In contrast, the residual lignin did not appear to affect the enzymatic hydrolysis of hybrid poplar pretreated at low severity. In the case of the lodgepole pine substrates, the residual lignin inhibited cellulosic hydrolysis through a combination of non-productive
interactions between lignin and cellulases and a decrease in the accessibility of cellulose to cellulases. Removing the inhibitory effect of lignin rendered the lodgepole pine substrates more susceptible to cellulytic hydrolysis (at low and high solids content) than the hybrid poplar substrates.
3.2 The Effect of Varying Pretreatment Chemicals on the Physicochemical Properties and Enzymatic Hydrolysis of Organosolv-Pretreated Lodgepole Pine

3.2.1 Background

As discussed in section 3.1, the susceptibility of organosolv-pretreated lodgepole pine to enzymatic hydrolysis appeared to be greatly influenced by the amount and nature of the residual lignin. The lignin inhibitory mechanism likely involved a combination of it restricting the accessibility of the cellulose to cellulases and the non-productive adsorption of the enzymes onto the residual lignin. It was also found that (as expected due to the greater accessibility of its cellulose component and ~50 % lower lignin content) when pretreated at low severity, organosolv-pretreated hybrid poplar was more readily hydrolyzed than was lodgepole pine. Moreover, the residual lignin remaining in the (low-severity) pretreated hybrid poplar did not appear to significantly influence its susceptibility to cellulolytic hydrolysis. Interestingly, lignin removal rendered the lodgepole pine substrates more susceptible to enzymatic hydrolysis than were the hybrid poplar substrates. This observation was more apparent when the solids content was increased from 2 % to 10 % (w/v). This strongly suggested that softwoods could be viable feedstock’s for the generation of second generation bioethanol. However, because complete delignification is likely not economically viable, we next focused on trying to elucidate the role that other substrate properties might play on influencing the susceptibility of organosolv-pretreated softwoods to enzymatic hydrolysis.

Previous research, which used mountain pine beetle-killed lodgepole pine (Pan et al, 2007) as a feedstock for organosolv pretreatment, showed that varying pretreatment variables (i.e. reaction time, temperature, solvent concentration, and catalyst concentration) resulted in
substrates with varying extents of delignification (from 5 % to 23 % residual lignin) and susceptibilities to enzymatic hydrolysis (from 35 % to 100 % cellulose conversion within 48 h). However, it was also found that the conditions required to obtain a substrate highly susceptible to enzymatic hydrolysis also resulted in lower sugar recoveries, most likely due to excessive carbohydrate degradation to furfural, 5-hydroxymethyl furfural (HMF), levulinic, and formic acids (Pan et al, 2007). It was also evident that the residual lignin content of a given substrate did not always correlate with its susceptibility to enzymatic hydrolysis.

Although previous work had provided us with valuable information with regard to process conditions, it should be noted that these earlier studies only used a single solvent (ethanol) and catalyst (H$_2$SO$_4$). Therefore, the more detailed study described below was undertaken with the objective of varying both the chemicals and solvent utilized during organosolv pretreatment of mountain-pine beetle-killed lodgepole pine. It was hypothesized that, by varying the pretreatment solvent and chemical additives, this would result in a range of physicochemical properties of the solid and liquid fractions and a range of susceptibilities to enzymatic hydrolysis at low cellulase loadings (2.5-10 FPU g$^{-1}$ cellulose). It was also hypothesized that it would be possible to correlate the differences in susceptibility to enzymatic hydrolysis to the various substrate properties.

### 3.2.2 Effect of Pretreatment Chemicals on the Chemical Composition of the Water-Soluble and Water-Insoluble Fractions

Organosolv pretreatments were performed as described previously (Ghose et al, 1983; Sahin, 2003; Yawalata and Paszner, 2004; Pan et al, 2007) utilizing both ethanol and butanol (EtOH and BuOH respectively) as solvents and acidic (H$_2$SO$_4$ and SO$_2$), neutral (MgCl$_2$),
and alkaline (NaOH) chemical additives (Table 4). The initial compositional analysis indicated that the different chemicals resulted in substrates with varying cellulose and hemicellulose contents (Table 13). For example, the amount of glucan ranged from 64.0 % to 77.3 % (NaOH EtOH and NAEM30 BuOH respectively) while the total hemicellulose content ranged from 1.6 % to 15.4 % (SO₂ BuOH and NaOH EtOH respectively, Table 13). However, the amount of lignin in the solid fraction was quite similar in the majority of the substrates (approximately 17 %) with the exception of substrates generated under alkaline conditions (NaOH EtOH, and NaOH BuOH), which contained 10.9 % and 7.5 % lignin, respectively (Table 13). This was expected since under alkaline conditions the hydrolysis of glycosidic bonds proceeds at a slower rate than do delignification reactions when compared to neutral and acidic conditions (Sjöström, 1993).

**Table 13.** Chemical composition (% w/w) and solids yield of mountain pine beetle-killed lodgepole Pine substrates generated by organosolv pretreatment, numbers in parentheses indicate the standard deviation (n = 3).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Glucan</th>
<th>Total hemicelluloses&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Lignin&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Solids yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAEM60 EtOH</td>
<td>72.5 (0.7)</td>
<td>5.5 (0.1)</td>
<td>18.1 (1.6)</td>
<td>45.1</td>
</tr>
<tr>
<td>NAEM30 EtOH</td>
<td>67.4 (1.2)</td>
<td>5.3 (0.1)</td>
<td>19.6 (3.7)</td>
<td>49.1</td>
</tr>
<tr>
<td>H₂SO₄ EtOH</td>
<td>74.8 (1.1)</td>
<td>3.6 (0.1)</td>
<td>17.6 (0.7)</td>
<td>43.5</td>
</tr>
<tr>
<td>SO₂ EtOH</td>
<td>73.8 (0.3)</td>
<td>3.9 (0.0)</td>
<td>18.6 (0.2)</td>
<td>43.9</td>
</tr>
<tr>
<td>NaOH EtOH</td>
<td>64.0 (2.8)</td>
<td>15.4 (0.3)</td>
<td>10.9 (0.9)</td>
<td>45.3</td>
</tr>
<tr>
<td>NAEM60 BuOH</td>
<td>75.0 (0.5)</td>
<td>2.1 (0.0)</td>
<td>18.5 (1.7)</td>
<td>46.2</td>
</tr>
<tr>
<td>NAEM30 BuOH</td>
<td>77.3 (1.3)</td>
<td>2.4 (0.2)</td>
<td>17.8 (0.4)</td>
<td>47.4</td>
</tr>
<tr>
<td>H₂SO₄ BuOH</td>
<td>74.6 (1.4)</td>
<td>2.4 (0.1)</td>
<td>18.3 (1.3)</td>
<td>45.0</td>
</tr>
<tr>
<td>SO₂ BuOH</td>
<td>77.3 (1.4)</td>
<td>1.6 (0.1)</td>
<td>17.5 (0.5)</td>
<td>44.5</td>
</tr>
<tr>
<td>NaOH BuOH</td>
<td>70.0 (0.7)</td>
<td>13.9 (0.0)</td>
<td>7.5 (1.5)</td>
<td>44.6</td>
</tr>
</tbody>
</table>

<sup>a</sup>Total hemicelluloses = Xylan + Arabinan + Mannan + Galactan. <sup>b</sup>Total lignin = AIL + ASL.

It was expected that the differences in the chemical composition of the solid fractions would be reflected in those of the liquid fraction (Tables 14 and 15). The water-soluble fraction was obtained following the precipitation of lignin from the spent pulping liquor (Pan et al, 2005). The water-soluble fractions from ethanol organosolv pulping of hardwoods have
been shown to be composed of mainly cellulose and hemicellulose-derived sugars (in mono- and oligosaccharide form), sugar degradation product such as 5-hydroxymethylfurfural (HMF) and furfural, and low MW (i.e. water soluble) lignin fragments (Lora et al, 1991). Our results were similar to previous work in that the pretreatment resulted in significant amounts of mono- and oligosaccharides in the liquid fraction. In contrast, the sugar concentrations in the liquid fractions of substrates generated under alkaline conditions were significantly lower (Tables 14 and 15). This was expected as acidic and neutral pretreatments result in the hydrolysis of the hemicellulosic component (Nguyen et al, 2000). It was apparent that for the most part, more than half the sugars in the water-soluble fraction were in an oligomeric form. This is of significance as hexose monomers (glucose, galactose, and mannose) are readily fermentable and can be used for ethanol production (Robinson et al, 2003). In contrast, oligosaccharides need to be further hydrolyzed to their monomeric components for further ethanol production (Shevchenko et al, 2000) or could be used to generate higher value co-products such as animal feed additives (Davis et al, 2002; Fernandez et al, 2002). It should also be noted that the pretreatments that used butanol yielded higher concentrations of sugars in the aqueous fractions. This was also expected since the volume of the aqueous layer in the butanol pretreatments was approximately 20% of the volume of the ethanol pretreated substrates due to the limited miscibility between butanol and water. Therefore, especially in the case of the pretreatments with H₂SO₄, the utilization of butanol resulted in relegation of the sugars to the limited aqueous layer resulting in their higher concentration. Since previous work showed that the H₂SO₄ catalyzed ethanol pretreatment of LPP resulted in significant sugar degradation, we next measured the formation of both furfural and HMF.
Table 14. Oligomeric sugar composition (mg ml\(^{-1}\)) of the water soluble fractions obtained from organosolv pretreatment of lodgepole pine wood chips, numbers in parentheses indicate the standard deviation (n = 3).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Glucan</th>
<th>Xylan</th>
<th>Arabinan</th>
<th>Mannan</th>
<th>Galactan</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAEM60 EtOH</td>
<td>bdl</td>
<td>bdl</td>
<td>bdl</td>
<td>0.4 (0.0)</td>
<td>bdl</td>
</tr>
<tr>
<td>NAEM30 EtOH</td>
<td>bdl</td>
<td>bdl</td>
<td>bdl</td>
<td>0.4 (0.0)</td>
<td>bdl</td>
</tr>
<tr>
<td>H(_2)SO(_4) EtOH</td>
<td>0.3 (0.0)</td>
<td>1.1 (0.1)</td>
<td>0.4 (0.0)</td>
<td>2.0 (0.1)</td>
<td>0.7 (0.0)</td>
</tr>
<tr>
<td>SO(_2) EtOH</td>
<td>0.9 (0.0)</td>
<td>1.0 (0.0)</td>
<td>0.3 (0.0)</td>
<td>1.3 (0.0)</td>
<td>0.5 (0.0)</td>
</tr>
<tr>
<td>NaOH EtOH</td>
<td>bdl</td>
<td>0.1 (0.0)</td>
<td>0.3 (0.0)</td>
<td>bdl</td>
<td>0.6 (0.0)</td>
</tr>
<tr>
<td>NAEM60 BuOH</td>
<td>0.3 (0.0)</td>
<td>0.1 (0.0)</td>
<td>bdl</td>
<td>1.1 (0.0)</td>
<td>0.2 (0.0)</td>
</tr>
<tr>
<td>NAEM30 BuOH</td>
<td>bdl</td>
<td>0.1 (0.0)</td>
<td>bdl</td>
<td>0.7 (0.0)</td>
<td>0.1 (0.0)</td>
</tr>
<tr>
<td>H(_2)SO(_4) BuOH</td>
<td>7.4 (0.1)</td>
<td>5.0 (0.0)</td>
<td>1.6 (0.1)</td>
<td>10.1 (0.1)</td>
<td>3.5 (0.0)</td>
</tr>
<tr>
<td>SO(_2) BuOH</td>
<td>7.8 (0.0)</td>
<td>1.7 (0.0)</td>
<td>0.7 (0.0)</td>
<td>4.7 (0.0)</td>
<td>2.0 (0.0)</td>
</tr>
<tr>
<td>NaOH BuOH</td>
<td>bdl</td>
<td>1.0 (0.0)</td>
<td>0.8 (0.0)</td>
<td>0.2 (0.0)</td>
<td>1.7 (0.0)</td>
</tr>
</tbody>
</table>

Table 15. Monomeric sugar composition (mg ml\(^{-1}\)) of the water soluble fractions obtained from organosolv-pretreated lodgepole pine wood chips, numbers in parentheses indicate the standard deviation (n = 3).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Glucose</th>
<th>Xylose</th>
<th>Arabinose</th>
<th>Mannose</th>
<th>Galactose</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAEM60 EtOH</td>
<td>bdl</td>
<td>0.1 (0.0)</td>
<td>bdl</td>
<td>0.2 (0.0)</td>
<td>bdl</td>
</tr>
<tr>
<td>NAEM30 EtOH</td>
<td>bdl</td>
<td>0.1 (0.0)</td>
<td>bdl</td>
<td>0.2 (0.0)</td>
<td>0.1 (0.0)</td>
</tr>
<tr>
<td>H(_2)SO(_4) EtOH</td>
<td>1.1 (0.0)</td>
<td>1.4 (0.0)</td>
<td>0.4 (0.0)</td>
<td>2.1 (0.1)</td>
<td>0.7 (0.0)</td>
</tr>
<tr>
<td>SO(_2) EtOH</td>
<td>1.8 (0.1)</td>
<td>0.9 (0.1)</td>
<td>0.3 (0.0)</td>
<td>1.3 (0.1)</td>
<td>0.5 (0.0)</td>
</tr>
<tr>
<td>NaOH EtOH</td>
<td>bdl</td>
<td>bdl</td>
<td>bdl</td>
<td>bdl</td>
<td>bdl</td>
</tr>
<tr>
<td>NAEM60 BuOH</td>
<td>0.3 (0.0)</td>
<td>0.2 (0.0)</td>
<td>bdl</td>
<td>0.4 (0.0)</td>
<td>0.1 (0.0)</td>
</tr>
<tr>
<td>NAEM30 BuOH</td>
<td>0.1 (0.0)</td>
<td>0.1 (0.0)</td>
<td>bdl</td>
<td>0.2 (0.0)</td>
<td>bdl</td>
</tr>
<tr>
<td>H(_2)SO(_4) BuOH</td>
<td>6.6 (0.2)</td>
<td>5.3 (0.2)</td>
<td>1.5 (0.1)</td>
<td>8.1 (0.3)</td>
<td>2.9 (0.1)</td>
</tr>
<tr>
<td>SO(_2) BuOH</td>
<td>7.0 (0.3)</td>
<td>2.7 (0.1)</td>
<td>0.8 (0.0)</td>
<td>4.0 (0.2)</td>
<td>1.7 (0.1)</td>
</tr>
<tr>
<td>NaOH BuOH</td>
<td>bdl</td>
<td>bdl</td>
<td>bdl</td>
<td>bdl</td>
<td>bdl</td>
</tr>
</tbody>
</table>

