THE INHIBITORY ROLE OF LIGNIN IN THE ENZYMATIC HYDROLYSIS OF SOFTWOODS

by:

Arwa Kurabi
B.Sc., Ryerson University, 2002

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ABSTRACT

Ethanol from biomass is one of the most promising technologies for the production of a renewable and environment friendly liquid biofuel. Currently, industrial production of ethanol is mostly based on sugar or starch-based substrates. However, ethanol produced through the bioconversion of lignocellulosic substrates such as woody and agricultural residues by enzymatic hydrolysis and fermentation, is a promising way of producing a green fuel.

Softwoods are characterized by a high lignin content, which makes their enzymatic hydrolysis and eventually conversion to ethanol extremely difficult. Pretreatment of softwoods makes the substrate more accessible to cellulose-degrading enzymes (cellulases) and facilitates the cellulose degradation by modifying the physicochemical properties of the substrate. Different studies have shown that the ability of cellulases to hydrolyze pretreated softwoods is limited by various substrate factors such as, porosity, cellulose crystallinity, available surface area and the physicochemical characteristics of the residual lignin. Although the influence of cellulose properties on the enzymatic hydrolysis of lignocellulose has been studied extensively, the role of lignin as a substrate limiting factor has been more difficult to elucidate.

This dissertation has focused on aspects relevant to the improvement of the enzymatic hydrolysis of softwood substrates. The first series of experiments were designed to address the possible inhibitory role of lignin in the enzymatic hydrolysis of lignocelluloses. A quantitative approach to compare the inhibition ability of lignin with other classical cellulase inhibitors was developed and assessed. In a related series of
experiments, novel cellulase complexes, characterized by their higher hydrolytic ability on lignocellulosic substrates were also evaluated.

A quantitative evaluation of the impact of lignin on the hydrolytic ability of various carbohydrases was performed. The study demonstrated that the magnitude of the lignin inhibition, on a concentration basis, was comparable to that of classical cellulase inhibitors. The inhibition by lignin followed a mixed-type pattern (competitive or uncompetitive, depending on the enzyme and substrate assayed). The second part of the research showed that a novel *Penicillium* sp. cellulase complex was more effective in the enzymatic hydrolysis of the pretreated softwood than commercially available cellulases. It was apparent that the *Penicillium* sp. cellulases yielded up to 2.5-fold more glucose from softwood substrates than was obtained when hydrolysis was carried out using *Trichoderma* sp. enzymes.

Thus, a novel enzyme complex with a particularly high hydrolytic ability was identified and its application to the hydrolysis of pretreated softwood was demonstrated. Naturally occurring high levels of xylanase and β-glucosidase activities and the presence of weaker lignin-binding cellulases were postulated to be the reasons for the better performance of the *Penicillium* sp. enzymes. It was suggested that the increase in β-glucosidase activity reduces the cellobiose end-product inhibition, while the increase in xylanase activity increases the enzyme accessibility to cellulose by removing the shielding xylan. Weaker lignin-cellulase interactions lead to more free enzymes in solution. If this hypothesis is confirmed in the future, it could be used as a basis for further improvement of the commercially available cellulase complexes for lignocellulose bioconversion.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AFEX</td>
<td>Ammonia fiber explosion treatment</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>β-G</td>
<td>Beta-glucosidase</td>
</tr>
<tr>
<td>CBD</td>
<td>Cellulose binding domain</td>
</tr>
<tr>
<td>CBU</td>
<td>Cellobiase units</td>
</tr>
<tr>
<td>CBU I</td>
<td>Cellobiohydrolase I</td>
</tr>
<tr>
<td>CBU II</td>
<td>Cellobiohydrolase II</td>
</tr>
<tr>
<td>CD</td>
<td>Catalytic domain</td>
</tr>
<tr>
<td>CMC</td>
<td>Carboxymethyl cellulose</td>
</tr>
<tr>
<td>cm</td>
<td>Centimeters</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>DNS</td>
<td>Dinitrosalicylic acid</td>
</tr>
<tr>
<td>DP</td>
<td>Degree of polymerization</td>
</tr>
<tr>
<td>EG I</td>
<td>Endoglucanase I</td>
</tr>
<tr>
<td>EG II</td>
<td>Endoglucanase II</td>
</tr>
<tr>
<td>EI</td>
<td>Enzyme inhibitor complex</td>
</tr>
<tr>
<td>ES</td>
<td>Enzyme substrate complex</td>
</tr>
<tr>
<td>ESI</td>
<td>Enzyme substrate inhibitor ternary complex</td>
</tr>
<tr>
<td>FPU</td>
<td>Filter paper unit</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transformed infrared spectroscopy</td>
</tr>
<tr>
<td>g</td>
<td>Grams</td>
</tr>
<tr>
<td>hr.</td>
<td>Hour</td>
</tr>
<tr>
<td>HEC</td>
<td>Hydroxyethylcellulose</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>H₂SO₄</td>
<td>Sulphuric acid</td>
</tr>
<tr>
<td>M</td>
<td>Molar concentration (mol/L)</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Matrix-assisted laser desorption and ionization time-of-flight</td>
</tr>
<tr>
<td>min</td>
<td>Minutes</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliters</td>
</tr>
<tr>
<td>µL</td>
<td>microliters</td>
</tr>
<tr>
<td>Na₂CO₃</td>
<td>Sodium carbonate</td>
</tr>
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<td>Sodium chloride</td>
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<td>Sodium hydroxide</td>
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<td>nm</td>
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<tr>
<td>µmol</td>
<td>Micromole</td>
</tr>
<tr>
<td>%</td>
<td>Percent</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>Psi</td>
<td>Pounds per square inch</td>
</tr>
<tr>
<td>rpm</td>
<td>Rotations per minute</td>
</tr>
<tr>
<td>SO₂</td>
<td>Sulphur dioxide</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per volume</td>
</tr>
<tr>
<td>w/w</td>
<td>Weight per weight</td>
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I dedicate this work to my beloved and lovely Nada
1. INTRODUCTION

Lignocellulosics-to-ethanol bioconversion can provide an environmentally friendly alternative fuel to gasoline, thereby reducing dependence on non-renewable energy sources and improving air quality. A range of lignocellulosic biomass sources, such as agricultural residues (straw, and corn stover), food-processing waste, forestry residues (thinnings, and mill wastes), municipal solid wastes (paper, cardboard, and yard trash), wastes from the pulp and paper industry, and herbaceous energy crops (e.g. switchgrass) have been identified to have the potential to serve as low cost, abundant feedstocks for the production of fuel ethanol. As a fuel, ethanol has limited emissions, and hence decreases the overall levels of emissions released into the environment. Also, it employs a feedstock that is renewable and otherwise would be land filled or considered waste. The bioconversion of biomass-to-ethanol can reduce the undesirable impacts of traditional waste management practices, such as field burning or wood waste incineration, which can result in air pollution and groundwater contamination, in addition to various other harmful effects.

Ethanol has been used as a fuel in the United States as far back as 1908. Nevertheless, early efforts to sustain a U.S. ethanol program failed because petroleum and natural gas fuels became available in large quantities at low cost, thus, leading to the elimination of the economic incentives for production of liquid fuels from crops. However, due to political and economical tribulations that occurred in the Middle East during the 1970s, oil supply disruptions and rising environmental concerns over the use of lead as a gasoline octane booster renewed interest in ethanol again. Major oil
companies began to phase out lead from gasoline and market ethanol as a gasoline volume extender and as an octane booster (Wyman and Hinman, 1990). Ethanol was blended directly into gasoline in a mix of 10 percent ethanol and 90 percent gasoline, called gasohol (http://www.nrel.gov/clean_energy/bioenergy.html, 2004).

Currently, ethanol is produced (from sugar cane and corn) in large quantities in Brazil and the United States to replace gasoline in motor vehicles. Ethanol production in the United States grew from 175 million gallons in 1980 to 1.4 billion gallons in 1998, with support from U.S. Federal and State ethanol tax subsidies and the mandated use of high-oxygen gasoline. Further, the United States plan to increase the production of biomass transportation fuels from 0.5% of U.S. transportation fuel consumption in 2001 to 4% by 2010, 10% by 2020, and 20% by 2030 (http://www.eere.energy.gov/biomass/, 2004). In Canada, government incentives such as tax breaks and legislations established in the late 1960s have increased the interest in the biofuels programs. Moreover, in 1997, Canada signed the Kyoto Protocol and made a commitment to reduce its carbon emissions to 6% below 1990 levels by 2008-2012 (www.greenfuels.org, 2002). Therefore, exploring renewable energy and reducing environmental impacts are the main tasks of the energy industry in the future for most developed and developing countries.

Currently, a significant end use of ethanol is its utilization as an ‘oxygenate’ in extending the volume of conventional gasoline. The higher octane number of ethanol increases the octane number of the mixture, reducing the need for the toxic, octane-enhancing additives. However, the cost of fuel ethanol must be approximately closer to the wholesale price of gasoline to succeed in current markets. Therefore, in order for ethanol to compete on its own merits, the cost of producing it must be reduced
substantially. Although the process is expensive at the present date, advances in contemporary process design and technology could decrease conversion costs notably.

It is evident that a large number of plants containing chemical building blocks, such as cellulose, starch and sugar, can be transformed into ethanol (IEA Bioenergy task, 2000). Sugar-containing plants, such as sugar cane, sweet sorghum and Nipa palm are the best candidates for the simple conversion of sugar-to-ethanol fuels due to the straightforwardness of the conversion process (Figure 1). The sugar can directly be fermented to ethanol. Starch-containing crops, such as cassava, sweet potatoes, yams, taro and tannia, are also good candidates for ethanol feedstock, but require an additional hydrolysis step to break down starch to its monomeric sugar form before fermentation (Figure 1). However, the high cost of such feedstocks is a major drawback. Substantial reductions in ethanol production costs can be made possible by using the less expensive cellulose-based feedstocks. Cellulosic feedstocks include agricultural wastes, grasses and woods, and other low-value biomass such as municipal waste. The work in this thesis has focused on the generation of ethanol from the more abundant, cellulose-containing biomass from softwood tree species.