Furfural and HMF are generated by the thermal decomposition of pentoses and hexoses under acidic and neutral conditions (Sjöström, 1993). The furfural and HMF concentrations were quite variable ranging from 0.3 to 1.0 mg ml\(^{-1}\) and 0.8 to 2.5 mg ml\(^{-1}\) for HMF and furfural, respectively (Table 16). It was apparent that pretreatment combining the NAEM salts with the ethanol solvent (NAEM60 EtOH and NAEM30 EtOH) resulted in a considerably higher concentration of furfural (2.2 and 2.5 mg ml\(^{-1}\), respectively) and a significantly lower concentration of pentoses in the liquid fraction. The increased level of furfural during the NAEM60 EtOH and NAEM30 EtOH pretreatments was likely caused by
the higher temperatures required for NAEM pretreatment (200 and 205 °C for NAEM60 and NAEM30, respectively) compared to the 170 °C utilized for the other pretreatments and the fact that pretreatment with NAEM salts is actually acidic (pH ~ 4.2).

Table 16. Concentration of sugar degradation products (mg ml\(^{-1}\)) of the water soluble fractions obtained from organosolv-pretreated lodgepole pine wood chips, numbers in parentheses indicate the standard deviation (n = 3).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Furfural</th>
<th>HMF</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAEM60 EtOH</td>
<td>2.2 (0.1)</td>
<td>0.7 (0.0)</td>
</tr>
<tr>
<td>NAEM30 EtOH</td>
<td>2.5 (0.1)</td>
<td>0.8 (0.0)</td>
</tr>
<tr>
<td>H(_2)SO(_4) EtOH</td>
<td>1.3 (0.0)</td>
<td>1.0 (0.0)</td>
</tr>
<tr>
<td>SO(_2) EtOH</td>
<td>0.8 (0.1)</td>
<td>0.8 (0.1)</td>
</tr>
<tr>
<td>NaOH EtOH</td>
<td>bdl</td>
<td>bdl</td>
</tr>
<tr>
<td>NAEM60 BuOH</td>
<td>1.3 (0.0)</td>
<td>0.3 (0.0)</td>
</tr>
<tr>
<td>NAEM30 BuOH</td>
<td>1.6 (0.1)</td>
<td>0.3 (0.0)</td>
</tr>
<tr>
<td>H(_2)SO(_4) BuOH</td>
<td>1.0 (0.1)</td>
<td>0.3 (0.0)</td>
</tr>
<tr>
<td>SO(_2) BuOH</td>
<td>1.8 (0.1)</td>
<td>0.5 (0.0)</td>
</tr>
<tr>
<td>NaOH BuOH</td>
<td>bdl</td>
<td>bdl</td>
</tr>
</tbody>
</table>

In contrast, (with the exception of the substrate generated with SO\(_2\)) all the pretreatments that utilized butanol resulted in lower furfural and HMF concentrations than those generated with ethanol (Table 16), which was indicative of lower levels of sugar degradation despite being carried out at the same temperatures. This was likely due to a combination of decreased acidity of the chemical environment as a result of the greater hydrophobicity of butanol as well as its ability to absorb a greater amount of heat than ethanol as evidenced by its higher specific heat capacity (111.46 vs. 176.86 J mol\(^{-1}\)K\(^{-1}\) for ethanol and butanol, respectively). With regards to the SO\(_2\) BuOH substrate, it is possible that the higher furfural concentration was due in part to the more thorough penetration of the wood chips by the gaseous SO\(_2\) since, these samples were impregnated 12 hours prior to pretreatment (Ewanick et al, 2007). Indeed, previous work by Eklund et al, (1995) showed that impregnation of Willow chips with H\(_2\)SO\(_4\) resulted in higher xylose recovery whereas
impregnation with SO₂ increased pentose degradation. Since it was apparent that the changes in the pretreatment solvent and chemical additives had a significant effect on the chemical composition of the liquid stream and the solid fraction, we next assessed whether the pretreatment parameters affected both physical properties and consequently the ease of saccharification of the resulting substrate by cellulases.

3.2.3 Effect of Pretreatment Chemicals on the Physicochemical Properties and Enzymatic Hydrolysis of the Solid Substrates

The enzymatic hydrolysis of the ten organosolv substrates was carried out at 2% solids (w/v), using low enzyme loadings (2.5, 5, and 10 FPU g⁻¹ cellulose) and the reaction was monitored over 72 h (Figure 17). It was hoped that by employing low enzyme loadings (2.5 and 5 FPU g⁻¹ cellulose) differences in the substrate’s ease of hydrolysis would be apparent, since higher dosages may mask differences between the substrates by saturating the substrate with enzyme. Furthermore, as enzymatic hydrolysis is one of the economic barriers for the commercialization of the bioconversion process (Merino and Cherry, 2007), it would be advantageous to identify substrates that hydrolyze at low enzyme dosages.

The hydrolytic potential for these substrates was assessed by measuring the amount of cellulose hydrolyzed to glucose during the first 12 h and is shown in Figure 17A. This time point was chosen from the enzymatic hydrolysis profiles because the differences in the extent of cellulose conversion were the more noticeable at this point compared to 72 h (Figure 17B) and 12 h was within the linear portion of the hydrolysis curve when using enzyme loadings of 10 FPU g⁻¹ cellulose. Significant differences were observed in the extent of hydrolysis (at 10 FPU g⁻¹ cellulose) after 12 h ranging from 21% (NaOH EtOH) to 82% (SO₂ BuOH) (Figure 17A). Substrates generated with butanol as the solvent hydrolyzed at a faster rate and
Figure 17. The effect of varying pretreatment additives and solvent on the cellulose conversion of organosolv-pretreated lodgepole pine after 12 (A) and 72 hours (B). Substrates were resuspended at 2% (w/v) solids content in 50 mM Na-acetate. Hydrolysis conditions: 50 °C, 150 rpm. Error bars indicate the standard deviation (n = 3).
to a greater extent than did those that were generated with ethanol. However, the differences in hydrolysis yields were less pronounced at enzyme loadings of 5 FPU g$^{-1}$ cellulose and virtually indistinguishable at 2.5 FPU g$^{-1}$ cellulose. A possible explanation for the lack of effectiveness at the lower enzyme loadings may be due to the non-productive binding of the cellulases to the lignin component of the substrate. Previous work has shown that cellulases bind to lignin via hydrophobic interactions or interactions between phenolic groups or a combination of both (Sewalt et al, 1997; Berlin et al, 2006b). However, it should be pointed out that, in the current study, the substrates that possessed the lowest amount of lignin (NaOH EtOH and NaOH BuOH, 10.9 % and 7.6 %, respectively) were also the least susceptible towards cellulolytic hydrolysis (21% and 34% 12 h cellulose hydrolysis yields at 10 FPU g$^{-1}$ cellulose, respectively, Figure 18A). This was in agreement with previous studies (Wong et al, 1988; Schell et al, 1998), which showed that partial delignification of steam-pretreated softwoods by alkaline post-treatment resulted in decreased hydrolysis yields. This decrease in hydrolysis yield has been attributed to redeposition of lignin on enzyme-accessible pores and cellulose surfaces (Wong et al, 1987). Moreover, Ishizawa et al, (2009) recently showed that complete delignification and removal of xylan from acid-pretreated corn stover also resulted in decreased hydrolysis yields. However, this particular study attributed the observed decrease in cellulose digestibility to the aggregation of cellulose microfibrils, resulting in decreased cellulase accessibility.

In addition to assessing the effect of the total lignin content, the influence of the substrates’ surface composition on enzymatic hydrolysis was also investigated using X-ray photoelectron spectroscopy (XPS). This technique enables the analysis of the substrate surface to a depth of approximately 5–10 nm. As mentioned in section 3.1.5, early studies on
surface analysis of paper and wood samples with this technique showed that the resulting spectrum was mainly composed of carbon and oxygen peaks (Dorris and Gray, 1978a and b; Gray, 1978; Palonen and Viikari 2004) and that the carbon peak was actually composed of four peaks representing the four oxidation states of carbon. Using the oxygen/carbon ratio of the substrate surface, XPS was used to estimate the amount of lignin and carbohydrates on the substrate’s surface (Laine et al, 1994; Palonen and Viikari, 2004). In addition, quantification of carboxylic carbons can be used as an indicator of a substrate’s hydrophilicity and swelling (Grethlein and Converse, 1991) both of which have been implicated in a lignocellulosic substrate’s susceptibility to cellulolytic hydrolysis (Chandra et al, 2008a). The XPS data showed that the O/C ratio ranges from 0.33 to 0.50 (Table 17), suggesting that all of the substrates’ surfaces are very rich in lignin as the theoretical O/C ratios are 0.33 for pure lignin and 0.83 for pure cellulose (Laine et al, 1994). The theoretical surface lignin coverage (TSLC) was also shown to range from 66% to 99%. These results were in agreement with those published earlier by Palonen and Viikari (2004) who used XPS to determine the surface composition of a steam-pretreated softwood mixture before and after treatment with cellulas and a laccase–cellulase mixture. This previous work showed that the proportion of lignin at the substrate surface was significantly higher than the lignin distributed throughout the substrate (66 % for the TSLC and 51% for lignin distributed throughout the substrate). In our own work we were unable to determine a relationship between ease of enzymatic hydrolysis and either the O/C ratio or the TSLC. However, our results indicate that, in agreement with the compositional data (Table 13), the NaOH EtOH and NaOH BuOH substrates displayed the lowest amount of surface lignin (O/C ratio 0.48 and 0.50 and TSLC 66% and 70%, respectively). We were also unable to detect any surface
carboxyl groups using this technique, suggesting that at the applied conditions, organosolv pretreatment did not promote the formation of carboxylic acids. Although the differences in the substrates’ susceptibility to enzymatic hydrolysis did not seem to be related to the surface/total lignin content, it is likely that this was due to the amount of lignin in eight of the ten substrates being quite similar with the exception of the substrates generated under alkaline conditions (Table 13). Thus, it is likely that the observed differences in hydrolysis yields were due to variations in other physicochemical properties, such as hemicellulose content, accessibility of cellulose to cellulases, and cellulose degree of polymerization (DP).

**Table 17.** Effects of varying pretreatment chemicals on the surface characteristics of organosolv-pretreated lodgepole pine substrates.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>C1 (%)</th>
<th>C2 (%)</th>
<th>C3 (%)</th>
<th>O/C</th>
<th>TSLCb (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAEM60 EtOH</td>
<td>66</td>
<td>31</td>
<td>3</td>
<td>ND</td>
<td>0.40</td>
</tr>
<tr>
<td>NAEM30 EtOH</td>
<td>68</td>
<td>29</td>
<td>3</td>
<td>ND</td>
<td>0.41</td>
</tr>
<tr>
<td>H₂SO₄ EtOH</td>
<td>69</td>
<td>28</td>
<td>3</td>
<td>ND</td>
<td>0.40</td>
</tr>
<tr>
<td>SO₂ EtOH</td>
<td>63</td>
<td>33</td>
<td>4</td>
<td>ND</td>
<td>0.40</td>
</tr>
<tr>
<td>NaOH EtOH</td>
<td>67</td>
<td>28</td>
<td>5</td>
<td>ND</td>
<td>0.48</td>
</tr>
<tr>
<td>NAEM60 BuOH</td>
<td>68</td>
<td>30</td>
<td>2</td>
<td>ND</td>
<td>0.35</td>
</tr>
<tr>
<td>NAEM30 BuOH</td>
<td>71</td>
<td>27</td>
<td>2</td>
<td>ND</td>
<td>0.34</td>
</tr>
<tr>
<td>H₂SO₄ BuOH</td>
<td>66</td>
<td>31</td>
<td>3</td>
<td>ND</td>
<td>0.38</td>
</tr>
<tr>
<td>SO₂ BuOH</td>
<td>70</td>
<td>28</td>
<td>2</td>
<td>ND</td>
<td>0.33</td>
</tr>
<tr>
<td>NaOH BuOH</td>
<td>57</td>
<td>36</td>
<td>7</td>
<td>ND</td>
<td>0.50</td>
</tr>
</tbody>
</table>

C1 = unoxidized carbon (C-C, C-H), C2 = carbon with one oxygen bond (C-O), C3 = carbon with two oxygen bonds (O-C-O or C=O), C4 = carbon with three oxygen bonds (O=C=O), ND = not detected. b TSLC = theoretical surface lignin coverage.

Similar to lignin, it has been shown that the residual hemicelluloses can act as a physical barrier restricting the accessibility of cellulose to cellulases (Boussaid et al, 2000; Fernandez-Bolanos et al, 2001). Our results indicate an inverse relationship between the 12 h cellulose conversion yields and the amount of hemicellulose present in the solid fraction (Figure 18B). This is in agreement with previous work, which showed that the removal of hemicelluloses during steam- and dilute acid pretreatment of softwoods increased their pore
Figure 18. Relationship between ease of enzymatic hydrolysis of organosolv-pretreated lodgepole pine substrates generated by varying the chemical additives and the solvent used during pretreatment and residual lignin (A) and hemicelluloses (B). Enzyme loading: 10 FPUg\(^{-1}\) cellulose.
volume, which in turn increased their ease of cellulolytic hydrolysis (Grethlein et al, 1984; Grethlein, 1985). Similarly, a more recent study by Mussatto et al, (2008) showed that removing 86.5% of the hemicelluloses and only 14% of the lignin present in brewer’s spent grain resulted in a 3.5-fold increase in the rate of cellulose hydrolysis, which was attributed to the increased accessibility of cellulose to cellulases. In contrast, Kumar and Wyman (2009) did not find a significant correlation between cellulose accessibility and lignin/xylan removal. However, unlike lignin, studies evaluating the effect of hemicelluloses on enzymatic hydrolysis have been less frequent (Chandra et al, 2007). This is likely due to the fact that hemicelluloses are highly sensitive to pretreatment conditions and vary widely in content and composition between different feedstocks. Recognizing the possible influence of hemicelluloses, Berlin et al, (2007) supplemented cellulase preparations with a variety of hemicellulose-degrading enzymes. This resulted in an increase in the rate of hydrolysis of dilute acid-pretreated corn stover and a twofold reduction in the amount of protein required to hydrolyze cellulose and xylan. Therefore, it is possible that supplementation of the substrates that were generated under neutral and alkaline conditions with suitable hemicellulases could improve their susceptibility to cellulolytic hydrolysis and overall fermentable sugar yield.

In addition to the chemical composition, physical properties, such as surface area, cellulose DP, and swelling, have been implicated in the substrate’s inherent recalcitrance to enzymatic hydrolysis (Mansfield et al, 1999). The surface area of pulp fibres can be further separated into the exterior surface area, which is determined by fibre dimensions such as length and width and interior surface area determined by pore volume, fissures, and micro-cracks (Chandra et al, 2007). Our results show an inverse relationship between initial fibre
length and the 12 h cellulose hydrolysis yields using 10 FPU g⁻¹ cellulose (Figure 19A). It should also be noted that, in general, the substrates generated under alkaline conditions (NaOH EtOH and NaOH BuOH) exhibited much larger fibre lengths (2.28 and 2.23 mm, respectively, Table 18) than those generated under acidic conditions. This increase in fibre length was most likely due to the greater efficiency of lignin removal and reduced fibre damage under alkaline conditions (which are two of the reasons alkaline processes are used in producing pulps for papermaking). It was also apparent that the substrates generated with butanol resulted in pulps with shorter fibres than did those generated with ethanol. This was likely caused by the limited miscibility of butanol and water resulting in the catalysts being allocated to the aqueous layer, thus increasing their active concentration and resulting in an increased hydrolysis of the cellulose. Similarly, Pan et al, (2007) showed that increasing the severity of the pretreatment conditions by increasing catalyst dosage during organosolv pretreatment of poplar chips led to a decrease in the average fibre size, which was attributed to possible chemical cutting of the fibres as shown using electron microscopy.

Table 18. Effect of varying pretreatment chemicals on the cellulose degree of polymerization (DP), fibre length (L_w) and water retention value, numbers in parentheses indicate the standard deviation (n = 3).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>DP_W</th>
<th>DP_N</th>
<th>PI</th>
<th>L_w (mm)</th>
<th>WRV</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAEM60 EtOH</td>
<td>1440 (31)</td>
<td>370 (4)</td>
<td>3.89</td>
<td>2.05 (0.02)</td>
<td>2.8 (0.3)</td>
</tr>
<tr>
<td>NAEM30 EtOH</td>
<td>1790 (21)</td>
<td>356 (6)</td>
<td>5.03</td>
<td>1.64 (0.06)</td>
<td>2.7 (0.0)</td>
</tr>
<tr>
<td>H₂SO₄ EtOH</td>
<td>1062 (46)</td>
<td>323 (9)</td>
<td>3.29</td>
<td>1.57 (0.04)</td>
<td>2.9 (0.4)</td>
</tr>
<tr>
<td>SO₂ EtOH</td>
<td>1200 (23)</td>
<td>308 (3)</td>
<td>3.90</td>
<td>1.51 (0.04)</td>
<td>2.9 (0.3)</td>
</tr>
<tr>
<td>NaOH EtOH</td>
<td>1512 (23)</td>
<td>293 (6)</td>
<td>5.17</td>
<td>2.28 (0.17)</td>
<td>3.2 (0.0)</td>
</tr>
<tr>
<td>NAEM60 BuOH</td>
<td>884 (22)</td>
<td>349 (6)</td>
<td>2.43</td>
<td>1.33 (0.01)</td>
<td>3.2 (0.2)</td>
</tr>
<tr>
<td>NAEM30 BuOH</td>
<td>820 (30)</td>
<td>354 (8)</td>
<td>2.32</td>
<td>0.97 (0.05)</td>
<td>3.1 (0.2)</td>
</tr>
<tr>
<td>H₂SO₄ BuOH</td>
<td>1060 (56)</td>
<td>445 (18)</td>
<td>2.38</td>
<td>1.51 (0.02)</td>
<td>3.2 (0.2)</td>
</tr>
<tr>
<td>SO₂ BuOH</td>
<td>769 (20)</td>
<td>331 (5)</td>
<td>2.33</td>
<td>1.24 (0.02)</td>
<td>3.5 (0.1)</td>
</tr>
<tr>
<td>NaOH BuOH</td>
<td>2159 (87)</td>
<td>471 (11)</td>
<td>4.59</td>
<td>2.23 (0.01)</td>
<td>3.1 (0.0)</td>
</tr>
</tbody>
</table>

DP_W = weight average degree of polymerization, DP_N = number average degree of polymerization, PI = polydispersity index (DP_W/DP_N), WRV = water retention value.
The relationship between initial fibre length and enzymatic hydrolysis was expected since it is generally accepted that smaller particles are more susceptible to cellulolytic hydrolysis as a result of their greater surface area (Ford, 1983; Ramos et al, 1993). Previously Mooney et al, (1999) had shown that the larger fibres isolated from Douglas-fir kraft pulp were hydrolyzed at a slower rate and to a lesser extent than was the whole pulp containing the “fines” (fibres < 0.2 mm) and small fibres. This was likely due to the increased surface area of the whole pulp provided by the fines and small fibres. However, while exterior surface area plays an important role in the enzymatic hydrolysis of lignocellulosic substrates, the information obtained from these measurements is limited, as it does not account for the substrate’s topology and porosity (interior surface area). Consequently, further information can be obtained from the measurement of interior surface area available to cellulases as measured by the Simon’s stain (SS) technique (Chandra et al, 2008).

In the current study, SS measurements of the organosolv pulps indicated that the ratio of orange to blue dye adsorption was quite diverse, ranging from 1.46 in the case of the substrate generated with NaOH and ethanol (NaOH EtOH) to 4.60 for the substrate pretreated using the combination of SO₂ and butanol (SO₂ BuOH). This seemed to indicate that there was a wide distribution of different surface areas between the various pretreatments. It was evident that the ratio of DO/DB adsorption by each substrate was a strong indicator of its susceptibility to enzymatic hydrolysis (Figure 19B). This suggested that the ease of enzymatic hydrolysis was heavily dependent on the accessibility of cellulose to cellulases, (i.e. the relative proportion of large pores) as has been suggested previously (Grous et al, 1986; Mansfield et al, 1999; Shevchenko et al, 2000; Fernandez-Bolanos et al, 2001 Selig et al, 2007; Ishizawa et al, 2009). Earlier it was suggested that the threshold pore
Figure 19. Relationship between ease of enzymatic hydrolysis of organosolv-pretreated lodgepole pine substrates generated by varying the chemical additives and the solvent used during pretreatment and fibre length (A) and cellulose accessibility as determined by the Simons’ stain method (B). Enzyme loading: 10 FPU g⁻¹ cellulose.
size for effective hydrolysis of lignocellulosic substrates is 5.1 nm (Grethlein, 1985).

However, it should be pointed out that Mooney et al, (1998) showed that increased substrate porosity of Douglas-fir refiner mechanical pulps only resulted in enhanced hydrolysis when accompanied by a delignification step (from 27.3% to 8.2%). In addition, a more recent study by Ishizawa et al, (2007) failed to find a significant correlation between substrate porosity and ease of hydrolysis of dilute acid-pretreated corn stover generated under varying pretreatment severities. This suggests that, in addition to substrate porosity, other factors such as the content and location of residual lignin and hemicelluloses contribute to the overall accessibility of cellulose to cellulases. Of note, in this study the two substrates containing the highest amount of hemicellulose and the lowest amount of lignin (NaOH EtOH and NaOH BuOH, 15.4% and 13.9% hemicellulose, and 10.9% and 7.5% lignin, respectively, Table 13) also showed low ratios of orange to blue dye adsorption (1.46 and 2.45, respectively, Figure 19B), implying that hemicellulose may act as a physical barrier preventing the orange dye (and by extrapolation cellulases) from accessing the cellulose component of the substrate. Indeed, it was apparent that the ratio of orange to blue dye adsorption was inversely proportional to the amount of hemicelluloses (Figure 20).

Furthermore, the substrates generated by pretreatment with butanol exhibited a larger orange to blue dye adsorption ratio than those generated with ethanol possibly due to the enhanced hemicellulose removal during the butanol pretreatment.

In addition to the distribution of large and small pores, other physical properties such as swelling of lignocellulosic substrates in aqueous solutions and cellulose DP have been shown to have an effect on cellulolytic hydrolysis (Chandra et al, 2007 and 2008a). Swelling is influenced by several factors, including charged groups in the interior and exterior of the
Figure 20. Relationship between cellulose accessibility as determined by Simons’ stain and the amount of residual hemicellulose in the organosolv-pretreated lodgepole pine substrates.

fibres, the pH of the medium and the presence of electrolytes (Lindstrøm and Carlsson, 1982). A widespread method used to estimate the swelling of lignocellulosic substrates is the centrifugal water retention value, (WRV) (Chandra et al, 2008a). Previous work by Bendzalova et al, (1996) as well as Ogiwara and Arai (1968 and 1969) attributed increased cellulase performance to increased porosity caused by swelling of cellulosic fibres. They found a linear correlation between initial hydrolysis rates and WRV when comparing sulfite, sulfate and semi-chemical pulps. From the WRV measurements of the organosolv pulps, it was evident that, although differences in WRV were not significant when comparing substrates generated with the same solvent, the substrates generated with butanol displayed a greater WRV than those generated with ethanol (Table 18). However, a notable exception was the NaOH EtOH substrate, which possessed a WRV comparable to that of the butanol substrates. This is likely because NaOH can enhance the swelling of lignocellulosic
substrates (Ogiwara and Arai, 1968 and 1969). Although we were unable to find a relationship between the ease of hydrolysis and the WRV of a particular substrate, it is possible that the greater swelling of the substrates generated with butanol contributes to their enhanced hydrolysis as compared to those generated with ethanol.

As noted earlier, the degree of polymerization (DP) of the cellulose has also been shown to have an effect on cellulolytic hydrolysis. However, the exact role of cellulose DP on cellulolytic hydrolysis is still somewhat unclear as some studies have not shown a clear relationship between ease of hydrolysis and initial DP (Sinistyn et al, 1991; Nazhad et al, 1993; Zhang and Lynd, 2006a) while other studies have shown that substrates with low initial DP hydrolyzed more quickly and to a greater extent than those with high DP (Puri, 1984; Martinez et al, 1997; Pan et al, 2008). In the current study, the initial cellulose DP was measured by cellulose derivatization by the tricarbanylation method with subsequent molecular weight measurement employing gel permeation chromatography (GPC).

It was evident that the average molecular weight distribution for the ten substrates was highly diverse ranging from 769 (SO₂ BuOH) to 2159 (NaOH BuOH) (Table 18). Similar to fibre length, substrates generated under acidic conditions exhibited significantly lower cellulose DP than those generated under alkaline conditions (Table 18). This is likely caused by the acid-catalyzed hydrolysis of the cellulose chains (Sjöström, 1993). The substrates generated with butanol using acidic conditions and NAEM salts showed a significantly lower initial cellulose DP than did their ethanol counterparts. As mentioned earlier, the butanol pretreatments were likely more severe due to the limited miscibility of butanol and water increasing the effective concentration of the catalysts in the aqueous layer. This is in agreement with previous studies (Pan et al, 2006b; 2007; 2008), which showed that
increases in organosolv pretreatment severity led to significant decreases in cellulose DP as well as increased hydrolysis yields. In addition, it was evident that those substrates that had a lower cellulose DP tended to be hydrolyzed to a greater extent after 12 h (Figure 21), suggesting that the substrates’ initial DP is an important factor in the cellulolytic hydrolysis of organosolv pretreated MPB-LPP.