Softwood lignocellulosic residues are considered to be a potential source of cheap and renewable feedstock for bioconversion in northern countries, such as Sweden, Canada and Western USA. In British Columbia, softwood forest residues comprise the most abundant source of raw materials available for bioenergy production. In 1998, the growing stock volume in B.C. totaled 10,041.5 million cubic meters, of which softwood account for 94.5% of total (www.cofi.org, 2000). Annually, British Columbia’s lumber industry generates some 76.9 million cubic meters of wood consisting mainly of
lodgepole pine (20%), spruce (13%) from the interior region and true firs (10%) and Douglas-fir (9%) from the coastal region. These results in around 8 million tones of wood residues generated annually. Approximately 5.7 million tones of residues are utilized in various forms (energy production and value-added uses) and 2.06 million tones per year are surplus to current production needs (www.nccp.ca/NCCP/pdf/Canada's_Wood_Residues.pdf, 1999). Therefore, there are sufficient amounts of residual woody biomass for the production of bioethanol in British Columbia.

Although, cellulosic materials are less expensive, they are more costly to convert to ethanol because of the necessary extensive processing (Figure 1, Figure 2). Due to the recalcitrant nature of these feedstocks, they need special pretreatment before being used to produce glucose that can be further fermented to alcohol (Hayn et al., 1993). The primary purpose of pretreatment is to maximize subsequent bioconversion yields. The enzymatic hydrolysis step is another major cost contributor to the overall softwood-to-ethanol process. These substrates are hydrolyzed very slowly by cellulases (Lee and Fan, 1983). In addition, cellulase enzymes used to convert cellulose to sugar are currently too expensive for commercial use. Nevertheless, current advances in biotechnology could lower costs by reducing the cost of enzymes (Fan et al., 1987, Gregg et al., 1998). Some of the approaches used to try to enhance the enzymatic hydrolysis step include improvement of specific activity of the enzymes, increase thermal stability of the enzymes; decrease non-specific binding, decrease feedback inhibition, increase catalytic activity, increase operational stability and finally, recovery and recycling (Walker and Wilson, 1991; Gregg and Saddler, 1997). A better understanding of the factors that
influence and limit the rate and extent of the enzymatic hydrolysis reaction would provide the basis for optimizing the cellulose bioconversion processes.

The hydrolysis of cellulose to glucose has been shown to be the rate-limiting step in the production of ethanol from cellulosic substrates. Cellulose hydrolysis limiting factors have been subjected to extensive studies in the past years. However, despite many years of research, a cost-effective bioconversion process for the enzymatic conversion of lignocellulosics has not yet been developed (Gregg et al., 1998). The complexity of the enzymatic hydrolysis reaction has been proven to be a major obstacle towards various

Figure 1. Overview of the biomass to ethanol process for different feedstocks. The figure shows the trade-off between feedstock costs vs. process complexity. (Source: Gregg, D., Faculty of Forestry-Dept. of Wood Science, 2004)
attempts trying to increase the rate of cellulose hydrolysis. The reaction progress is convoluted by enzymatic changes and substrate structural modification, which occur throughout the hydrolysis process. Nevertheless, unproductive binding of cellulases in general and particularly by lignin, is considered by many researchers to be one of the main limiting factors of enzymatic hydrolysis of softwoods (McMillan, 1994; Mooney et al., 1998). It is believed, and in some cases, it has been confirmed, that a reduction of this negative factor leads to a significant enhancement of the enzyme effective activity (Alksrawi et al., 2003; Berlin et al., 2004).

As suggested previously, the difficulty in converting softwood residue to ethanol is related to the complex nature of this feedstock. In order to understand the softwood-to-ethanol process treatment problems, the following section contains essential background information on the substrate structural characteristics.

**Figure 2.** Steps in the bioconversion of lignocellulosic residues to ethanol.
1.1 Structural features of softwood lignocellulosic materials

Structural and chemical features of the woody lignocellulosic substrates influence the rate and extent of their enzymatic degradation. The chemical construction of woody cells reflects on wood’s function as a structural and supportive material. Woody cell walls are composed of three major organic polymers: cellulose, lignin and hemicellulose, and the minor extractives, which combine to form the woody cellular structure. Variations in the characteristics and volume of the four components and differences in cellular structure depend on several factors, including species and age, and the part of the tree from which it was extracted. These natural variations result in some woods being hard and heavy and some light and soft, some strong and some weak, some naturally durable and some prone to decay. Differences in chemical composition account for the great variation in wood and its properties. A brief description of each of the chemical components follows.

1.1.1 Cellulose

Cellulose is the most widely distributed plant skeletal polysaccharide. It constitutes about half of the cell wall material of wood (Table 1). This linear polymer is made of D-glucose units joined by β-1,4-glycosidic bonds. The successive glucose residues are rotated by 180° relative to each other (Fengel and Wegener, 1983), and thus the repeating unit of the cellulose chain is the cellobiose unit (Figure 3). The rotation causes the cellulose to be highly symmetrical with equal number of hydroxyl groups on each side of the chain. The degree of polymerization (DP) is high, about 10,000 glucose units depending on the source (Haygreen and Bowyer, 1996). The cellulose chain functional groups are the hydroxyl groups, which are able to interact with each other or with other functional groups. Cellulose fibers are arranged in parallel polysaccharide chains, with
crystalline and amorphous regions, held together by hydrogen bonds which give the fibers their high mechanical strength. It has been proposed that highly crystalline materials are less prone to enzymatic breakdown than amorphous materials (Sasaki et al., 1979). Amorphous regions form where irregular cellulose chain organization occurs, and have the ability to absorb water due to available hydrogen bonding.

In the cell wall, cellulose chains are aligned into bundles that are laid down in a spiral around the walls of each cell forming the elementary fibrils. The, elementary fibrils are organized into several layers of microfibrils with different orientations or microfibril angles. Bunches of the elementary fibrils are embedded in a matrix of hemicellulose. The exterior of the microfibriles formed closely association with hemicelluloses and lignin via covalent bonds (Haygreen and Bowyer, 1996). Consequently the isolation of cellulose requires intensive chemical treatments. In view of that, the close association of the highly ordered cellulose skeleton with lignin and hemicellulose makes the woody rigid and protected structure highly resistant to hydrolytic attacks (Kadla and Gilbert, 2000; Hon and Shiraishi, 2001).

![Figure 3. Cellulose is comprised of linear chains of glucose linked by β-1,4-glycosidic bonds. (http://www.unilim.fr/theses/2002/sciences/2002limo0025/these_body.html)](http://www.unilim.fr/theses/2002/sciences/2002limo0025/these_body.html)
1.1.2 Hemicellulose

Hemicellulose is a semi-amorphous heteropolysaccharide made of several sugars such as glucose, galactose, xylose, arabinose, and mannose. Unlike cellulose, which is made only from glucose, hemicellulose consists of glucose and several other water-soluble sugars produced during photosynthesis. At different sites, the linear sugar backbone is substituted with acetyl or glucuronic acid groups. Hemicellulose has a low molecular weight with a degree of polymerization between 120-250 and it makes up 15 to 25 percent of the dry weight of wood (Haygreen and Bowyer, 1996). As a supporting material in cell walls, it associates with lignin in a matrix surrounding cellulose bundles. Furthermore, hemicellulose is species dependent and therefore is different in type and amount between hardwoods and softwoods. Hemicellulose in hardwoods and annual plants is mainly xylan (15–30%), whereas softwood hemicelluloses consist of galactoglucomannans (15–20%) and xylans (7–10%) (Sjöström, 1993).

Table 1. Structural and chemical composition of some lignocellulosic feedstocks (Sjöström, 1993).

<table>
<thead>
<tr>
<th>Chemical Composition (% dry weight)</th>
<th>Hardwoods</th>
<th>Softwoods</th>
<th>Straw</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose</td>
<td>45</td>
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<tr>
<td>Hemicellulose</td>
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<td>25</td>
<td>36</td>
</tr>
<tr>
<td>Lignin</td>
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<td>30</td>
<td>15</td>
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<table>
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<th>Softwoods</th>
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<td>90-95</td>
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<tr>
<td>Vessel elements</td>
<td>60-70</td>
<td>&lt;2</td>
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</tr>
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</table>

n/a-not applicable
1.1.3 Lignin

Lignin is a three-dimensional, highly branched amorphous resin (non-carbohydrate). Its basic building unit is phenylpropane and it is synthesized by free radical polymerization of phenylpropanoid monomers initiated by the enzyme peroxidase (Haygreen and Bowyer, 1996). The functional groups affecting the reactivity of lignin include free phenolic hydroxyl, methoxyl, benzylic hydroxyl, benzyl alcohol, non-cyclic benzyl ether and carbonyl groups. The degree of polymerization is fairly low, approximately 50-500dp and it is formed last during cell development. Lignin occurs in the matrix of the plant cell walls where it permeates the entire cell wall but the cellulose/lignin ratio varies between each layer. For instance, lignin makes 65% of the middle lamellae and primary wall, where it acts as a glue binding cells together (Haygreen and Bowyer, 1996). Lignin is also present in the secondary cell walls after the cells have differentiated and ceased growing (Hon and Shiraishi, 2001). The complex heterogeneous polymer associates with cellulose and hemicellulose to provide strength and rigidity to plant cells. The lignin coating the cellulose fibers provides a further barrier to hydrolytic attack by cellulases (Gharpuray et al., 1983; Sinitsyn et al., 1991).