![Figure 21. Relationship between ease of enzymatic hydrolysis of organosolv-pretreated lodgepole pine substrates generated by varying the chemical additives and solvent used during pretreatment and cellulose degree of polymerization (DPw). Enzyme loading: 10 FPU g⁻¹ cellulose.](image)

**3.2.4 Conclusions**

Variations in the organosolv pretreatment conditions of MPB-LPP resulted in the generation of substrates with a range of chemical compositions. Substrates generated under acidic conditions were readily hydrolyzed despite more selective delignification resulting after alkaline pretreatment. This was most likely due to a combination of hemicellulose
removal and cellulose depolymerization. The use of butanol produced substrates that were more readily hydrolyzed compared to using ethanol as the solvent. This is likely caused by the higher severity encountered during butanol pretreatments due to the limited miscibility of butanol and water. This increased severity resulted in the generation of substrates with less hemicellulose, a smaller initial fibre length, increased ratio of large vs. small pores, lower initial DP and increased swelling. In addition to creating a substrate which was more amenable to subsequent hydrolysis, butanol pretreatments appears to minimize sugar degradation. These results suggest that, in addition to the substrate’s chemical composition, other factors such as the accessibility of cellulose to cellulases both internally (porosity, cracks, delamination) and externally (particle size, fibre length, fines content), have a significant effect on the susceptibility of the organosolv pretreated substrates to enzymatic hydrolysis.
3.3 The Effect of Fibre Length on the Enzymatic Hydrolysis of Organosolv-Pretreated Lodgepole Pine

3.3.1 Background

The work presented in section 3.2 showed a strong correlation between the initial fibre length and the susceptibility of organosolv pretreated substrates to enzymatic hydrolysis. These results are in general agreement with previous studies (Mooney et al, 1999; Pan et al, 2008; Yeh et al, 2010) which showed a good relationship between particle size and enzymatic hydrolysis. However, the interpretation of these previous studies has been somewhat inconclusive, likely due to differences in the methods used to change one of the substrates characteristics also influencing other substrate characteristics. For example, observed changes in fibre length resulting from the use of different pretreatment chemicals and solvents or increased pretreatment severity are often accompanied by changes in chemical composition, cellulose degree of polymerization (DP) and accessibility of cellulose to cellulases (Pan et al, 2008; Del Rio et al, 2010). Similarly, trying to reduce initial size via milling also decreases cellulose crystallinity (Yeh et al, 2010). Consequently, it is difficult to ascertain how each of these substrate characteristics independently and collectively influences recalcitrance.

3.3.2 Size-Fractionation and Characterization of Organosolv-Pretreated Lodgepole Pine

Fibre fractionation was carried out using a Bauer-Mcnett fibre classifier consisting of five chambers each fitted with a progressively smaller screen mesh that act as mechanical barriers to separate the fibres based on their length. The majority of the fibres (approximately 53%) were found in the R28 and R48 fractions, with 30% in the P200 fraction, while only
about 4% were retained in the R14 fraction (Table 19). In contrast, the majority (usually 50-60%) of the fibres present in softwood kraft pulp (the predominant pulping process used in the paper industry) are found in the R14 fraction with a very small amount of fibres (< 5%) found in the P200 fraction (Lapierre et al, 2009).

Table 19. Gross fibre characteristics of fractionated organosolv pretreated lodgepole pine., numbers in parentheses indicate the standard deviation (n = 3).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Percent of original substrate</th>
<th>Average Fibre length (mm)</th>
<th>Fines content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>numerical</td>
<td>length weighted</td>
</tr>
<tr>
<td>Unfractionated</td>
<td>100</td>
<td>0.53</td>
<td>1.71</td>
</tr>
<tr>
<td>R14</td>
<td>3.6 (0.1)</td>
<td>2.92</td>
<td>3.43</td>
</tr>
<tr>
<td>R28</td>
<td>41.6 (0.1)</td>
<td>2.26</td>
<td>2.92</td>
</tr>
<tr>
<td>R48</td>
<td>11.7 (0.5)</td>
<td>1.77</td>
<td>2.11</td>
</tr>
<tr>
<td>R100</td>
<td>8.7 (0.1)</td>
<td>0.84</td>
<td>1.00</td>
</tr>
<tr>
<td>R200</td>
<td>3.9 (0.4)</td>
<td>0.45</td>
<td>0.54</td>
</tr>
<tr>
<td>P200</td>
<td>30.4 (0.2)</td>
<td>0.12</td>
<td>0.20</td>
</tr>
</tbody>
</table>

The likely explanation for this difference in fibre distribution is that, unlike the kraft process which is performed using alkaline conditions, organosolv pretreatment is typically carried out under acidic conditions, which results in acid-catalyzed fibre damage and an increase in surface area (Pan et al, 2008). Although undesirable during the papermaking process (due to decreased paper strength), the increased surface area available to cellulases as a result of acid-catalyzed fibre damage is thought to be one the reasons why the organosolv process is effective in reducing the recalcitrance of softwoods (Pan et al, 2008).

The fibre length distribution of the fractionated organosolv substrates was measured with a fibre quality analyzer (FQA). As expected, the fibres’ retained in the R14 fraction were the longest (3.4 mm length weighted average fibre length, $L_w$) while the P200 fraction contained the smallest fibres (0.20 mm $L_w$) (Table 19). As was also expected, the numerical and length weighted fibre length ($L_n$ and $L_w$, respectively) values were similar for the
different fractions due to removal of the fines, which skew \( L_n \) towards a lower value. However, removal of the fines only has a minor effect on \( L_w \) since the fibre length values are squared thus increasing the influence of the larger fibres on the average fibre length (Table 19). Although the fractionated substrates showed substantial differences in their average fibre lengths, the fibre length distribution overlapped between adjacent fractions (i.e. R14 overlaps with R28 while R28 overlaps with R14 and R48, etc) (Figure 22). This overlap was likely caused by the fact that, although fibre separation in the Bauer-McNett classifier is primarily due to fibre length, fibre separation by flexibility also plays a significant role (Karnis, 1997). Therefore it is probable that some fibres that would typically belong in one fraction based on their size, would find their way to the adjacent fraction due to their ability to pass through a smaller mesh opening.

**Figure 22.** Fibre length distribution of unfractionated and R14 to R200 organosolv pretreated substrates. Inset: fibre length distribution of the P200 fraction.
In addition to fibre length, other factors that influence enzymatic hydrolysis of the cellulose component of pretreated lignocellulosic substrates include, the amount and location of lignin and hemicellulose, fibre swelling, accessibility to cellulas, crystallinity and degree of polymerization of cellulose itself. When the cellulose, hemicellulose and lignin content of the fractionated and unfracrionated substrates were compared it was apparent that the vast majority of the lignin was associated with the P200 fraction (Table 20). In contrast, the chemical composition of the remaining fractions was relatively uniform with cellulose and lignin contents ranging from 90.7 to 96.9% and 2.4 to 7.6% respectively, and very small (<3.5%) amounts of hemicelluloses (Table 20).

**Table 20.** Chemical composition (% w/w) of size-fractionated organosolv pretreated lodgepole pine, numbers in parentheses indicate the standard deviation (n = 3).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Arabinan</th>
<th>Galactan</th>
<th>Glucan</th>
<th>Xylan</th>
<th>Mannan</th>
<th>Lignin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfractionated</td>
<td>bdl</td>
<td>bdl</td>
<td>82.6 (1.3)</td>
<td>bdl</td>
<td>1.4 (0.2)</td>
<td>19.0 (0.5)</td>
</tr>
<tr>
<td>R14</td>
<td>bdl</td>
<td>bdl</td>
<td>90.7 (2.7)</td>
<td>0.3 (0.01)</td>
<td>3.1 (0.2)</td>
<td>7.6 (1.8)</td>
</tr>
<tr>
<td>R28</td>
<td>bdl</td>
<td>bdl</td>
<td>94.6 (1.3)</td>
<td>bdl</td>
<td>1.0 (0.02)</td>
<td>5.1 (0.4)</td>
</tr>
<tr>
<td>R48</td>
<td>bdl</td>
<td>bdl</td>
<td>96.9 (3.6)</td>
<td>bdl</td>
<td>3.3 (0.04)</td>
<td>2.4 (0.6)</td>
</tr>
<tr>
<td>R100</td>
<td>bdl</td>
<td>bdl</td>
<td>95.0 (3.2)</td>
<td>0.3 (0.06)</td>
<td>3.1 (0.04)</td>
<td>4.9 (0.6)</td>
</tr>
<tr>
<td>R200</td>
<td>bdl</td>
<td>bdl</td>
<td>93.2 (0.6)</td>
<td>bdl</td>
<td>2.6 (0.2)</td>
<td>7.1 (0.1)</td>
</tr>
<tr>
<td>P200</td>
<td>0.1 (0.01)</td>
<td>0.1 (0.01)</td>
<td>30.2 (0.8)</td>
<td>0.4 (0.01)</td>
<td>1.1 (0.1)</td>
<td>71.1 (0.8)</td>
</tr>
</tbody>
</table>

The uniform chemical composition observed between most of the fibre fractions, was likely due to the fact that delignification during organosolv pretreatment has been shown to occur primarily at the middle lamella where lignin is highly concentrated. In contrast, delignification during the kraft process involves the uniform removal of lignin from the middle lamella and the secondary cell wall (Pazsner and Behera, 1989, Hallac et al, 2010a). This difference in the topochemistry of delignification is one of the reasons organosolv pretreatment results in satisfactory fibre separation even though high levels of lignin may remain in the substrate. Because of the topochemistry of organosolv delignification, it is
possible that some of the lignin associated with the P200 fraction originated from the lignin that was removed from the middle lamella during pretreatment, and re-precipitated onto the fibre’s surface during cooling before being removed during fractionation. This proposed mechanism is supported by previous work (Del Rio et al, 2011) where over 50% of the lignin present in organosolv-pretreated lodgepole pine was extracted with 65% aqueous ethanol under reflux for 3h.

Unlike lignin and hemicellulose that physically and/or chemically inhibit the enzymatic hydrolysis of cellulose, the degree of polymerization (DP) and crystallinity are properties associated with the cellulose chain length and the arrangement of cellulose molecules. There have been varying reports in the literature regarding the effects of cellulose DP on the ease of enzymatic hydrolysis (Cateto et al, 2011, Pan et al, 2008). In the current study, using the intrinsic viscosity technique, the majority of the fibre fractions exhibited similar DP values ranging from 874 to 1046, while the unfractionated substrate had a DP value of 787 and the R200 and P200 fractions exhibited lower DP values (722 and 402 respectively) (Table 21).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>DPv</th>
<th>Crystallinity (A_{1428}/A_{898})</th>
<th>Cellulose Accessibility (A_{max,DO}/A_{max,DB})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfractionated</td>
<td>787 (27)</td>
<td>0.80 (0.02)</td>
<td>1.38</td>
</tr>
<tr>
<td>R14</td>
<td>874 (26)</td>
<td>0.75 (0.01)</td>
<td>1.45</td>
</tr>
<tr>
<td>R28</td>
<td>1064 (40)</td>
<td>0.72 (0.01)</td>
<td>1.46</td>
</tr>
<tr>
<td>R48</td>
<td>919 (50)</td>
<td>0.71 (0.01)</td>
<td>1.50</td>
</tr>
<tr>
<td>R100</td>
<td>1046 (27)</td>
<td>0.73 (0.01)</td>
<td>1.51</td>
</tr>
<tr>
<td>R200</td>
<td>722 (56)</td>
<td>0.75 (0.02)</td>
<td>1.52</td>
</tr>
<tr>
<td>P200</td>
<td>402 (33)</td>
<td>0.97 (0.00)</td>
<td>1.20</td>
</tr>
</tbody>
</table>

Table 21. Cellulose degree of polymerization, crystallinity, and cellulose accessibility (Simon’s stain) of size-fractionated organosolv-pretreated lodgepole pine, numbers in parentheses indicate the standard deviation (n = 3).
The results shown here contrast those of Lapierre et al, (2006) who found a proportional relationship between cellulose DP and fibre length. However, it is likely that these earlier results were only coincidental as the cellulose chain length is approximately three orders of magnitude lower than the fibre length (Lapierre et al, 2006). The lower cellulose DP of the P200 fraction was likely due to the acidic conditions utilized in the organosolv pretreatment resulting in some of the cellulose in the original feedstock undergoing significant acid-catalyzed hydrolysis, which would have resulted in both a decrease in fibre length and a decrease in cellulose DP.

In the same way that there has been considerable debate on the effect of cellulose DP on the enzymatic hydrolysis of cellulose, the influence of cellulose crystallinity has also not been fully resolved. However, many of the different interpretations of the overall influence of crystallinity on the enzymatic hydrolysis of cellulose can be attributed to variations in measurement techniques utilized and the use of model cellulose substrates rather than real biomass (Park et al, 2010). In this study, it was apparent that in addition to lower cellulose DP, the cellulose crystallinity of the P200 fraction was significantly higher (0.97) than that of the other fractions (from 0.71 to 0.75, Table 21). This increase in cellulose crystallinity is likely due to the hydrolysis of some of the amorphous cellulose during pretreatment resulting in an enrichment of crystalline cellulose in the P200 fraction (alongside a concomitant reduction in cellulose DP).

While the cellulose DP and crystallinity are believed to influence the effectiveness of enzymatic hydrolysis, it has been proposed that the overall accessible surface area of the pretreated biomass (i.e. the combination of exterior surface area, such as fibre size and interior surface area, such as the fiber porosity) to the cellulases is the key substrate
characteristic that limits hydrolysis (Chandra et al 2008a). Therefore, we next assessed the influence of fibre size fractionation on the cellulase-accessible surface area as determined by Simon’s stain. With the exception of the P200 fraction, which showed a decrease in cellulose accessibility when compared to the unfractionated substrates, (Table 21), the other fractions exhibited a minor increase in accessible surface area (ranging from 1.45 to approximately 1.52, Table 21). It is likely that the higher lignin content of the P200 fraction contributed to its lower amount of accessible cellulose. Overall, it was evident that, although the fibre fractions differed significantly in size from 0.2 to 3.4 mm, with the exception of the P200 fraction, they were quite similar with regard to their chemical composition, DP, crystallinity and accessible cellulose.

3.3.3 Enzymatic Hydrolysis of the Size-Fractionated Substrates

When the hydrolysis profiles of the unfractionated and fractionated substrates were compared (Figure 23), it was evident that, with the exception of the P200 fraction, fractionation resulted in improved hydrolysis yields. This was from 82% to near complete cellulose conversion after 72 hours at an enzyme loading, of 5 FPU g⁻¹ (likely due to the removal of the lignin rich-fines). However, the different fractions did not vary substantially in their susceptibility to enzymatic hydrolysis despite considerable differences in fibre size. In contrast, the P200 fraction was considerably more recalcitrant (42% cellulose hydrolysis yields after 72 hours) despite its significantly smaller fibre length. The similar hydrolysis yields obtained with the size-fractionated substrates (excluding the P200 fraction) were likely due to the comparable accessibility of their cellulose component to cellulolytic enzymes, as mentioned in section 3.3.2. Conversely, it is also likely that the greater recalcitrance of the
P200 fraction was due to its higher lignin content, significantly lower cellulase-accessible cellulose and higher crystallinity.

Figure 23. Enzymatic hydrolysis of size-fractionated organosolv pretreated lodgepole pine at 5 FPU g\(^{-1}\) cellulose. Hydrolysis conditions: 2% solids content, 50mM Na-acetate buffer pH 4.8, 50 °C, 150 rpm. Error bars indicate the standard deviation (n=3).

These results agree with those of Peters and coworkers (1991), who showed that size fractionation of microcrystalline cellulose, did not have an effect on the extent of enzymatic hydrolysis. In contrast, Yeh et al, (2010) found significant improvements in their substrate’s susceptibility to enzymatic hydrolysis after size reduction via milling. However, unlike this study, Yeh and coworkers also observed a significant decrease in cellulose crystallinity as a result of milling. In addition, it should be noted that the range of fibre sizes of the substrates used in the work by Yeh and coworkers was three orders of magnitude smaller than the fibres used in this study (0.78 μm to 25 μm compared to 0.198 mm to 3.431 mm respectively) and
slightly smaller than those used in the study by Peters and coworkers (38 μm to 105 μm). Therefore, it is possible that the differences in fibre length observed in the work reported here were not sufficient to elicit an effect on enzymatic hydrolysis as even the shortest fibres are orders of magnitude larger than cellulases (5.1 nm, Grethlein, 1985). It is likely that the previously observed relationship between fibre length and ease of enzymatic hydrolysis of organosolv pretreated substrates (Del Rio et al, 2010; Pan et al, 2008) was due to the effects of pretreatment conditions on other substrate properties such as swelling, degree of polymerization and the overall accessibility of cellulose to cellulases. This possible mechanism is supported by Sinitsyn et al, (1991) who showed that the particle size of sugar cane bagasse had no effect on enzymatic hydrolysis whereas increasing cellulose accessibility increased glucose conversion.

Since the initial fibre size did not seem to affect the ease of enzymatic hydrolysis of the size-fractionated substrates, we next assessed whether the enzymes themselves were changing the substrate’s fibre length during hydrolysis by assessing the changes in fibre length (fibre fragmentation) of each substrate during the course of hydrolysis (Figure 24A). The most significant changes occurred during the first 3 h of enzymatic hydrolysis with prolonged incubation times showing little change in the average fibre length despite continued cellulose hydrolysis. The fibre length of nearly all the substrates was virtually identical after approximately 12 h regardless of the initial fibre length. It was apparent that the substrates with the longer initial fibre lengths fragmented to a greater extent than did the substrates with smaller fibre lengths. Again, a notable exception to this observation was the P200 substrate, which did not show any measurable changes in fibre length during the course of enzymatic hydrolysis, likely due to the greater recalcitrance of this fraction. Thygesen et
Figure 24. Changes in average fibre size during the course of enzymatic hydrolysis of size-fractionated organosolv pretreated lodgepole pine (A) and changes in the fines content of selected size-fractionated organosolv pretreated lodgepole pine substrates during the course of enzymatic hydrolysis (B).
al, (2010), showed that, during the initial stages of hydrolysis, fibre fragmentation occurs at irregular regions of the fibre wall known as dislocations. It is probable that the longer fibres will have more dislocation sites along their surface compared to the shorter fibres accounting for the differences in fragmentation between the different fractions.

It was also apparent that during enzymatic hydrolysis of the size fractions, the fibre population rapidly shifted towards the accumulation of fines (fibres between 0.07-0.2 mm), regardless of the initial fibre length (Figure 24B). This observation was in agreement with previous work (Park et al, 2007), which showed that enzymatic hydrolysis of softwood and hardwood kraft pulps resulted in the generation of significant amounts of fines during the early stages of hydrolysis.

3.3.4 Characterization and Enzymatic Hydrolysis of Small Fibre-Enriched Substrates

Although the work reported in section 3.3.3 suggests that initial fibre size does not influence a substrate’s susceptibility to enzymatic hydrolysis, it should be noted that the majority of the fines were removed during the fractionation process. Since previous research (Lu et al, 2010) showed that because of their higher exterior surface area, the fines fraction was up to six times more susceptible to enzymatic hydrolysis than were the longer fibres, we next assessed the effect of an increased fines content on the effectiveness of enzymatic hydrolysis. However, the high lignin content (71%) of the P200 fraction, which contained >93% fines (Table 19) seemed to complicate the comparison with the other fibre fractions that were isolated from the original organosolv pretreated lodgepole pine substrate.

Therefore, we decided to exploit the ability of cellulases to increase the fines content in the substrate during the initial stages of hydrolysis by pre-hydrolyzing the organosolv-pretreated lodgepole pine to varying extents (from 0% to 80% cellulose conversion, PreH-1 to PreH-4,
Table 22). The prehydrolyzed substrates and the initial substrate (UnH) were delignified to determine if enrichment in fines could enhance enzymatic hydrolysis while removing the inhibitory effect of lignin. Prehydrolysis of the original organosolv-pretreated substrate resulted in an increase in the fines content that was proportional to the extent of cellulose hydrolysis (from 8.9% to 55.4% based on the weighted average fines, Table 22). The chemical composition of the unhydrolyzed and prehydrolyzed substrates showed that these substrates varied within a narrow range containing 97-99% glucan, 2-3% mannan, and 0-1% xylan and 0.2-0.8% galactan.

### Table 22. Gross fibre characteristics of sodium chlorite-delignified organosolv-pretreated lodgepole pine before and after prehydrolysis to various extents, numbers in parentheses indicate the standard deviation (n = 3).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Cellulose conversion (%)</th>
<th>Ln(^a) (mm)</th>
<th>Lw(^b) (mm)</th>
<th>Width (μm)</th>
<th>% Fines (^c)</th>
<th>% Fines (^d)</th>
<th>Crystallinity ((A_{1425}/A_{808}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>UnH</td>
<td>0</td>
<td>0.64</td>
<td>1.96</td>
<td>31.2</td>
<td>51.3</td>
<td>8.9</td>
<td>0.77 (0.01)</td>
</tr>
<tr>
<td>PreH-1</td>
<td>15.6 (1.4)</td>
<td>0.28</td>
<td>0.82</td>
<td>32.2</td>
<td>62.3</td>
<td>26.1</td>
<td>0.74 (0.02)</td>
</tr>
<tr>
<td>PreH-2</td>
<td>34.1 (1.0)</td>
<td>0.21</td>
<td>0.54</td>
<td>32.9</td>
<td>73.0</td>
<td>39.1</td>
<td>0.73 (0.03)</td>
</tr>
<tr>
<td>PreH-3</td>
<td>61.2 (2.1)</td>
<td>0.18</td>
<td>0.49</td>
<td>32.1</td>
<td>79.1</td>
<td>48.4</td>
<td>0.93 (0.04)</td>
</tr>
<tr>
<td>PreH-4</td>
<td>80.0 (1.0)</td>
<td>0.16</td>
<td>0.33</td>
<td>34.1</td>
<td>79.4</td>
<td>55.4</td>
<td>0.95 (0.01)</td>
</tr>
</tbody>
</table>

\(^a\)Ln = number average fibre length, \(^b\)Lw = weighted average fibre length, \(^c\)n = numerical percent fines content, \(^d\)w = length weighted percent fines content.

Although the prehydrolyzed substrates exhibited an increase in fines content, it was evident that they were undergoing other changes to their structure as an increase in cellulose crystallinity was observed for the preH-3 (0.93) and preH-4 (0.95) substrates, which had been prehydrolyzed to 61.2% and 80.0% cellulose conversion respectively. As mentioned in section 3.3.2, there have been conflicting reports in the literature regarding the changes in crystallinity during cellulose hydrolysis as some researchers have concluded that amorphous cellulose is preferentially hydrolyzed resulting in increases in crystallinity during enzymatic hydrolysis (Hall et al, 2010) while others have shown that crystallinity does not increase during hydrolysis (Wang et al, 2006).
In addition to changes in cellulose crystallinity, pre-hydrolysis resulted in a substantial (60%) decrease in cellulose DP (from 792 to 478, for the UnH and PreH-1 substrates respectively). Further cellulose hydrolysis of up to 80% (w/w) resulted in an increasingly slow decrease in cellulose DP (Figure 25). These results are consistent with work by Cao and Tan (2002), who found that treatment of softwood dissolving pulp with purified endoglucanase I (Cel 7b) resulted in an initial rapid reduction of cellulose DP during the early stages of enzymatic hydrolysis followed by no further changes in DP. This suggested that, in the early stages of hydrolysis, rapid endoglucanase action results in considerable cleavage along the cellulose chain, generating new chain ends for the action of exoglucanases. Other workers (Pala et al, 2007) have suggested that a combination of both the initial susceptibility to endoglucanase action followed by a layer by layer cellulose solubilization pattern is the reason why a significant portion of high DP cellulose remained unchanged after significant cellulose conversion.

Figure 25. Effect of enzymatic hydrolysis on the cellulose degree of polymerization (DPv) of organosolv pretreated lodgepole pine. Error bars indicate the standard deviation (n=3).
It was apparent that despite having a higher fines content and lower DP, the hydrolysis yields for the preH-1 and PreH-2 substrates were identical to that of the UnH substrate (Figure 26). This suggested that cellulose DP and fines content do not have a significant influence on the hydrolysis of the small fibre enriched substrates. To gain further insight into the lack of an effect of cellulose DP on enzymatic hydrolysis while limiting the influence of swelling and crystallinity, the UnH and PreH-4 substrates (representing the extremes in initial DP) were subjected to phosphoric acid swelling. When the ATR-FTIR spectra of the starting material and the phosphoric acid swollen cellulose substrates were compared, the peaks commonly associated with crystalline cellulose (1098 and 1425 cm\(^{-1}\))

**Figure 26.** Enzymatic hydrolysis at 2 FPU g\(^{-1}\) cellulose of delignified organosolv pretreated lodgepole pine prehydrolyzed to various extents. Hydrolysis conditions: 2% (w/v) solids content, 50 mM Na-acetate buffer pH 4.8, 50 °C, 150 rpm. Error bars indicate the standard deviation (n=3).
disappeared (Figure 27), suggesting that in addition to increasing swelling, the dissolution and regeneration process disrupted cellulose crystallinity.

**Figure 27.** ATR-FTIR spectra of delignified organosolv-pretreated lodgepole pine prehydrolyzed to approximately 80% cellulose conversion (PreH-4) before (A) and after swelling with phosphoric acid (B). Arrows show the peaks associated with crystalline cellulose (1098 and 1425 cm$^{-1}$).

When the phosphoric acid-treated UnH and PreH-4 substrates were subjected to enzymatic hydrolysis it was evident that disrupting cellulose crystallinity and increasing swelling resulted in significant improvements in the ease of enzymatic hydrolysis of both substrates. Interestingly, nearly identical hydrolysis yields were observed for the phosphoric acid-treated UnH and PreH-4 substrates of 82% and 78%, respectively (Figure 28) despite their marked differences in DP (792 Vs 375 respectively). These results strongly support the observation that, rather than cellulose DP, other factors such as swelling and increased
accessibility of the cellulose structure as a result of the phosphoric acid treatment play a more significant role in influencing the organosolv-pretreated substrates’ susceptibility to enzymatic hydrolysis at low enzyme loadings.

**Figure 28.** Enzymatic hydrolysis at 2 FPU g\(^{-1}\) cellulose of phosphoric acid swollen cellulose (PASC) produced from delignified organosolv-pretreated lodgepole pine (UnH PASC) and delignified organosolv-pretreated lodgepole pine prehydrolyzed to approximately 80 % cellulose conversion (PreH-4 PASC). Hydrolysis conditions: 2% (w/v) solids content, 50 mM Na-acetate buffer pH 4.8, 50 °C, 150 rpm. Error bars indicate the standard deviation (n=3).