Lignin is thermoplastic and becomes pliable at high temperatures and hard again when it cools. Therefore, the chemical structure of native lignin is essentially changed under high temperature and acidic conditions, such as the conditions used during steam pretreatment. The subsequent formation of the melted lignin into globules may expose cellulose further to cellulase attack (Saddler et al., 1982; Wong et al., 1988). Lignin has also shown to be agglomerated into smaller particles and separate from cellulose at high temperatures (200°C). Hence, isolation of native lignin is complicated, when at all
possible (Fengel and Wegener, 1983; Tanahashi et al., 1983). Recent observations provide indications that lignin is not homogeneous in structure. In fact, lignin seems to consist of amorphous regions and structured forms, such as oblong particles and globules (Novikova et al., 2002).

The cellulose and hemicellulose content is higher in hardwoods than softwoods. Consequently, the lignin content of softwoods is usually higher than that in hardwoods. Table 1 shows the chemical composition differences between agricultural residues, softwood, and hardwood. Fundamentally, they have similar cellulose content; however, agricultural residues have more hemicellulose (up to 35%) than softwoods (25%) and hardwoods (27%). The main difference amongst them is the lignin content, with softwoods containing 30%, while hardwoods contain 23% (Haygreen and Bowyer, 1996). Several past studies have found that an increase in the enzymatic hydrolysis rate was related to a decrease in the lignin content of the substrates (Gharpuray et al., 1983; Sinitsyn et al., 1991). The lignin types and distribution are also different between softwoods and hardwoods. Guaiacyl lignin occurs in almost all softwoods, and a mixture of guaiacyl and syringyl lignin is typical of hardwoods. In addition, guaiacyl lignin contains more phenolic hydroxyl groups than does syringyl based lignin (Sjöström, 1993). It has been suggested that guaiacyl lignin are more resistant to chemical pretreatment than syringyl lignin (Ramos et al., 1992). A more resistant structure of guaiacyl lignin has also been observed in degradation studies of synthetic lignins by the lignin-degrading fungus *Phanerochaete chrysosporium* (Faix et al., 1985).

In softwoods, almost 70% of the lignin is located in the secondary wall. For hardwoods, there is no certain lignin content in the secondary wall due to the
heterogeneous nature of wood. However, the lignin in the secondary wall of hardwoods has a high content of syringyl units in fiber and ray cells, whereas guaiacyl units are present in the middle lamella in vessel cells. As a result, it has been found that softwoods are more resistant to lignin removal by alkaline extraction in comparison to hardwoods (Ramos et al., 1992). It was observed that the residual substrate remaining after extensive hydrolysis of steam pretreated aspen and eucalyptus was mainly composed of vessel elements which are known to have a greater guaiacyl to syringyl ratio than other cells found in hardwood (Ramos et al., 1992). However, to date, little work has been done on characterizing the lignin types after substrate pretreatment, which in all probability has a major affect on lignocellulosic hydrolysis and cellulases inhibition.

1.2 Pretreatment of lignocellulosic biomass

The physical structure of cellulose in native lignocellulose is intrinsically resistant to enzymatic attack and cellulose is further protected by the surrounding matrix of lignin, hemicellulose and pectin. During the bioconversion of lignocellulosic substrates, the pretreatment methods influence the hydrolysis efficiency significantly (Saddler et al., 1993). Pretreatment removes and degrades the hemicellulose and lignin, and facilitates the accessibility of cellulases to cellulose. Different pretreatment methods would produce different lignin contents and types (Lynd et al., 2002). Pretreatment of lignocellulosic biomass is crucial to enzymatic hydrolysis. Ideally, pretreatment of wood residues should lead to optimal recoveries of hemicellulose sugars in the monomeric form, easily-hydrolysable water-insoluble cellulosic residues, and high product yields in the subsequent fermentation step, with minimal costs. The formation of degradation products from lignin and sugars, such as furfural and hydroxymethyl furfural, which are known to
inhibit the fermenting organisms, should be minimized. Pretreatment should also facilitate the recovery of lignin and other non-fermentable constituents for their conversion to co-products and simplify downstream processing of pretreated raw material. However, in practice not all of these goals can be achieved with any current pretreatment methods. Instead, a compromise is sought between all of these desirable characteristics to a certain magnitude (Nguyen et al., 1998, Wu et al., 1999).

Numerous pretreatment options are now being explored to fractionate, solubilize, hydrolyze, and separate cellulose, hemicellulose and lignin components. These include mild acid or alkaline, hot water, SO₂, hydrogen peroxide, ammonia fiber explosion (AFEX), enzymatic hydrolysis, and organic solvent treatments (McMillan, 1994). Several of these pretreatment processes are now under techno-economic evaluation (Gregg et al., 1998; Wingren et al., 2003).

Pretreatment effectiveness is highly dependent on the structural, chemical and physical properties of the feedstock. Agricultural residues, such as corn stover, contain low lignin content and are more readily pretreated than softwood residues that contain higher amounts of lignin and have a much more rigid and compact cellular structure, composed mainly of tracheid fiber cells. Hardwoods are also easier to pretreat due to the presence of vessels that permit greater penetration of heat, chemicals, and enzymes into the biomass matrix (McMillan, 1994). Presently, the costs for pretreatment and delignification, and the cost of enzymes for cellulose hydrolysis, are the two main economic obstacles deterring commercial bioconversion (Gregg et al., 1998; Wingren et al., 2003).
1.3 Enzymatic hydrolysis of lignocellulose

1.3.1 Cellulase systems

Microorganisms such as bacteria, actinomycetes, and fungi produce a complex array of hydrolytic enzymes during growth on cellulosic substrates. However, the cellulase system of *T. reesei* (initially named *Trichoderma viride*) has been the early focus of extensive research for the past 50 years (Nevalainen, 1980; Bailey et al., 1981; Olsson et al., 2003; Lemos et al., 2003). Microorganisms of the genera *Trichoderma* and *Aspergillus* are generally considered to be cellulase producers, and crude enzymes produced by these microorganisms are commercially used in textile, food and detergent industries. Microorganisms of the genus *Trichoderma* produce relatively large quantities of endo-β-glucanase and exo-β-glucanase, but only low levels of β-glucosidase, while those of the genus *Aspergillus* produce relatively large quantities of endo-β-glucanase and β-glucosidase with low levels of exo-β-glucanase production (Dekker, 1985). In general, the total mass of cellulases secreted by filamentous fungi is greater than that secreted by cellulolytic bacteria. More recently however, interest in bacterial cellulases has grown, partly due to novel advances in molecular biology techniques. To date, most of the cellulase genes cloned are from bacterial sources; partly due to the relative ease of the cloning process. Cellulases from *Cellulomonas fimi* has be one of the best characterized bacterial cellulases (Gilkes et al., 1991).

Typically, cellulase enzyme profiles are dominated by exoglucanases and endoglucanases (cellbiohydrolases) that are directly involved in cellulose hydrolysis. The complete degradation of cellulose to glucose requires the cooperative action of at least three different kinds of enzymes: cellbiohydrolases, endoglucanases and β-
glucosidases (Esteghlalian et al., 2001). It is believed that the hydrolysis starts with endoglucanases (EGs) randomly attacking the β,1-4 glycosidic linkages in the amorphous regions of the cellulose. This significantly reduces the degree of polymerization of the substrate and creates new chain ends that facilitate the accessibility for the exoglucanases (CBHs) to split off glucose, cellobiose, and small oligosaccharide units (Figure 4). Subsequently, β-glucosidases hydrolyze cellobiose and cellotriose into single glucose molecules (Kleywegt et al., 1997). Accordingly, β-glucosidases are not classified as cellulases because they hydrolyze cellobiose not cellulose.

Cellulases also include a range of accessory enzymes, including some responsible for the hydrolysis of hemicellulose and the pectin matrix (Lynd, 2002). β-glucosidase, together with most cellobiohydrolases and xylanases, are known to hydrolyse their substrate with retention of the anomeric configuration, as was first demonstrated by Parrish and Reese. Mechanistically, this implies that these enzymes operate by the double displacement mechanism (Trimbur et al., 1993).

A study done by Clarke et al. (1993) concluded that β-glucosidases from different sources, utilize a double displacement mechanism to hydrolyze their substrates. Two acidic residues (Asp/Glu) at the active center participate in the hydrolysis and there is a covalently linked enzyme-substrate intermediate. One functions as a nucleophile in the formation of the covalent enzyme-glycosyl intermediate and the other as the acid-base catalyst to protonate the leaving group (glucose) and then deprotonate the water molecule. Another study done by Trimbur et al. (1993) concluded that the substrate (aglycon) binding and catalytic sites are different and distinct because enzyme inactivated by substrate analogues can continue catalysis.
1.3.1.1 *Trichoderma reesei* cellulases

Filamentous fungi, *Trichoderma sp*, are usually the preferred source of industrial cellulase complexes, primarily because their capacity for extracellular protein production is greater than that of cellulolytic bacteria. These organisms are capable of secreting an excess of 30 g/L of protein into the extracellular medium (Conesa et al., 2001). *T. reesei* produces three groups of enzymes involved in the hydrolysis of cellulosic substrates. These include cellobiohydrolases (E.C.3.2.1.91; CBHs, exoglucanases), endoglucanases (E.C.3.2.1.4, EGs) and β-glucosidases (E.C. 3.2.1.21). *T. reesei* produces at least two exoglucanases (CBH I and CBH II), five endoglucanases (EG I, EGIII, EGIV, EGIV and EGV), and two β-glucosidases (β-G) (Kubicek, 1992). CBH I and CBH II hydrolyze the reducing and non-reducing ends of cellulose chains of microcrystalline cellulose respectively. Moreover, they are the major components of *T. reesei* cellulase, representing 60% and 20% of total protein content correspondingly (Teeri, 1997). Endoglucanases (EGs) make-up less than 20% of the total protein content of *T. reesei* cellulase complex. Meanwhile, *T. reesei* complex is deficient in β-glucosidase and usually requires supplementation. β-glucosidase plays an important role in the hydrolytic process. In the past, the break down of cellobiose to two glucose molecules was considered to probably be the rate limiting step in cellulose hydrolysis. This is because β-glucosidase is inhibited by its end product, glucose. Therefore, the substrate cellobiose accumulates and in turn inhibits the cellulase complex. If the rate of cellobiose hydrolysis could be increased, cellulose hydrolysis rate would be substantially improved (Dekker, 1985).
All *T. reesei* cellulases, except EG III, as well as many other cellulases from other fungi, have a two domain structure consisting of a larger catalytic domain (CD) and a smaller cellulose binding domain (CBD), held together by a flexible glycosylated peptide linker (Gilkes et al., 1991). It has been elucidated that both catalytic domain and binding domain of CBH I bind specifically to the cellulose surface (Linder et al., 1995). The CBD mainly improves the binding of the catalytic domain to crystalline cellulose and enhances the enzymatic hydrolysis of insoluble substrates. In addition, it has been reported that CBD from *T. reesei* could improve the hydrolytic activities of cellobiohydrolase I (CBH I) and endoglucanases (Lemos et al., 2003). The catalytic domain without CBD would lose most of its hydrolytic ability towards insoluble cellulose substrates but not on soluble cellulose substrates.

Thus far, crystal structures have been determined for the catalytic domains of *T. reesei* CBH I, CBH II and EG I (Divne et al., 1994; Kleywegt et al., 1997; Rouvinen et al., 1990). Three-dimensional structures of CBD's are also available and their structure-function interactions with cellulose have been studied extensively. Both CBH I and CBH II have an enclosed tunnel structure. It is believed that the presence of the tunnel structure allows for close interaction between the catalytic domain and the loose chain ends appearing on the crystalline cellulose surface (Divine et al., 1994). In contrast, the catalytic domain in EGs is more open in structure and resembles a cleft. This reveals the aromatic residues and enables them to interact with the amorphous cellulose surface more effectively.

X-ray crystallography has elucidated to the three dimensional structure of CBH I, and revealed that this enzyme has a large globular head connected to a flexible tail by a serine
and threonine-rich hinge (Schmuck et al., 1986). The 50 Å long active site tunnel contained 11 different glucosyl-binding sites. Four tryptophan residues evenly spread along the tunnel are the major determinants for the formation of the -7, -4,-2 and +1 glucosyl-binding sites. Also, cellobiose was found to bind almost exclusively to the +1 and +2 subsites (Teeri, 1997; Kleywegt et al., 1997). In CBH II, two well-ordered loops form a 20 Å long tunnel (Rouvinen et al., 1990).

Different cellulases have been shown to synergistically hydrolyze microcrystalline cellulose. Endo-exo synergism has been found during the hydrolysis of Avicel and bacterial microcrystalline cellulose (Henrissat et al., 1985). Further, CBH I and CBH II have demonstrated exo-exo synergism (Medve et al., 1994, Barr et al., 1996). During hydrolysis, some synergism could be lost due to the inactivation of certain components. Eriksson et al. (2002b) reported that, for the hydrolysis of steam-exploded spruce by the reconstitution of CBH I and EG I, the hydrolysis rates could be recovered significantly by adding fresh EG I after 24 hours hydrolysis. EG I activity leads to an increase in chain ends which CBH I can act on. This helps explain the declining rate observed during lignocellulose hydrolysis.

1.3.1.2 *Penicillium sp.* cellulases

*Penicillium verruculosum* cellulase complex comprises: five endoglucanases (EG I, EG II, EG III EG IV and EG V) and two cellobiohydrolases (CBH I and CBH II). Components of the cellulase systems were classified based on their mode of catalytic action. The molecular weights and isoelectric points varied from 19,100-114,800 daltons and 3.70-4.38 respectively (Kastelyanos et al., 1995). Cellobiase (β-D-glucosidase) with a molecular weight of 100kDa and pI 5.2 was also recently isolated and its biochemical
properties and kinetic parameters were determined - $K_m$ was 0.36 mM using cellobiose as a substrate (Zorov et al., 2001). Also, cellulases from *Penicillium notatum* had been characterized by Pettersson (1969). All studies show that the organizational and biochemical properties of the *Penicillium sp.* cellulases are very similar to other known acidic cellulases isolated from other cellulase complexes. Castellanos et al. (1995 a and b) have shown that their cellulolytic activity exceeds that of commercial preparations from *T. reesei*. The authors also optimized the reaction conditions for enzymatic hydrolysis of various lignocellulosic substrates. These cellulases show the maximal activity at pH 4.5–5.0, temperature 55-60°C.

1.3.2 Cellulose hydrolysis

Acid hydrolysis of cellulose has been used in the past years as an effective approach to accomplish cellulose saccharification (Himmel, 2004). This method occurs at a fast rate but involves the risk of sugar degradation under non-optimal reaction conditions. Most of the degraded compounds were also shown to be inhibitory to the subsequent fermentation microorganisms. In addition, research evaluating the commercial employment of acid hydrolysis process showed that the equipment and chemicals needed for acid hydrolysis are very costly, due to corrosion and high acid consumption levels during the process (Galbe and Zacchi, 2002). Therefore, enzymes were chosen as an alternative to the deleterious and corrosive acid method. The use of enzymes for the saccharification of cellulose has been intensively studied over the past years (Converse et al., 1990; Nidetzky et al., 1994; Yu and Saddler, 1995; Tengborg et al., 2001). Presently, enzymatic hydrolysis is considered the most promising technology for converting
biomass-to-ethanol due to the continual decrease in enzyme costs, lack of equipment corrosion, and the potential for enzyme recycling.

A typical cellulose hydrolysis pattern in a batch enzyme-based process is characterized by a two-phase curve, with an initial logarithmic phase followed by an asymptotic phase. The cause of this gradual plateau in reaction rates is not fully understood yet but it has been postulated that both enzyme and substrate related properties contribute to this effect. The enzymes must be able to penetrate the very compact lignocellulosic matrix for effective hydrolysis to occur (Mansfield, et al., 1999). Once in solution, the enzymes must partition between the liquid phase and the insoluble cellulosic substrate. The dynamics of desorption and readsorption onto cellulose affect the rate and the extent of hydrolysis, as well as the possibility of enzyme recycling. Substrate structural features also undergo significant changes during the course of the reaction (Väljamäe et al., 1998). These changes are evident by the decreasing rate of hydrolysis and the incomplete hydrolysis at shorter reaction times. The structural intricacy of lignocellulosic substrates adds to the complexity of the cellulose hydrolysis. Moreover, the physiochemical properties of the substrate affect hydrolysability at different morphological levels:

- Particle level: size, fibril dimension etc.
- Fiber/fibril level: porosity, accessible surface area, residual lignin, hemicellulose content, cell wall thickness, etc.
- Microfibril/Molecular level: degree of polymerization (DP), crystallinity, Lattice structure, lignin vs. hemicellulose distribution, etc.

Considered individually, none of these factors has been able to provide a universal explanation for the drop in the rate of cellulose hydrolysis that is observed after the initial
rapid reaction. The physical and structural features of lignocellulosics, combined with the chemical composition of the material, control the susceptibility of the feedstock to enzymes. In the case of model or pure cellulosic substrates, such as Avicel, it has been suggested that the crystallinity and the degree of polymerization both play a dominant role in determining the rate and yield of glucose release (Sasaki et al., 1979; Fan et al., 1987). Hydrogen bonding packs crystalline regions of cellulose tightly rendering it impermeable to water and enzymes. It should be noted, however, that model feedstocks cannot be considered indicative of the realistic feedstocks behavior because a realistic lignocellulosic substrate derived from agricultural or wood residues would contain hemicellulose and lignin, as well as cellulose. When the cellulose within these feedstocks is subjected to enzymatic hydrolysis, the presence of other chemical structures may act as contaminants, either by adsorbing active enzymes or by physically hindering the accessibility of enzymes to cellulose. Therefore, removal of these contaminants would result in the exposure of larger surface area of the cellulose and enhancement of fiber swelling. It has been shown that lignin content and distribution, particle size, pore size/volume etc. are the major factors that control the digestibility of substrate at each of the fiber, fibril and microfibril levels (Mansfield et al., 1999; Boussaid and Saddler, 1999). It is probable that these factors have much more of an effect on the overall efficiency of the enzymatic hydrolysis than simple characteristics, such as the DP and crystallinity, which maybe only influential at the microfibril/molecule level (Ramos and Saddler, 1994b).

In spite of intensive research in the past years, a comprehensive model that can fully describe the mechanisms by which cellulases disintegrate and depolymerize cellulose has
not been developed. As mentioned previously this is largely due to the intricacies of physiochemical nature of the substrates, as well as the lack of methodology to define the rate determining intermediates. The interactions between the enzyme and substrate are very complex. For instance, there are enzyme factors that limit the efficiency of hydrolysis, such as end-product inhibition, lost synergy and deactivation of enzymes during hydrolysis (Lee and Fan, 1983; Hogan, 1987).

**Figure 4.** Enzymatic hydrolysis of lignocellulosic substrates by multi-component cellulase systems. BG - beta-glucosidase; EG (I, II, III) - endoglucanases; CBH (I, II) - cellobiohydrolases; R - cellulose chain reducing ends; NR - cellulose chain non-reducing ends.

1.4 **Factors limiting cellulose hydrolysis**

There are many limiting factors for the enzymatic hydrolytic process that have been identified in the literature. These factors can be divided into two main categories: substrate and enzymatic limiting factors.
The substrate limiting factors can still be subdivided into:

- Cellulosic limiting factors: pore size, available surface area, DP, crystallinity, nonproductive binding, etc.
- Non cellulosic limiting factors: lignin, lignin distribution, hemicellulose, etc.