### 3.3.5 Conclusions

This work was carried out to clarify the role that the initial fibre length and cellulose DP might have on the enzymatic hydrolysis of organosolv-pretreated softwood substrates while maintaining other substrate properties as uniform as possible. Surprisingly, it was apparent that, neither fibre length (over the size range of 0.2 mm to approximately 4.5 mm) nor reductions in cellulose DP had an appreciable effect on the substrates’ susceptibility to
enzymatic hydrolysis. Increasing the fines content via prehydrolysis also did not have a significant effect on enzymatic hydrolysis despite significantly higher surface area. It is likely that the overall accessibility of cellulose to cellulases is the most important substrate characteristic rather than any single component such as fibre length, cellulose degree of polymerization and crystallinity.
3.4 The Effect of Chemical and Mechanical Treatments on the Properties and Susceptibility of Organosolv-Pretreated Lodgepole Pine to Enzymatic Hydrolysis

3.4.1 Background

The work reported in section 3.1.4 showed that we were able to obtain near-complete cellulose hydrolysis at relatively low enzyme loadings and solids content (7.5 FPU g\textsuperscript{-1} cellulose and 2\% respectively). However, we were unable to achieve significant cellulose conversion when very low (2.5 FPU g\textsuperscript{-1} cellulose) enzyme loadings were used. This decrease in cellulose hydrolysis was likely caused by the residual lignin (approximately 18 \% w/w of the substrate), which prevented the accessibility of cellulose to cellulases and decreased their availability through non-productive binding. In agreement with previous work (Jeoh et al, 2007; Chandra et al, 2008b; Arantes and Saddler, 2010 and 2011; Rollin et al, 2011), the studies in sections 3.2 and 3.3 strongly suggested that the overall accessibility of cellulose to cellulases played a key role in governing the susceptibility of the organosolv-pretreated lodgepole pine substrates to enzymatic hydrolysis. In light of these findings, it was hypothesized that mild chemical or physical pretreatments could be used to increase the overall cellulose accessibility without significant lignin removal.

In the work reported here, organosolv-pretreated lodgepole pine was subjected to either chemical (neutral sulphonation) or mechanical (refining) treatments to try to increase the overall accessibility of cellulose to cellulases (and hopefully its susceptibility to enzymatic hydrolysis at low enzyme loadings), without significantly changing its chemical composition. Overall, it was hoped that this would provide some insights into the interactions between cellulases and lignocellulosic substrates and identify the reasons why we could not obtain significant hydrolysis yields at low enzyme loadings.
3.4.2 Characterization of Organosolv-Pretreated Lodgepole Pine Subjected to Chemical and Mechanical Treatments

As mentioned in section 3.4.1, the primary goal of this study was to gain a better insight into the factors that govern the efficacy of enzymatic hydrolysis by increasing the accessible surface area, swelling, and hydrophilicity of an organosolv pretreated softwood substrate via physical and chemical treatments.

Compositional analyses of the sulphonated, untreated and control substrates (OSLPP-Sulph, OSLPP and Sulph-control respectively, Table 23) revealed that the OSLPP-Sulph substrate contained approximately 21% less lignin than did the OSLPP substrate. This was in apparent contrast to previous work by Mooney et al, (1998) who found that the sulphonation of Douglas-fir refiner mechanical pulp under neutral conditions increased the total lignin content due to the incorporation of sulphonate groups. However, this apparent contradiction was likely caused by the fact that the lignin in the refiner mechanical pulp was highly unmodified as it had not been exposed to the solvolytic chemicals used during organosolv pretreatment. For example, recent work by Sannigrahi et al, (2010b) showed that the residual lignin from organosolv-pretreated loblolly pine contained three times more phenolic hydroxyl groups than did milled wood lignin obtained from the same source (due to the solvolytic cleavage of the α-O-4 and β-O-4 ether bonds). Moreover, earlier work (Heitner et al, 1982; Beatson et al, 1984) showed that at neutral and alkaline conditions, sulphonation (and subsequent delignification) occurs primarily via the formation of quinone methide intermediates and therefore requires free phenolic hydroxyl groups (Figure 29). Therefore it is possible that the lignin removal that was observed as a result of sulphonation was partly promoted by the increase in phenolic hydroxyl groups generated during the organosolv
pretreatment. Alternatively, sulphonation carried out under acidic conditions can result in greater delignification as it is independent of phenolic hydroxyl groups (Sjöström, 1993). On the other hand, increasing the amount of sodium sulphite from 0.25 M to 1.0 M did not have an effect on the degree of delignification and extent of sulphonation suggesting that the amounts of sodium sulphite used in this study were greater than what is required to sulphonate all of the available sites in the substrate.

![Proposed reaction mechanism for the sulphonation of lignin under neutral and alkaline conditions. Modified from Beatson et al, (1984).](image)

**Figure 29.** Proposed reaction mechanism for the sulphonation of lignin under neutral and alkaline conditions. Modified from Beatson et al, (1984).

In contrast to the neutral sulphonation treatment, mechanical treatment in the form of PFI-mill refining ranging from 2500 to 20000 revolutions (Ref-1 to Ref-3 respectively) did not result in significant changes to the chemical composition of the substrates (Table 23). However, it was expected that an increase in swelling, porosity, disorder at the fibril level and a decrease in particle size resulting from fibre cutting and delamination would occur, as observed previously (Laine et al, 2004; Cullis et al, 2004). Indeed, it was apparent that refining the OSLPP substrate resulted in a decrease in the average particle size and an increase in the fines content (fibres ranging in length from 0.07 to 0.20 mm) which was
proportional to the extent of refining (Table 24). However, since refining was conducted under conditions that minimize fibre cutting (10 % solids) it is likely that the observed decrease in particle size was mostly caused by fibre delamination. This was supported by the fact that fibre width also decreased proportionally with the extent of refining (from approximately 32 µm to 23 µm for the OSLPP and Ref-3 substrates respectively, Table 24). In contrast to the refining results, sulphonation of the OSLPP substrate did not result in appreciable changes in fibre length or width (data not shown).

**Table 23.** Chemical composition of organosolv pretreated lodgepole pine before and after chemical and mechanical treatments, numbers in parentheses indicate the standard deviation (n=3).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Glucan (% solids)</th>
<th>Xylan</th>
<th>Arabinan</th>
<th>Mannan (% solids)</th>
<th>Galactan</th>
<th>Lignin (% solids)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OSLPP</td>
<td>82.6 (1.3)</td>
<td>bdl</td>
<td>bdl</td>
<td>1.4 (0.2)</td>
<td>bdl</td>
<td>19.0 (0.5)</td>
</tr>
<tr>
<td>Sulph-control</td>
<td>81.7 (1.2)</td>
<td>bdl</td>
<td>bdl</td>
<td>1.3 (0.2)</td>
<td>bdl</td>
<td>17.8 (0.2)</td>
</tr>
<tr>
<td>OSLPP-Sulph</td>
<td>88.7 (3.2)</td>
<td>bdl</td>
<td>bdl</td>
<td>0.9 (0.1)</td>
<td>bdl</td>
<td>15.1 (0.9)</td>
</tr>
<tr>
<td>Ref-1</td>
<td>81.8 (0.5)</td>
<td>bdl</td>
<td>bdl</td>
<td>1.2 (0.3)</td>
<td>bdl</td>
<td>18.8 (0.3)</td>
</tr>
<tr>
<td>Ref-2</td>
<td>82.2 (0.8)</td>
<td>bdl</td>
<td>bdl</td>
<td>1.4 (0.2)</td>
<td>bdl</td>
<td>18.4 (0.2)</td>
</tr>
<tr>
<td>Ref-3</td>
<td>82.4 (0.6)</td>
<td>bdl</td>
<td>bdl</td>
<td>0.9 (0.2)</td>
<td>bdl</td>
<td>18.6 (0.6)</td>
</tr>
<tr>
<td>OS-LPP EX</td>
<td>92.5 (2.3)</td>
<td>bdl</td>
<td>bdl</td>
<td>0.2 (0.1)</td>
<td>bdl</td>
<td>7.6 (0.1)</td>
</tr>
<tr>
<td>OS-LPP DL</td>
<td>92.6 (1.1)</td>
<td>bdl</td>
<td>bdl</td>
<td>0.8 (0.04)</td>
<td>bdl</td>
<td>8.1 (0.8)</td>
</tr>
</tbody>
</table>

The influence of refining on the accessibility of cellulose to cellulases was assessed by the Simon’s stain (SS) method, as modified by Chandra et al, (2008b). It was apparent that refining had a small but measurable effect on the ratio of adsorbed DO to DB ranging from 1.56 to 1.76 for OSLPP and Ref-3, corresponding to an increase of approximately 15% (Table 25). It should also be noted that the larger increases in the ratios of adsorbed dyes were observed when the substrates were refined at 2,500 and 10,000 revolutions (Ref-1 and Ref-2, respectively) whereas further refining resulted in negligible increases in dye adsorption (Table 25). Although it appeared that mechanical damage caused by refining resulted in a significant decrease in particle size through fibre delamination, refining did not appear to increase cellulose accessibility to any significant extent. Although these results
were unexpected they can be partially explained by the fact that, unlike kraft fibres, organosolv fibres have been shown to be relatively uniform in their chemical composition (Nikhil and Pazsner, 1989; Suurnäkki et al, 1996). Therefore, it is likely that as a result of this uniform chemical composition, mechanical damage caused by refining did not result in increased cellulose exposure or accessibility to cellulases.

**Table 24.** Effect of refining on the gross fibre characteristics of organosolv pretreated lodgepole pine.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Ln⁷ (mm)</th>
<th>Lw⁸ (mm)</th>
<th>% Fines (Ln)</th>
<th>% Fines (lw)</th>
<th>Width (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OSLPP</td>
<td>0.87</td>
<td>1.84</td>
<td>50.0</td>
<td>6.5</td>
<td>32.3</td>
</tr>
<tr>
<td>Ref-1</td>
<td>0.50</td>
<td>1.31</td>
<td>57.9</td>
<td>17.1</td>
<td>28.7</td>
</tr>
<tr>
<td>Ref-2</td>
<td>0.43</td>
<td>1.06</td>
<td>58.0</td>
<td>19.7</td>
<td>25.3</td>
</tr>
<tr>
<td>Ref-3</td>
<td>0.35</td>
<td>0.75</td>
<td>60.1</td>
<td>25.0</td>
<td>22.9</td>
</tr>
</tbody>
</table>

⁷Ln = number average length, ⁸Lw = weighted average length.

Neutral sulphonation also resulted in negligible changes being observed with regard to the ratio of direct orange to direct blue dye adsorbed, despite removing approximately 21% of the lignin present in the substrate (Table 25). This is likely due to the fact that the Simon’s stain technique uses sulphonated dyes which may be prevented from adsorbing to the cellulose adjacent to the sulphonated lignin due to electrostatic repulsion by the negative charges in the dye and the residual lignin in the substrate.

In addition to particle size and pore size distribution, as mentioned previously, cellulose crystallinity has been suggested to play an important role in the enzymatic hydrolysis of cellulose, with the amorphous fractions being less recalcitrant than the crystalline regions (Zhang and Lynd, 2004). To try to assess the influence of the treatments on cellulose crystallinity we recorded the infrared spectra of the substrates before and after treatment. From these spectra, cellulose crystallinity was calculated as the ratio of absorption at 1425 cm⁻¹ and 898 cm⁻¹, which correspond to CH₂ scissoring (found in crystalline cellulose) and the vibrational mode of C1 with four atoms attached to it (characteristic of
amorphous $\beta$-glycosidic linkages) respectively (Jeihanipour et al., 2010). Treatment with sodium sulphite appeared to have no effect on the crystalline structure of cellulose (Table 25). However, it was apparent that refining had a small but measurable effect on cellulose crystallinity as the CI decreased with the extent of refining from 0.85 to 0.78 for the untreated substrate and the substrate refined at 20,000 revolutions (OSLPP and Ref-3 respectively). This decrease in CI was likely caused by the increased fibre disorder resulting from fibre beating (Chandra et al., 2007).

Table 25. Cellulose accessibility, crystallinity and fibre swelling of organosolv-pretreated lodgepole pine before and after neutral sulphonation and PFI-mill refining, numbers in parentheses indicate the standard deviation$^a$.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Cellulose accessibility ($A_{\text{max}DO}/A_{\text{max}DB}$)</th>
<th>Crystallinity ($A_{1425}/A_{898}$)</th>
<th>WRV</th>
</tr>
</thead>
<tbody>
<tr>
<td>OSLPP</td>
<td>1.56</td>
<td>0.85 (0.02)</td>
<td>2.5 (0.1)</td>
</tr>
<tr>
<td>Sulph-control</td>
<td>1.52</td>
<td>0.83 (0.01)</td>
<td>2.4 (0.1)</td>
</tr>
<tr>
<td>OSLPP-Sulph</td>
<td>1.61</td>
<td>0.85 (0.5)</td>
<td>2.8 (0.0)</td>
</tr>
<tr>
<td>Ref-1</td>
<td>1.67</td>
<td>0.83 (0.02)</td>
<td>3.0 (0.0)</td>
</tr>
<tr>
<td>Ref-2</td>
<td>1.73</td>
<td>0.80 (0.01)</td>
<td>3.1 (0.0)</td>
</tr>
<tr>
<td>Ref-3</td>
<td>1.76</td>
<td>0.78 (0.02)</td>
<td>3.2 (0.0)</td>
</tr>
</tbody>
</table>

$^a$Crystallinity measurements are the average of 10 separate measurements, WRV’s are the average of 3 separate experiments.

Besides cellulose crystallinity, fibre swelling has been shown to play a major role in a substrate’s susceptibility to hydrolysis by cellulases (Chandra et al., 2007; Luo and Zhu, 2010). Previous work by Bendzalova et al., (1996) showed that wood chips exposed to a variety of swelling agents prior to mechanical pulping exhibited a linear correlation between WRV and susceptibility to cellulolytic hydrolysis. Both treatments used in this study resulted in increased WRVs (and therefore swelling) (Table 25). However, compared to refining sulphonation did not impart a significant increase in WRV (from 2.5 in the untreated substrate to a maximum of 2.8 and 3.2 for the sulphonated and refined substrates respectively). These results suggest that the relatively small amounts of sulphonate groups
(approximately 32 mmol kg⁻¹, Table 26) that were introduced into the residual lignin during sulphonation were not sufficient to increase fibre swelling to the same extent as the mechanical damage undergone by the substrate during refining. Alternatively, it is possible that increased fibre swelling could be observed if the measurements were to be performed at higher temperatures (due to the thermal softening of the residual lignin). Indeed a previous study on the effects of temperature on fibre swelling (Eriksson et al, 1991) showed that the maximum swelling of spruce CTMP fibres was achieved at 50-60 °C while in the case of spruce TMP fibres, swelling continued above 95 °C.