The enzymatic limiting factors include different susceptibility to end-products inhibition and/or to pseudoinhibition by lignin and substrate, differences in pH-, thermo- and operational stability, different specific activities of the cellulolytic components, synergism, etc. Most studies have also shown that cellobiose, glucose and ethanol are major inhibitors of cellulases (Saddler, 1986). Particularly, the activity of CBH I is strongly inhibited by its product cellobiose. The results from FTIR and circular dichroism showed that cellobiose could combine with the tryptophan residue located at the active site of cellobiohydrolase and produce steric hindrance. This prevents cellulose chains from diffusing further into the active site of the enzyme. In addition, the change in conformation of cellobiohydrolase caused by cellobiose binding also reduced the activity of cellobiohydrolase during hydrolysis (Zhao et al., 2004).

Lignin is also believed to be a strong inhibitor of cellulases. This assumption is based on the fact that usually a reduction of the lignin content leads to an improvement in the hydrolysis process efficiency (Lu et al. 2002; Varga, 2003). Since cellulose is an insoluble substrate, cellulase enzymes must first adsorb to it before they can start the hydrolysis. However, cellulases have the ability to also adsorb to lignin as well as cellulose. The cellulases adsorbed on lignin will not hydrolyse cellulose; they will thus be nonproductively bound. In the past decades, the adsorption of cellulases from *T. reesei* on different cellulosic substrates has been studied extensively (Karlsson et al., 1999; Banka and Mishra, 2002; Palonen et al., 2003; Pinto et al., 2004). However few articles
focused on the adsorption and interaction between cellulase and lignin (Palonen et al., 2003). Generally, proteins adsorb on solid particles by specific or non-specific binding, and the driving forces for binding could be hydrogen bonding, electrostatic or hydrophobic interactions. The main interaction between cellulases and lignin is probably the hydrophobic interaction (Palonen et al., 2003). The residual lignin in softwood could bind cellulases and reduce the accessibility of enzyme to cellulose (Mooney et al., 1998). Unproductive binging of cellulases by lignin is considered by many eminent researchers as one of the main substrate-enzyme limiting factors of the softwood enzymatic hydrolysis (Eriksson et al., 2002b; Alkasrawi et al., 2003).

1.4.1 Lignin as an inhibitor

Lignin is the most abundant non-carbohydrate constituent of wood and as stated previously, it is widely accepted that lignin interferes with cellulose hydrolysis during biomass conversion. Lignin has been shown to act as an inhibitor during enzymatic hydrolysis of lignocellulosic substrates (Kong et al., 1992, Excoffier et al., 1991). An extensive modeling study of 147 lignocelluloses concluded that lignin content was a key factor in determining biomass digestibility (Chang and Holtzapple, 2000) and enzyme recovery (Lu et al., 2002). Furthermore, it has been suggested that lignin limits cellulose hydrolysis by forming a physical barrier that restricts enzyme access. Consequently, it was observed that lignin removal prior to enzymatic hydrolysis improved the rate and yield of hydrolysis substantially (Wong et al., 1988; Ooshima et al., 1990; Excoffier et al., 1991). Numerous lines of evidence indicate that lignin-enzyme interactions make a significant contribution to the decline in rate observed during hydrolysis of lignocellulose substrates. Several studies reported improved hydrolysis yields of lignocellulosic
substrates in the presence of surfactants (Ooshima, et al., 1986; Park et al., 1992; Helle et al., 1993). This effect has also been attributed to a reduction in non-productive binding of cellulases to the lignin component (Eriksson et al., 2002a). A similar mechanism has been used to explain the enhancement of lignocellulose hydrolysis seen following addition of exogenous protein (Tengborg et al., 2001). Studies on model lignocellulosic systems have led to the same conclusion: pre-incubation of a cellulase complex with various isolated lignins reduced activity on filter paper and lowered the level of soluble protein in the reaction mixture indicating non-productive binding (Sewalt et al., 1997).

1.5 Novel approaches to improve the hydrolysis of lignocellulosic materials

Recently, significant reductions in enzyme costs have been obtained using genetic engineering. Recent efforts by leading manufacturers of industrial enzymes have reduced the cost of cellulase for hydrolysis of pretreated corn stover by about thirty-fold (Genencor International, 2004). This reduction was achieved through a combination of enzyme production and performance improvements (Genencor International, 2004). However, the high costs for delignification and enzymatic hydrolysis still hinder commercialization of softwood bioconversion processes. A study done by Wolfenden et al. (1998), of the spontaneous hydrolysis of $\beta$-glycosides indicated that the prospects of further improvements of the catalytic rates maybe limited because enzymes involved in the hydrolysis of $\beta$-1,4-glucans are already exceptionally efficient enzymes.

In the past, there has been an intensive search and screening for new microorganisms that would produce cellulolytic enzymes of higher specific activity, higher thermostability and lower sensitivity to end product inhibition and lignin. Strategies to
improve the performance of cellulase complexes typically involve random mutagenesis of fungal strains, genetic engineering of individual 1,4-β-glucanases, and optimization of the ratio of the various 1,4-β-glucanase components, followed by screening for increased specific activity or stability. Generally, screening of cellulase complexes for improved activity uses a standard assay proposed by the Union of Pure and Applied Chemists to measure hydrolysis of filter paper (Ghose, 1987). *Penicillium* sp. cellulases were shown to have higher levels of cellobiase activity and higher operational stability (Castellanos et al., 1995a). Castellanos et al. (1995b) observed that *Penicillium* cellulase outperformed cellulases from *T. reesei* and *A. foetidus* for the hydrolysis of paper industry waste materials and waste cellolignin. Also, the authors noted that transglycosylation did not significantly reduce the glucose yield during the hydrolysis of cellulose. The work described in this thesis will evaluate the abilities of two novel mutant fungal preparations of *Penicillium* sp. (MSUBC1) and *T. reesei* (MSUBC2) and two commercial cellulase preparations from *T. reesei*, Celluclast 1.5 L (TR2) and Fibrilase (TR1) to hydrolyze cellulose in D. fir pretreated by steam explosion or ethanol organosolv.

1.6 Research objectives and aims

It is recognized that processing costs, particularly the cost of pretreatment and subsequent enzymatic hydrolysis, continue to impede full-scale commercialization of a biomass-to-ethanol process. So far, a fair amount of information about the effect of cellulose properties on the action of enzymes has been accumulated (Lee et al., 1982; Saddler et al., 1982, Sinitsyn et al., 1991; Chang and Holtzapple, 2000). However, very few studies have touched on the role of lignin as a substrate limiting factor during the
enzymatic hydrolysis step. Therefore, one of the major research objectives of this thesis was to address this area and to further explore its relevance.

As previously mentioned, the amount, distribution and chemical reactivity of lignin on the fiber surface, and the interaction of lignin with cellulolytic enzymes, have been identified as primary factors affecting enzyme accessibility and catalytic efficiency (Gharpuray et al., 1983; Saddler et al., 1982). Qualitative data indicates that lignin is a strong inhibitor of cellulases, but no quantitative analysis of enzyme inhibition by lignin has yet been reported. As part of a systematic study to evaluate the impact of lignin on hydrolysis, we quantified the inhibition of a *T. reesei* cellulase complex and a β-glucosidase from *A. niger* by classical cellulase inhibitors, such as palladium, δ-gluconolactone and glucose, plus various residual lignin preparations from various different sources. We also chose to work with purified, well characterized enzyme components. The study focused on CBH I because it is the major carbohydorase and constitutes about 60-70% of the cellulases in a *T. reesei* culture filtrate (Ramos et al., 1994; Nidetzky et al., 1994). The endoglucanase EG II was also used, as it comprises about 10% of the cellulases in *T. reesei*. Some mechanistic information regarding the cellulase-lignin interaction was also obtained. The hardwood tree specie chosen for this study was Poplar which is known for its rapid growth. The softwood specie used was D. fir which is the historical substrate in the Saddler group.

The specific aim of this dissertation was to evaluate the role of unproductive binding of cellulases by lignin and its significance in reducing the cellulose hydrolysis rates and carbohydrate yields. It was desired that a better understanding of these types of interactions would aid in the development of a novel strategy for improving the
economics of lignocellulosic bioconversion processes based on reducing the amount of enzyme used in the enzymatic hydrolysis step. This reduction could be achieved through finding cellulases with improved activities and lower affinity to lignin. This means that the effective concentration of enzymes in solution would increase due to the decrease in non-productive binding to lignin.

The catalytic performances of two commercial cellulase complexes from *Trichoderma* sp. (TR1 and TR2) and two novel complexes obtained following selection and random mutagenesis of *Penicillium* and *Trichoderma* strains in the laboratory (called MSUBC1 and MSUBC2 respectively) were evaluated. We hoped to find in nature cellulases that have higher hydrolytic rates and lower non-productive affinity to lignin. All enzymes were tested on the basis of similar specific cellulase activities or filter paper activities. These experiments described are a part of a program to develop a cost-effective process for the pretreatment and enzymatic hydrolysis of softwood residues.
2. MATERIALS AND METHODS

2.1 Enzymes

2.1.1 Cellulase preparations

The complete commercial enzyme preparations from *Trichoderma reesei* cellulase complex used for cellulose degradation were Celluclast® 1.5L (Novozymes, USA) and Fibrilase® (Iogen Corporation, Canada). The commercial β-glucosidase preparation used was Novozym 188® (Novozymes, USA) from *Aspergillus niger*. The activities of Celluclast®, Fibrilase® and Novozym 188® were 60.1 FPU/mL, 55.5 FPU/mL and 341.3 CBU/mL respectively, as measured by standard NREL and IUPAC procedures (IUPAC, 1987). Further, two preparations produced in the laboratory from mutant strains of *Trichoderma* sp. and *Penicillium* sp., MSUBC1 (740.0 FPU/g) and MSUBC2 (382.0 FPU/g), were evaluated. It should be noted that the enzymes were loaded based on filter paper units (FPU) activity.

2.1.2 Enzyme purification

The four major components in the *T. reesei* cellulase complex CBH I, CBH II, EG I and EG II were purified by FPLC using our group's automated multidimensional fast protein liquid chromatographic scheme for preparative-scale purification (Duoflow Maximizer, Bio-Rad, USA). The separation system uses six sequential ion-exchange and gel-filtration columns and allows automated preparation of the target enzymes in large-scale quantities within few hours. Enzyme identities were confirmed by peptide sequencing and MALDI-TOF mass spectrometry. The first stage of this procedure comprised removal of non-protein low molecular weight contaminants (insoluble...
substances, carbohydrates, pigments, etc). The insoluble residues were removed from the concentrated liquid culture by means of protein precipitation through the addition of ammonium sulfate (80% saturation) and then redissolving the precipitate in 50 mM acetate buffer. The subsequent chromatographic steps resulted in group and then individual enzyme purification. During purification, eluted proteins concentrations were monitored by UV detection at 280nm. The purity of the isolated monocomponents was confirmed by SDS-PAGE and IEF.

2.1.3 Enzyme activities

All enzyme activities were determined at 50°C, except Avicelase and β-glucosidase, which were analyzed at 40°C. Filter paper activity was determined using Whatman No. 1 filter paper as substrate, as recommended by the International Union of Pure and Applied Chemists (IUPAC, 1987), and is expressed as filter paper units (FPUs). Briefly, 1.0 mL of 50 mM acetate buffer, pH 4.8, was added over a 1 cm x 6 cm rolled piece of filter paper in 15mL glass test tube. The samples were pre-incubated at 50°C for 5 min then mixed with 0.5 mL diluted enzyme. After incubation for 50 min at 50°C, 3 mL of dinitrosalicylic acid (DNS) reagent was added, and the mixture was boiled for 5 min. After cooling, 0.2 mL of the reaction mixture was added to 2.5ml distilled deionized water in a plastic cuvette. The amount of reducing sugar release was calculated from the absorbance at 560 nm with reference to a glucose standard curve. Enzymes were diluted so that 2.0 mg of glucose were released. Therefore, the estimated amount of enzyme that releases 2.0 mg glucose in the FPU reaction contains 0.37 units. Buffer, enzyme and substrate blanks were also included.
Carboxymethyl cellulase activity (CMCase) was measured by incubating the enzyme for 10 min in 50mM sodium acetate buffer at pH 4.8 containing 0.5% CMC. The CMC powder (medium viscosity) from Sigma (USA) was dissolved in acetate buffer using a magnetic stirrer and left to stand over night at 4°C. Activities were calculated from reducing ends, which were measured using the dinitrosalicylic acid method. Reducing sugars produced were calculated by measuring the absorbance at 560nm with reference to glucose/CMC standard. Enzymes were diluted so that the absorbance of the final reaction mixture was less than 1.0 absorbance unit. One unit of CMCase activity corresponds to 1.0 μmol of the substrate glucosidic bonds cleaved in 1.0 minute at the given assay conditions (Ghose, 1987).

Avicelase activity was assayed with a 1% (w/v) suspension of Avicel from Sigma (USA), in 0.1 M sodium acetate buffer, pH 4.8. The reaction mixture consisting of 1% suspension of Avicel, enzyme, and water to a total volume of 2.0 mL, was incubated at 50°C for 2 hours. The tubes were centrifuged and the supernatant analyzed for soluble sugars by DNS colorimetric method at 560 nm. Enzyme and reagent blanks were incorporated (Wood and Bhat, 1988).

β-glucosidase activity was assayed by monitoring the release of p-nitrophenol from p-nitrophenol-β-D-glucoside (Sigma, USA). 1.0 mL of the 10mM p-nitrophenyl-β-D-glucoside substrate was mixed with 1.8 ml of 0.1 M sodium acetate buffer, pH 4.8 and equilibrated to 50°C in a water bath. Diluted enzyme solution of 200 μL was added to the tubes and then incubated for 30 min at 50°C. To stop the reaction, 4.0 ml of glycine buffer was added, and the liberated p-nitrophenol was measured at 430 nm using a standard graph relating micromoles of nitrophenol to optical absorbance. The usual
enzyme and reagent blanks were included. One unit of β-glucosidase activity is the amount of enzyme required to release 1 μmol p-nitrophenol per minute under the conditions of the assay (Wood and Bhat, 1988).

Hydroxyethylcellulose (HEC) was used to assay endoglucanase activity. 1% medium viscosity HEC (Fluka, AG) was dissolved in 0.05 M citrate buffer, pH 4.8 for 1 hour and let to clarify for another hour. The assay consisted of incubating 1.8 ml HEC substrate solution plus 0.2 mL enzyme solution at 50°C for 10 minutes. The reaction was terminated by adding 3 ml DNS reagent. After boiling the tubes for 5 minutes, the color formed was measured against the spectro zero at 540 nm, after correction for the enzyme blank. The unit of HEC activity was expressed as the number of μmoles of D-glucose liberated per minute per ml of enzyme under the assay conditions (Ghose, 1987).

Xylanase activity was measured using birchwood xylan and monitoring the release of reducing sugars by the Somogyi-Nelson method (Somogyi, 1952). Briefly, 0.15 mL of 50 mM acetate buffer, pH 5.0, plus 0.25 mL of 1% (w/v) xylan solution was pre-incubated at 50°C for 5 min then mixed with 0.1 mL of diluted enzyme. After incubation for 10 min at 50°C, 0.5 mL of Somogyi reagent was added and the mixture was boiled for 40 min. After cooling, 0.5 mL of Nelson reagent was added, and the total volume adjusted to 5.0 mL with distilled water. The amount of reducing sugar released was calculated from the absorbance at 610 nm with reference to a xylose standard curve. One unit of xylanase activity is the amount of enzyme required to release 1 μmol of reducing sugars (as [xylose equivalents]/min). β-glucanase activity was measured using the same procedure, with barley β-glucan replacing xylan. Pectinase and mannanase activities were measured using polygalacturonic acid and galactomannan, respectively, as previously described.
(Semenova et al., 2002; Baraznenok et al., 1999). The overall cellulolytic activity, measured for these cellulase systems, is summarized in Table 2 in the results section.

The protein concentration of enzyme preparations was determined by the BSA method (Pierce, USA) with BSA as standard according to the manufacturer's directions. The purified monocomponents concentration were determined spectrophotometrically at 280 nm using the molar extinction coefficients 78,800; 64,000 for CBH I and EG II respectively (Väljamäe, 2002). The protein concentration of four enzyme preparations produced in the laboratory from mutant strains of Trichoderma sp. and Penicillium sp., MSUBC1 and MSUBC2, were also determined by the Lowry's method following precipitation with trichloroacetic acid (Lowry et al., 1951).

2.2 **Cellulosic and lignocellulosic substrates**

2.2.1 **Softwood samples**

Representative samples of coastal D. fir (*Pseudotsuga menziesii*) sapwood and heartwood were collected in British Columbia. Samples were chipped to approximately 2 x 2 x 0.5 cm, screened for size uniformity, and equilibrated at 4°C in sealed plastic bags to appropriate moisture content prior to pretreatment.

2.2.2 **Softwood pretreatment**

The two lignocellulosic substrates used were D. fir steam-exploded wood and ethanol organosolv pulp. Organosolv pretreatment of was carried out in a 1 L-stainless steel pressure reactor (Parr Instrument Co., Moline, IL, USA). The chips were subjected to 50% (w/w) of 95% ethanol, adjusted to pH 2.42 with 10% (v/v) sulfuric acid. The solvent to wood ratio was 7:1 (w/w). The Pressure reactor conditions were 195°C and
pressure of 460 psi (3.2MPa), set for 40 minutes. The time required to reach the target cooking temperature was approximately 53 min in all cases. After cooking, the reactor was cooled by immersion in ice until the interior temperature was less than 55°C. After pulping, the brown liquor was decanted and the pulp was homogenized for 5 minutes in 70% (v/v) ethanol at 70°C (pulp to ethanol was 9:1) in a British disintegrator (TMI, Montreal, Canada). Further, the pulp was washed with 3-fold (v/v) 70% ethanol (at 70°C) and rinsed extensively with water. After washing, the pulp was filtered and stored in sealed plastic bags at 4°C for further use.

The steam-exploded was subjected to SO$_2$-catalyzed steam-explosion at medium severity conditions (4.5 minutes, 195°C). The dry chips were impregnated overnight with 4.5% (w/w) SO$_2$ per original dry wood and then they were subjected to the steam gun at the above conditions. After pretreatment, the liquid fraction was removed by decantation and filtration. Solids were washed with distilled water until neutrality and then samples were stored in sealed plastic bags at 4°C until use.

### 2.2.3 Lignin isolation

In order to start the design of a model for the proposed study, two different high purity residual lignin samples were isolated one from hardwoods, yellow poplar, and the other from softwoods. The lignin fractions were first extracted by ethanol organosolv-pretreatment, as described above. This type of pulping method is currently considered in the Saddler's group the most prospective for the softwood-to-ethanol bioconversion process. Then, an enzymatic method was used to purify the extracted lignin and remove the residual carbohydrates. The extensive enzymatic digestion of cellulose was carried out for 72 hours at 50°C using commercial cellulose and hemicellulose degrading enzyme
preparations. A protease treatment with further extensive buffer wash was then used in an effort to remove as much residual protein as possible. The residual lignin was pelleted out of solution by centrifugation at 6000g for 20 minutes. The residual lignin pellet was further rinsed twice with sodium acetate buffer pH 4.8 and centrifuged to remove any contaminants. The final lignin residues were collected and freeze-dried overnight. Then, lignin preparations were manually ground and screened through a 180 μm mesh to reduce variation in particle size. The carbohydrate composition and lignin content of the purified residual lignins were determined using the Klason lignin method described in the following section. The final preparation had an average particle size of approximately 5 μm, determined by light microscopy combined with automated image analysis, and a surface area of 11.72 m²/g determined by BET. Lignin characterization using ¹H and ¹³C NMR and lignin elemental analysis were also performed (data not shown). Please note that hence forward in this thesis, these model isolated and purified ethanol pulp residual lignins will be referred to as “lignin” unless otherwise indicated.