**Table 26.** Amount of anionic charges present in organosolv pretreated lodgepole pine substrates before and after chemical treatments, numbers in parentheses indicate the standard deviation (n = 3).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Sulphonate groups (mmol kg⁻¹)</th>
<th>Carboxylic groups (mmol kg⁻¹)</th>
<th>Total charged groups (mmol kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OS-LPP</td>
<td>bdl</td>
<td>127.2 (3.0)</td>
<td>127.2 (3.0)</td>
</tr>
<tr>
<td>Sulph-control</td>
<td>bdl</td>
<td>121.5 (5.6)</td>
<td>121.5 (5.6)</td>
</tr>
<tr>
<td>OSLPP-Sulph</td>
<td>32.0 (3.5)</td>
<td>167.6 (6.6)</td>
<td>199.6 (7.5)</td>
</tr>
<tr>
<td>OS-LPP EX</td>
<td>bdl</td>
<td>69.8 (10.0)</td>
<td>69.8 (10.0)</td>
</tr>
<tr>
<td>OS-LPP DL</td>
<td>bdl</td>
<td>226.3 (2.9)</td>
<td>226.3 (2.9)</td>
</tr>
</tbody>
</table>

**3.4.3 Effects of PFI-Mill Refining of Enzymatic Hydrolysis**

To measure the effects of mechanical post treatments on hydrolysis rates and yields, the refined substrates were hydrolyzed at 5 and 2.5 FPU g⁻¹ cellulose, 50 °C, and 2 % (w/v) solids content (Figure 30). Despite slight increases in swelling and decreases in crystallinity index and particle size, it was found that refining did not have a significant effect on the substrate’s susceptibility to cellulosic hydrolysis (approximately 80 % and 40 % after 72 h
Figure 30. Effects of PFI-mill refining on the enzymatic hydrolysis of organosolv-pretreated lodgepole pine at 5 FPU g\(^{-1}\) cellulose (A) and 2.5 FPU g\(^{-1}\) cellulose (B). Substrates were resuspended at 2% (w/v) solids content in 50 mM Na-acetate buffer at pH 4.8. Hydrolysis conditions: 50 °C, 150 rpm. Error bars indicate the standard deviation (n=3).
for 5 and 2.5 FPU g⁻¹ cellulose respectively). These results are in direct contrast to previous work by Laine and coworkers (2004), which showed that PFI-mill refining of fully bleached pine kraft pulp resulted in significant increases in its susceptibility to cellulolytic hydrolysis. However as previously mentioned, the chemical composition of kraft pulp fibres is much less uniform than that of organosolv fibres. Therefore, it is possible that refining of kraft pulps results in a significant exposure of cellulose whereas refining organosolv fibres may not increase the exposure of cellulose to any significant degree. This suggestion is partially supported by the relatively small (approximately 15%) increase in the ratio of adsorbed dyes (Simon’s stain) for the refined substrates (Table 25).

3.4.4 Effects of Neutral Sulphonation on Enzymatic Hydrolysis

In contrast, chemical treatment of OSLPP (in the form of sulphonation) resulted in significant increases in the extent of enzymatic hydrolysis (from 80% to 95% and 35% to 80% after 72 hours for 5 and 2.5 FPU g⁻¹ cellulose respectively, Figure 31) despite a smaller effect on fibre swelling. In addition, sulphonation resulted in significant (from 68% to 86%) improvements in the 72 h enzymatic hydrolysis yields at a 10% (w/v) substrate concentration (Figure 32). This is of particular importance as it is generally accepted that increases in solids loadings employed during enzymatic hydrolysis will be required to decrease operation costs (Kristensen et al, 2009, Wingren et al, 2003). These results are in agreement with previous work (Mooney et al, 1998) which showed that, in addition to increased fibre swelling, sulphonation of Douglas-fir refiner mechanical pulp resulted in a doubling of the 72 h cellulose hydrolysis yields, to 40% at 20 FPU g⁻¹ cellulose.
Figure 31. Effects of sulphonation on the enzymatic hydrolysis of organosolv-pretreated lodgepole pine at 5 FPU g\(^{-1}\) cellulose (A) and 2.5 FPU g\(^{-1}\) cellulose (B). Substrates were resuspended at 2% (w/v) solids content in 50 mM Na-acetate buffer at pH 4.8. Hydrolysis conditions: 50 °C, 150 rpm. Error bars indicate the standard deviation (n=3).
As it was clear that some delignification occurred during sulphonation, the influence of partial lignin removal on enzymatic hydrolysis was examined in greater detail by subjecting the starting OSLPP substrate to either a mild extraction with 65 % aqueous ethanol under reflux or a partial delignification with sodium chlorite in 1 % acetic acid. Although the hot aqueous ethanol extraction was effective in removing lignin from organosolv pretreated substrates a disadvantage of this type of extraction is that only the lignin that has precipitated on the fibre’s surface is usually removed (Zhang et al, 2007b). Alternatively, partial delignification using sodium chlorite should result in a more homogenous removal of lignin from all parts of the substrate (Sjöström, 1993).

![Figure 32](image)

**Figure 32.** Effects of sulphonation on the 72 hours cellulose hydrolysis yields of organosolv-pretreated lodgepole pine at 10% (w/v) solids content and 5 FPU g⁻¹ cellulose. Substrates were resuspended in 50 mM Na-acetate buffer at pH 4.8. Hydrolysis conditions: 50 °C, and 150 rpm. Error bars indicate the standard deviation (n=3).

The lignin content of the extracted and sodium chlorite-delignified substrates (OSLPP-EX and OSLPP-DL respectively) was approximately 8% corresponding to a
removal of almost 60% of the lignin present in the original substrate (OSLPP) (Table 23). It was found that the hydrolysis profiles of OSLPP and OSLPP-EX were very similar at an enzyme loading of 5 FPU g⁻¹ cellulose and 2% (w/v) solids loading with only marginal improvements in the final hydrolysis yields of OSLPP-EX (from 80% to 85% after 72 hours, Figure 33). In contrast, the hydrolysis profile of the OSLPP-DL substrate showed a significant increase in hydrolysis yields when compared to that of the untreated substrates (from 80% to 96% after 72 hours, Figure 33) despite having a similar chemical composition to that of the OSLPP-EX substrate. These results strongly suggest that the improvements in hydrolysis yields after sulphonation of OSLPP were caused by factors other than delignification.

**Figure 33.** Effects of lignin removal on the enzymatic hydrolysis of organosolv-pretreated lodgepole pine. Substrates were resuspended at 2% (w/v) solids content in 50 mM Na-acetate buffer at pH 4.8. Hydrolysis conditions: 50 °C, 150 rpm. Error bars indicate the standard deviation (n=3).
A possible explanation for the increases in hydrolysis yields after either sulphonation or partial delignification with sodium chlorite is that the increased hydrophilicity of the residual lignin through the introduction of negative charges limited the non-specific binding of cellulases. This decrease in non-specific binding is possibly due to electrostatic repulsion between the sulphonated lignin and the major components in the cellulase system, which are negatively charged at pH 4.8 (Hui et al, 2001). Supporting evidence for this possible mechanism is that the total amount of charged groups increased from approximately 127 mmol kg\(^{-1}\) to 200 mmol kg\(^{-1}\) for OSLPP and OSLPP-Sulph respectively (Table 26). It should also be noted that the number of carboxyl groups in the OSLPP-EX substrate decreased to approximately 70 mmol kg\(^{-1}\), likely as a result of lignin removal. In contrast, the number of carboxyl groups in the OSLPP-DL substrate increased to approximately 226 mmol kg\(^{-1}\) despite having almost the same amount of lignin as OSLPP-EX. This discrepancy is likely due to the formation of carboxyl groups during the oxidation of lignin by chlorine dioxide generated from chlorite under acidic conditions (Sjöström, 1993).

Further evidence that the incorporation of negative charges into lignin prevented non-specific binding via electrostatic repulsion is presented in Table 27. The data in this table show that the maximum adsorption capacity for direct blue 1 (DB-1), which is a negatively charged dye with high affinity for cellulose and a molecular weight of 992.8 g mol\(^{-1}\) (Chandra et al, 2008b) decreased from 140 mg g\(^{-1}\) to 120 mg g\(^{-1}\) for OSLPP and OSLPP-Sulph respectively (approximately 15 %). These results agree with those of Palonen and Viikari (2004) who showed that modification of steam-pretreated softwood lignin by laccase increased the number of carboxylic acid groups with a concomitant decrease in the non-specific adsorption of cellulases, and an increase in hydrolysis yields of 13 %. In addition,
Nakagane et al. (2011b) showed that carboxylic acid-enriched dehydrogenative polymer (DHP) lignin derived from ferulic acid exhibited a significant decrease in non-specific adsorption of cellulases compared to DHP lignin derived from coniferyl alcohol. That study also showed that the enzymatic hydrolysis of Avicel (a commercially available cellulose preparation) was not affected by the presence of ferulic acid-derived DHP lignin whereas coniferyl alcohol-derived DHP lignin had an adverse effect on the enzymatic hydrolysis of Avicel.

Table 27. Maximum adsorption capacities of direct blue 1 (DB) for organosolv-pretreated lodgepole pine before and after sulphonation, numbers in parentheses indicate the standard deviation (n = 3).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>A_{max}DB (mg g(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>OS-LPP</td>
<td>141 (0.5)</td>
</tr>
<tr>
<td>Sulph-control</td>
<td>139 (0.2)</td>
</tr>
<tr>
<td>OSLPP-Sulph</td>
<td>120 (0.9)</td>
</tr>
</tbody>
</table>

To determine the effects of increasing anionic charges on cellulase adsorption during enzymatic hydrolysis, batch hydrolysis experiments were conducted at 5 FPU g\(^{-1}\) cellulose and the amount of protein in solution was determined over the course of hydrolysis. It was apparent that, during the early stages of hydrolysis, the OSLPP-DL and OSLPP-Sulph (containing approximately 226 and 200 mmol kg\(^{-1}\) of anionic charges and 8 % and 15 % lignin respectively) substrates exhibited slightly higher amounts of free protein than did the OSLPP and Sulph-control substrates (containing approximately 127 and 121 mmol kg\(^{-1}\) of anionic charges and 19 % and 18 % lignin respectively). However, the differences in the amount of free protein became more pronounced at the later stages of hydrolysis, with almost 80 % of the protein added to OSLPP-DL and OSLPP-Sulph remaining free after 72 hours compared to only 50 % for the OSLPP and Sulph-control substrates (Figure 34).
A possible explanation for the more pronounced differences in protein adsorption during the later stages of hydrolysis is that, as cellulose is removed from the insoluble substrate, the proportion of lignin increases, thus increasing the likelihood of cellulase-lignin interactions. However, these interactions are less likely to occur in substrates containing a greater amount of anionic charges in their lignin such as the OSLPP-DL and OSLPP-Sulph. These observations provide further support to the suggestion that increases in enzymatic hydrolysis rates and yields during sulphonation are primarily due to decreased cellulase-lignin interactions, likely mediated through electrostatic repulsion.

![Graph showing protein content in supernatant over time](image)

**Figure 34.** Effects of sulphonation, and oxidative delignification on cellulase adsorption during enzymatic hydrolysis of organosolv pretreated lodgepole pine 5 FPU g⁻¹ cellulose). Substrates were resuspended at 2% (w/v) solids content in 50 mM Na-acetate buffer at pH 4.8. Hydrolysis conditions: 50 °C, 150 rpm. Error bars indicate the standard deviation (n=3).

### 3.4.5 Conclusions

Surprisingly, mechanical treatment of organosolv-pretreated lodgepole pine substrate did not result in significant changes to its susceptibility to enzymatic hydrolysis despite
increases in swelling, fines content, fibre delamination and decreased cellulose crystallinity. In contrast, sulphonation removed of approximately 25% of the residual lignin and significantly improved hydrolysis yields as well as enzyme recovery at low enzyme loadings. These results suggest that, rather than delignification, the improvements in hydrolysis after sulphonation were caused by reduced interactions between lignin and cellulases, likely induced by electrostatic repulsion as a result of increased anionic charges in the residual lignin. This indicated that, aside from complete delignification, (which is likely to be both technically and economically impractical), by increasing the anionic charge of the lignin, it might be possible to improve the overall hydrolysis of pretreated biomass substrates.
Chapter 4: Conclusions and Future Work

4.1 Conclusions

The overall goal of the work described in this thesis was to investigate, and hopefully improve, the interactions between cellulases and lignocellulosic substrates generated by the organosolv process. The working hypothesis was that (through a combination of physical and chemical effects), the residual lignin prevented the efficient enzymatic hydrolysis of organosolv-pretreated softwoods and hardwoods. At the same time we assessed alternative strategies to complete delignification, such as assessing the substrate-related properties that influenced the pretreated substrate’s susceptibility to enzymatic hydrolysis. We anticipated that, if these properties were better characterized, they could be altered to generate substrates that displayed superior susceptibility to enzymatic hydrolysis.