2.2.4 Composition analysis of pretreated softwoods and isolated residual lignins

The carbohydrate composition and lignin content of the pretreated softwood samples and purified lignins were determined using a modified Klason lignin method derived from the TAPPI standard method T222 om-88. The substrates used for Klason analysis were initially oven dried overnight at 110°C and then ground to pass a 40-mesh size screen in a Wiley mill. Later, a sample 0.2 g (dry) was incubated at 20°C with 3mL of 72% (v/v) sulfuric acid (H₂SO₄) for 2 hours, with mixing every 10 minutes to hydrolyze the polysaccharides. The reaction was then diluted with 112 ml of deionized water, to a final acid concentration of 4% H₂SO₄, and then transferred to a serum bottle. The solution
was then autoclaved at 121°C for 1 hour. Acid-insoluble lignin fraction was determined gravimetrically after filtration of the hydrolyzate through a medium coarseness vacuum filter. A sample of the filtrate was removed and stored in a Falcon tube for analysis of carbohydrates and acid soluble lignin. Ultraviolet absorption at 205 nm was used to determine the concentration of soluble lignins released during hydrolysis according to Tappi method UM-250. The lignin content is calculated using an expression of Beer’s Law:

\[
\text{Lignin} = \frac{\text{Absorbance (A)}}{b \text{ (light path in cm)}} \times a \text{ (absorptivity in 1/gcm)}
\]

Furthermore, the carbohydrate content of the acid hydrolyzate was determined by HPLC with fucose as an internal standard, as later described. Each determination was run in triplicate.

2.3 Enzymatic hydrolysis

2.3.1 Batch hydrolysis of pretreated softwood

Hydrolysis experiments were run in triplicates in 125 mL flasks. Flasks contained 0.1 M acetate buffer, pH 4.8, 2% and 5% (w/w) substrate and 10 FPU of cellulase complex per gram of cellulose, to a total reaction volume of 50 mL. Samples were taken at 3, 6, 12, 24, 48 and 72 hours. Microbial contamination was prevented by adding tetracycline (Sigma, USA) and cycloheximide (Sigma, USA) at concentrations of 40 µg/mL and 30 µg/mL, respectively, to the reaction medium at a final concentration of 0.017% (w/w). The flasks were incubated with continuous shaking (150 rpm) at 45°C. Sample aliquots of 500 µL were taken at various time intervals and were immediately boiled for 5 minutes to inactivate the enzymes. The samples were then micro-centrifuged at 15,000 rpm for 5 minutes and the supernatant liquid was then taken for sugar analysis. Moreover, the
effectiveness of the enzymatic hydrolysis was evaluated by measuring the conversion of cellulose (%) after 72 hours and the initial hydrolytic rates.

2.3.2 Lignin effect on enzymatic hydrolysis

The influence of softwood and hardwood lignin preparations on the enzymatic hydrolysis of α-cellulose by cellulase complexes from *T. reesei* (0.91 FPU/mg protein, 14.1 CMCase U/mg protein, 2.18 Avicelase U/mg protein) and *Penicillium sp.* (0.88 FPU/mg protein, 16.9 CMCase U/mg protein, 2.03 Avicelase U/mg protein) was examined. α-Cellulose was used as a model substrate to study the kinetics of the enzymatic hydrolysis by cellulases. Reaction mixtures (containing 20 g/L α-cellulose, 3.0 g/L lignin and 0.7 mg/mL cellulase in a total volume of 1.2 mL were incubated at 45°C, pH 4.80, for 30 minutes with agitation. The reaction was stopped by the addition of 100 μl of 1.0 M sodium carbonate and the mixture was centrifuged at 15,000 rpm for 10 minutes. Also, hydrolysis products were analyzed by HPLC. High purity lignin samples were prepared from and yellow poplar by ethanol organosolv pretreatment, followed by enzymatic hydrolysis as described in the former section. All experiments were done in triplicate and the results were expressed as averages.

2.4 Enzyme inhibition studies by classical inhibitors and lignins

The focus of this thesis was primarily on the quantitative evaluation of the model lignin inhibitory effect on cellulases. The kinetic experiments were carried out in a 2 mL screw-cap tubes in a thermostated air-incubator with constant agitation by inversion using a rotational mixer at 25 rpm. The reactions were initiated by mixing the enzyme solution with the substrate. Later the reactions were terminated by adding 1.0 M Na₂CO₃ to a final
pH of 12. The effect of glucose, cellobiose, γ-gluconolactone, palladium chloride and lignin on the catalytic activity of the specified enzyme was measured by including a given amount of the inhibitor solution in the assay mixture. Due to the insoluble nature of lignin in buffer, a 30 mg/ml suspension was prepared in 0.05 M sodium acetate buffer, pH 4.8 by stirring with a magnetic stirrer. The suspension was stirred for at least 60 minutes before dispensing it into reaction tubes. This suspension simulated a "homogenous lignin solution".

β-glucosidase (from A. niger) activity was assayed at 40°C, pH 4.8, for 10 min using 20 mM cellobiose at a final enzyme concentration of 0.03 mg enzyme/mL. All reaction volumes were 1.0mL. To determine the kinetic parameters $K_m$ and $V_{max}$ of cellobiose hydrolysis, substrate concentration were varied from 17 mM to 1 mM. In the inhibition study, a definite amount of glucose, or γ-gluconolactone or PdCl$_2$ or lignin was added into the reaction system at the start of the reaction. Reaction mixtures contained 0-3.0 mM glucose, 0-6.0 mM γ-gluconolactone, 0-1.6 mM Pd$^{2+}$ (from PdCl$_2$), or 0-15.0 mg/mL lignin. The reactions were terminated by adding 100 μL of 1.0 M sodium carbonate to a final pH of 12. The initial rate of reaction for each cellobiose initial concentration was directly obtained from hydrolysed cellobiose concentration divided by time.

T. reesei cellulase complex was assayed at 45°C, pH 4.8, for 30 min using 2% (w/w) α-cellulose and a final protein concentration of 0.09 mg protein/mL. Due to the fine nature of the α-cellulose powder, a suspension of 20 g/L substrate in 0.05M sodium acetate buffer at pH 4.8 was first prepared and agitated for a 60min with magnetic stirring. Later, exact volumes were dispensed in a series of tubes. All reaction volumes were 1.0 mL. The effect of glucose, or γ-gluconolactone or PdCl$_2$ or lignin on the
catalytic activity of \textit{T. reesei} cellulase was measured by introducing the inhibitor to the assay mixture. Inhibitors concentrations were 0-2.0 mM glucose, 0-5.0 mM $\gamma$-gluconolactone, 0-1.0 mM, \text{PdCl}_2, or 0-3.13 mg/mL lignin. The reaction was initiated by addition of enzyme followed by vortex mixing for few seconds and stopped at selected times by addition of 150$\mu$L of 1.0M NaOH to a final pH of 12. The cellulose residue was pelleted by centrifugation at 15,000 rpm for 10 minutes and the concentration of the total sugar in the supernatant was determined. Each experimental point was done in duplicate and was represented by the average.

\textit{T. reesei} CBH I initial hydrolytic activity was assayed at 45°C, pH 4.8, for 60 min using 20 g/L $\alpha$-cellulose and 0.09 mg protein/mL. The $\alpha$-cellulose slurry was prepared in 0.05 M sodium acetate buffer pH 4.8. The inhibitors concentration ranges where as follows: cellobiose concentration 0-1 mg/mL, and lignin concentrations were between 0 to 1.5 g/L. The reactions were terminated by adding 200 $\mu$L of 1.0 M sodium carbonate to a final pH of 12. \textit{T. reesei} EG II activity was assayed at 40°C, pH 4.8, for 10 min using 1 mg/mL cellopentaose and 0.45 $\mu$g protein/mL. The effect of cellobiose and lignin on the catalytic activity of EGII was measured by including a given concentration of the complex solution, at pH 4.8, in the assay mixture. The reaction mixtures contained 0-1.5 g/L cellobiose or 0-3.3 g/L lignin. The products of the enzymatic cellulose hydrolysis were quantified by HPLC in all cases. Michaelis constants ($K_m$) and inhibitory constants ($K_i$) were obtained from the straight-line equation, attained by linear regression analysis of double reciprocal plots. Then, fitting of the experimental points was made using Excel (Microsoft, USA) program.
2.5 Calculation of $V_{\text{max}}$ and $K_m$ from kinetic data

Microsoft Excel XP has built-in functions that easily carry out line fitting for linear and certain types of curved data using the Solver add-in.

2.5.1 Outline of Microsoft Excel spreadsheet

The data analysis was performed based on the results obtained from the kinetic studies. The data in column A represent the substrate concentration values, and in B the sets of velocity data containing error. The values of $K_m$ and $V_{\text{max}}$ are reset to 1 in cells B1 and B2 respectively. Solver uses a complex mathematical algorithm that keeps changing the values of $K_m$ and $V_{\text{max}}$ until it obtains the smallest possible value of the sum of squares. This would then be the computer generated best fit line.

Column C contained the calculated velocity from each substrate concentration using the Michaelis' equation and the values of $K_m$ and $V_{\text{max}}$. The final column (D) uses the POWER function of Excel to calculate the square of the difference between the experimental and calculated velocities for each substrate concentration. Evidently, the closer these two values are to each other the smaller the square of the difference will be indicating a good fit.