The preliminary work confirmed the inhibitory role that the residual lignin played in limiting the enzymatic hydrolysis of lodgepole pine and hybrid poplar (representing softwoods and hardwoods respectively). As expected, the hybrid poplar displayed greater delignification selectivity and lower recalcitrance than did the lodgepole pine. However, after near-complete delignification, the lodgepole pine substrates were significantly more susceptible to cellulolytic hydrolysis than were the delignified hybrid poplar substrates. This apparent contradiction was due in part to the greater cellulose depolymerization undergone by the lodgepole pine substrates during pretreatment. In addition to confirming the mechanisms behind the inhibitory role of the residual lignin, this work indicated that softwoods could be used as a feedstock for the generation of second generation bioethanol provided that the residual lignin was modified or removed. In an attempt to circumvent the need to completely remove the residual lignin, the role that the other substrate characteristics
might have on increasing the susceptibility of organosolv-pretreated softwoods to enzymatic hydrolysis was next investigated.

By varying the pretreatment solvent and chemical additives, a set of substrates with diverse physicochemical properties and varying degrees of susceptibility to enzymatic hydrolysis were generated. It was hoped that it would be possible to correlate the differences in susceptibility to enzymatic hydrolysis to the various substrate properties. It was evident that the substrates generated under acidic conditions were more susceptible to cellulolytic hydrolysis despite the more selective delignification achieved during alkaline pretreatment. This was likely due to increased cellulose accessibility through a combination of the removal of hemicelluloses, acid-catalyzed cellulose depolymerization and decreased particle size. Overall, these results showed that, although a substrate’s chemical composition is of great importance, it should not be considered in isolation but rather as one of several factors that influences the overall accessibility of cellulose to cellulases (and consequently a substrate’s susceptibility to enzymatic hydrolysis).

Subsequent work investigated the possible effect that fibre length and cellulose DP might have on enzymatic hydrolysis. Surprisingly while the removal of the lignin-rich fines improved the overall cellulose hydrolysis yields, decreases in fibre length did not have a significant effect on the substrates’ susceptibility to enzymatic hydrolysis despite the increase in exterior surface area. Similarly, reductions in cellulose DP did not improve the substrate’s ease of enzymatic hydrolysis. These results support the concept that, rather than a single substrate characteristic such as chemical composition, fibre length or cellulose DP, effecting the ease of hydrolysis it is rather the overall enzyme-accessible surface area (which is influenced in part by those characteristics) that is the key property governing a specific
substrate’s susceptibility to enzymatic hydrolysis. Thus the possibility of utilizing mechanical and chemical treatments to increase cellulose accessibility without altering the substrate’s chemical composition was further investigated.

As expected, the fibre damage caused by the mechanical treatment increased fibre swelling and decreased cellulose crystallinity both of which have been previously associated with the ease of hydrolysis of lignocellulosic substrates. However, because of the uniform nature of the organosolv substrates, the substrate’s accessibility to cellulases and susceptibility to enzymatic hydrolysis were not affected. In contrast a mild chemical treatment in the form of neutral sulphonation increased fibre swelling, removed approximately 25 % of the residual lignin and significantly improved hydrolysis yields as well as enzyme recovery. Further studies revealed that those improvements were due to decreased non-productive interactions between the sulphonated lignin and cellulases rather than because of the partial delignification. This suggested that, while it may be difficult to increase the accessibility of the cellulose component, increasing the hydrophilicity of the residual lignin could be a suitable strategy to reduce the recalcitrance of organosolv-pretreated softwood.

The work described in this thesis provides insights into the interactions between cellulases and lignocellulosic substrates generated by the organosolv process (especially those derived from softwoods). However because of the relatively low price of sugar and ethanol, the organosolv process will likely better suited as the “front-end” of an integrated biorefinery specializing in the production of high value commodity chemicals and cellulose derivatives rather than as a pretreatment for the production of second generation bioethanol.
4.2 Future Work

As mentioned in section 4.1, the work described in the thesis addressed some of the key aspects governing the interactions between cellulases and organosolve treated lignocellulosic substrates. However, several questions remain unanswered from both a fundamental and an applied perspective. The following suggested work might help guide future research.

4.2.1 Development of a Two-Stage Organosolv Pretreatment to Include a Sulphonation Step

The high pretreatment severity required for effective organosolv delignification, results in the formation of significant amounts of sugar degradation products, which in addition to decreasing the fermentable sugar yield can inhibit the fermentation process. However, attempts to decrease pretreatment severity have resulted in substrates that require higher enzyme loadings and extended reaction times to achieve effective cellulose hydrolysis yields (as a result of less extensive delignification). Therefore a possible strategy to decrease the pretreatment severity could be to develop a two-stage organosolv pretreatment. The first stage would be a lower severity organosolv pretreatment to remove the hemicelluloses and partially delignify the substrate (producing the sulphur-free lignin that is characteristic of organosolv process). The second stage would be a sulphonation step, which would increase the hydrophilicity of the residual lignin and decrease the non-productive binding with cellulases. The sulphonation step could be carried out under acidic and neutral conditions, which would likely result in different degrees of sulphonation and delignification. The different substrates would likely exhibit different degrees of ease of enzymatic hydrolysis and cellulase recovery.
4.2.2 Enhancing the Recovery of Hemicellulosic Sugars through Prehydrolysis

As an alternative to the two-stage organosolv pretreatment described in section 4.2.1, it could be possible to increase the hemicellulosic sugars through the incorporation of a prehydrolysis step followed by the organosolv pretreatment. Advantages of this type of two-stage pretreatment would include the ability to alter the prehydrolysis conditions to obtain the hemicellulosic sugars in polymeric or monomeric form. While the monomeric sugars could be used to produce commodity chemicals such as succinic acid, the polysaccharides could be used to develop higher value products such as hydrogels and microgels.

4.2.3 Investigating the Effect of the Initial Feedstock Moisture Content on the Efficiency of Delignification and Cellulolytic Hydrolysis

When the results obtained in section 3.2 (using LPP-B as the feedstock) were compared to those of sections 3.1, 3.3 and 3.4 (using LPP-A as the feedstock), it was evident that the different feedstocks produced substrates with different susceptibilities towards enzymatic hydrolysis (70-80 % cellulose conversion at 5 FPU g\(^{-1}\) cellulose compared to 25 % for substrates produced from LPP-A and LPP-B respectively) despite being subjected to the same pretreatment conditions. A possible explanation for these differences in cellulose conversion is that the two feedstocks exhibited different permeability to the pretreatment liquor as a result of their different moisture contents (14 % vs. 5 % for LPP-A and LPP-B respectively). Moreover, previous work (Cullis et al, 2004) showed that increasing the initial moisture content of softwood chips (from 12 % to 30 %) prior to steam pretreatment resulted in increased ease of delignification, susceptibility toward enzymatic hydrolysis and decreased the formation of sugar degradation products. Therefore it may be possible to improve the efficiency of the organosolv process (and possibly decrease pretreatment severity) by
increasing the moisture content of the wood chips prior pretreatment. Indeed our preliminary work showed that presoaking LPP-A in the pretreatment liquor 24 h before pretreatment produced a substrate with lower lignin content (11 %, Table A2) and somewhat increased susceptibility to enzymatic hydrolysis (81 % after 72 h, Figure A3).

4.2.4 Elucidating the Mechanism of Improved Enzymatic Hydrolysis after Sulphonation

The work reported here showed that sulphonation of the residual lignin improved cellulose conversion yields primarily as a result of decreased non-productive binding between lignin and cellulases. However, it is still not clear if the improvements in hydrolysis yields were caused by the increased hydrophilicity of the residual lignin or by electrostatic repulsion between the negatively charged cellulases and the (also negatively charged) sulphonated lignin. A possible strategy to address this lack of understanding would be to use enzymes such as laccases to graft a variety of hydrophilic groups (anionic, neutral and cationic) onto previously purified residual lignin. The effects of the various modifications on the non-productive binding to cellulases could then be measured. In addition, the use of purified cellulase components would help us elucidate their adsorption and desorption properties.

4.2.5 Altering the Composition of the Pretreatment Liquor to Increase the Hydrophilicity of the Residual Lignin

The work reported in this thesis showed that increasing the hydrophilicity of the residual lignin via neutral sulphonation improved the cellulose conversion yields by decreasing the non-productive binding of cellulases to lignin. One of the features of the ethanol organosolv process is the incorporation of the ethanol and water into the lignin
during pretreatment. Therefore it may be beneficial to alter the pretreatment liquor to create a ternary mixture composed of water, ethanol and a third solvent that after its incorporation into the residual lignin could increase its hydrophilicity. Possible solvents include low molecular weight polyalcohols and hydroxy acids such as ethylene glycol, glycerol and glycolic acid respectively. The greater enzyme recovery in the hydrolysate could also simplify future enzyme recycling strategies.

4.2.6 Confirming the Uniform Chemical Composition of Organosolv-Pretreated Substrates

The work reported in the thesis strongly suggested that the most important substrate characteristic affecting the ease of enzymatic hydrolysis of the organosolv-pretreated substrates is their overall accessibility of cellulose to cellulases. However, in this study, we were unable to increase cellulose accessibility through a mechanical treatment (PFI-mill refining). This contradicts previous studies that had shown that refining of kraft pulps and steam pretreated substrates improved their susceptibility to enzymatic hydrolysis. A possible explanation for those findings was that the organosolv-process generates substrates that have a uniform chemical composition and, as a result (and unlike kraft pulps and steam pretreated substrates) the removal of the surface material through refining would not have resulted in increased cellulose accessibility. The chemical characterization of the organosolv-pretreated substrates before and after removal of the surface material through a combination of surface analysis techniques such as atomic force microscopy and XPS as well as bulk characterization techniques such as compositional analysis, and Simons’ stain would provide support to this hypothesis.
4.2.7 Extending Organosolv Pretreatment to Include Other Softwoods and Enzyme Preparations

The main goal of the work described in the thesis was to further understand the influence of substrate-related factors on the susceptibility of organosolv-pretreated softwoods to enzymatic hydrolysis. This objective was met using a single cellulase preparation and mountain pine beetle-killed lodgepole pine as the feedstock. Having established the beneficial effects of increasing the hydrophilicity of the residual lignin and the difficulties associated with attempting to increase the cellulose accessibility, it would be of interest to see if similar effects are observed when using other softwood feedstocks such as Douglas-fir, or radiata pine. Moreover, the current generation of commercial cellulase preparations (such as Novozymes’ Cellic® Ctec3) has been formulated to include significant hemicellulolytic activity. Therefore, it would of interest to determine whether it would be possible to increase the recovery of hemicellulosic sugars in the solid fractions (though a decrease in pretreatment severity), while still achieving effective cellulose conversion yields.
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Appendices

Appendix A  Supplementary Data

Table A1. Chemical composition (% w/w) of untreated mountain pine beetle killed lodgepole pine and hybrid poplar feedstocks\(^a\).

<table>
<thead>
<tr>
<th>Component</th>
<th>Arabinan</th>
<th>Galactan</th>
<th>Glucan</th>
<th>Xylan</th>
<th>Mannan</th>
<th>Lignin(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPP-A</td>
<td>1.6 (0)</td>
<td>2.7 (0.1)</td>
<td>48.2 (0.3)</td>
<td>5.2 (0.1)</td>
<td>8.7 (0.2)</td>
<td>29.4 (0.3)</td>
</tr>
<tr>
<td>LPP-B</td>
<td>1.1 (0.1)</td>
<td>1.9 (0.1)</td>
<td>47.5 (2.8)</td>
<td>6.3 (0.4)</td>
<td>10.9 (0.5)</td>
<td>26.3 (0.4)</td>
</tr>
<tr>
<td>POP</td>
<td>0.6 (0.1)</td>
<td>0.7 (0.1)</td>
<td>49.3 (0.9)</td>
<td>17.6 (0.2)</td>
<td>2.7 (0.3)</td>
<td>25.4 (0.6)</td>
</tr>
</tbody>
</table>

\(^a\) % dry extractive-free wood.

\(^b\) = acid soluble lignin + insoluble (klason) lignin.
Figure A1. Sample determination of maximum adsorption capacity of the OSLPP-LS substrate for bovine serum albumin (BSA). A) Langmuir adsorption isotherm and B) linear regression (Lineweaver-Burk plot) of the adsorption isotherm. The y-intercept of the Lineweaver-Burk plot corresponds to the maximum adsorption capacity of the OSLPP-LS substrate for BSA. Error bars on the isotherm indicate the standard deviation of three separate experiments (n = 3).
Figure A2. Sample plot of $\eta_{sp}/c$ vs. $c$ (substrate concentration) for the OSLPP-LS substrate. The y-intercept (determined by extrapolation of the straight line) corresponds to the substrate’s intrinsic viscosity.
**Table A2.** Chemical composition (%, w/w) of presoaked organosolv-pretreated lodgepole pine (OSLPP-LS presoaked). Numbers in parenthesis indicate the standard deviation (n = 3). Pretreatment conditions: 65 % (v/v) EtOH, 1.1 % (w/v) H₂SO₄, 170 °C, 60 min.

<table>
<thead>
<tr>
<th></th>
<th>Arabinan</th>
<th>Galactan</th>
<th>Glucan</th>
<th>Xylan</th>
<th>Mannan</th>
<th>Lignin^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>bdl^a</td>
<td>bdl</td>
<td>89.6 (2.7)</td>
<td>1.7 (0.1)</td>
<td>1.5 (0.0)</td>
<td>11.0 (0.4)</td>
<td></td>
</tr>
</tbody>
</table>

^a = below detection limit  
^b = acid soluble lignin + insoluble (klason) lignin.
Figure A3. The effect of preincubating the feedstock with the pretreatment liquor prior to pretreatment on the enzymatic hydrolysis of organosolv-pretreated loblolly pine. Hydrolysis conditions: 2% (w/v) solids content, 50 mM Na-acetate buffer pH 4.8, 50 °C, 150 rpm. Error bars indicate the standard deviation (n=3).