2.5.2 The graph

The dots on the graph are a plot of the experimental velocity data against substrate concentration, while the line is a plot of the calculated velocity data against substrate concentration.
2.6 Analyzing carbohydrate composition of samples

The soluble sugar concentration for all hydrolysis experiments were determined by high-performance liquid chromatography (HPLC) using a Dionex DX-500 (Dionex, Sunnyvale, CA) equipped with an ion-exchange CarboPac PA1 analytical column, and pulsed amperometric detector (Dionex ED40) with a gold electrode. Prior to injection, collected samples were filtered through a 0.45 μm syringe filter (Millipore, Bedford, MA). Fucose was used as an internal standard to detect the amounts of glucose and/or cellobiose in the hydrolyzates. A volume of 20 μL was loaded using a spectra system AS3500 autosampler (Spectra-Physics, CA).

D-glucose (Sigma, USA), cellobiose (Sigma, USA), and cellotriose (Sigma, USA) were used as external standards for hydrolysis experiments. Elution was performed with 100 mM NaOH isocratically at a flow rate of 1.0 ml/min with a postcolumn addition of 200 mM NaOH flow at a rate of 0.5 ml/min. For the substrates chemical composition analysis, the sugars fucose, arabinose, galactose, glucose, xylose and mannose were used as external standards. Mono-, di- and oligosaccharides in hydrolysates were analyzed on the HPLC by modifying the mobile phase with 1 M sodium acetate. Substrate conversion was based on the cellulose content in the substrate. Soluble sugars were then measured in the filtrates and the sum of the produced sugars was used to calculate the percent cellulose conversion.
3. RESULTS

3.1 Activities of cellulase preparations on model substrates

The protein contents and hydrolytic activities of the enzymatic preparations on a range of model cellulosic, hemicellulosic and related substrates are shown in Table 2. The specific FPA of *Penicillium* cellulase was approximately the same as that of *Trichoderma* cellulases (0.7-1.0 FPU/mg protein). All cellulase preparations demonstrated similar specific CMCase activity (14.1-20.7 U/mg), and specific Avicelase activity (1.55-2.4 U/mg). The preparations showed considerably greater differences in their levels of endogenous specific β-glucosidase activity (0.15-1.16 U/mg) and in the levels of the three hemicellulase and pectinase activities examined (xylanase, mannanase, pectinase). *Penicillium* cellulase had significantly higher xylanase, mannanase, and pectinase activities in comparison to all other cellulase preparations. Cellobiase activity of *Penicillium* cellulase (MSUBC 1) was 7.7 times higher than that of commercial *T. reesei* (TR 2) and 1.8 times higher than that of commercial *T. reesei* (TR 1). In addition, all preparations had similar levels of β-glucanase activity.

Table 3 summarizes the characteristics and activities of the purified enzymes. Bound proteins were eluted with a NaCl gradient. The purified enzymes were kindly provided by Dr. Alex Berlin. The enzymes identities were confirmed by partial peptide sequencing, substrate specificity, and protein characteristics. The purity was confirmed by SDS-PAGE and IEF as mentioned previously.
Table 2. Enzyme activities in commercial and laboratory cellulase preparations.

<table>
<thead>
<tr>
<th>Protein^1</th>
<th>Penicillium MSUBC1</th>
<th>T. reesei MSUBC2</th>
<th>TR 1 Fibrilase</th>
<th>TR 2 Celluclast 1.5L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulase activities (U/mg protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibrilase</td>
<td>837.5</td>
<td>556.0</td>
<td>130.0</td>
<td>129.3</td>
</tr>
<tr>
<td>Celluclast 1.5L</td>
<td>129.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMC</td>
<td>0.88</td>
<td>0.69</td>
<td>0.91</td>
<td>1.04</td>
</tr>
<tr>
<td>Avicelase</td>
<td>16.89</td>
<td>19.19</td>
<td>14.11</td>
<td>20.71</td>
</tr>
<tr>
<td>Other enzyme activities (U/mg protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Glucosidase</td>
<td>2.03</td>
<td>2.37</td>
<td>2.18</td>
<td>1.55</td>
</tr>
<tr>
<td>β-Glucanase</td>
<td>1.16</td>
<td>0.6</td>
<td>0.15</td>
<td>0.66</td>
</tr>
<tr>
<td>Xylanase</td>
<td>17.03</td>
<td>14.59</td>
<td>16.81</td>
<td>19.22</td>
</tr>
<tr>
<td>Pectinase</td>
<td>39.12</td>
<td>9.25</td>
<td>3.51</td>
<td>11.76</td>
</tr>
<tr>
<td>Mannanase</td>
<td>0.64</td>
<td>0.25</td>
<td>0.07</td>
<td>0.05</td>
</tr>
</tbody>
</table>

^1 Protein concentration – mg/g, except TR1 and TR2 (mg/ml)

Table 3. Protein contents and specific activities of the T. reesei purified monocomponents.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>CBH I</th>
<th>CBH II</th>
<th>EG I</th>
<th>EG II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mw, Da (Väljamäe, 2002)</td>
<td>57,209</td>
<td>57,706</td>
<td>48,309</td>
<td>44,329</td>
</tr>
<tr>
<td>(\varepsilon_{280} M^{-1} cm^{-1}) (Väljamäe, 2002)</td>
<td>86,000</td>
<td>92,000</td>
<td>67,000</td>
<td>78,000</td>
</tr>
<tr>
<td>C Protein 280 (mg/ml)</td>
<td>2.773</td>
<td>1.540</td>
<td>0.268</td>
<td>0.668</td>
</tr>
<tr>
<td>pI</td>
<td>4.5</td>
<td>6.1</td>
<td>4.8</td>
<td>4.9</td>
</tr>
<tr>
<td>Purity</td>
<td>≥0.98</td>
<td>≥0.95</td>
<td>≥0.95</td>
<td>≥0.95</td>
</tr>
<tr>
<td>Content (%)</td>
<td>≥55</td>
<td>≥20</td>
<td>≥8</td>
<td>≥9</td>
</tr>
<tr>
<td>FPA (U/g)</td>
<td>35</td>
<td>125</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CMCase (U/mg)</td>
<td>0.096</td>
<td>0.317</td>
<td>1.90</td>
<td>0.732</td>
</tr>
<tr>
<td>Avicelase (U/g)</td>
<td>9.07</td>
<td>14.0</td>
<td>18.9</td>
<td>29.5</td>
</tr>
<tr>
<td>Hydroxyethyl cellulose (n) (nkat/mg)</td>
<td>0.891</td>
<td>6.740</td>
<td>48.4</td>
<td>36.5</td>
</tr>
</tbody>
</table>
3.2 Composition of isolated residual lignin, pretreated softwood samples and α-cellulose

Using the same extraction and isolation methods described above, the residual lignin from had a high degree of purity (98%) and its carbohydrate content was less than 2%. The residual lignin from poplar had a lower degree of purity (80%) and it had a higher carbohydrate content (20%).

The composition of pretreated softwood samples and cellulosic material (% of dry weight) are displayed in Table 4. The yields of substrate components varied between the pretreatments. The glucose contents of the steam-pretreated samples and the organosolv samples were around 47% and 85% respectively, corresponding to 42% and 76% cellulose, assuming that all glucose is derived from cellulose. The lignin content of steam-exploded (44%) was higher than that of untreated wood (data shown in Table 1) due to the hemicellulose solubilization in the pretreatment process. Also, the organosolv-pretreated sample contained significantly higher amount of cellulose and considerably lower amount of lignin, than the samples pretreated by steam-explosion due to solubilization of hemicellulose and lignin during pretreatment. The total hemicellulose-derived sugars (arabinose, galactose, mannose and xylose) content was low in both pretreated lignocellulosic substrates (2.5-3%). It is important to note that SO₂ catalyzed steam explosion has been shown to enhance the enzymatic hydrolysis of softwoods. Steam-explosion results in greater disruption of the fiber morphology and greater enzyme accessibility to the substrate. However, as the organosolv process removes most of the lignin and hemicellulose, leaving behind a substrate with high cellulose content, it was anticipated that organosolv pretreated samples would also have high hydrolysis initial rates and yields. The role of pretreatment in the enzymatic hydrolysis is described in the
following section. No lignin was detected in α-cellulose; with very little residual hemicellulose detected.

### Table 4. Monosaccharide and lignin composition of steam-exploded and organosolv pretreated softwoods (% dry weight).

<table>
<thead>
<tr>
<th>Component</th>
<th>Steam-exploded D. fir</th>
<th>Organosolv pretreated D. fir</th>
<th>α-Cellulose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabinose</td>
<td>0.14 ± 0.00</td>
<td>ND</td>
<td>0.67</td>
</tr>
<tr>
<td>Galactose</td>
<td>0.34 ± 0.00</td>
<td>ND</td>
<td>0.90</td>
</tr>
<tr>
<td>Glucose</td>
<td>47.39 ± 1.11</td>
<td>84.80 ± 2.60</td>
<td>97±1.31</td>
</tr>
<tr>
<td>Xylose</td>
<td>0.58±0.00</td>
<td>1.12 ± 0.19</td>
<td>0.45</td>
</tr>
<tr>
<td>Mannose</td>
<td>2.05 ± 0.07</td>
<td>1.38 ± 0.28</td>
<td>0.98</td>
</tr>
<tr>
<td>Klason Lignin</td>
<td>44.35 ± 0.19</td>
<td>12.69 ± 0.40</td>
<td>ND</td>
</tr>
<tr>
<td>Acid Soluble Lignin</td>
<td>1.20 ± 0.03</td>
<td>0.33 ± 0.01</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND – not detected

### 3.3 The effect of pretreatment method on enzymatic hydrolysis

As it has been mentioned previously, the development of a techno-economically viable bioconversion process of lignocellulosics is greatly hindered by the recalcitrant nature of the substrate. In connection with this matter, a comparison, both in terms of hydrolytic rate and yield between a lignocellulosic feedstock pretreated by two types of
pretreatment methods, steam-explosion catalyzed by SO2 and ethanol organosolv pulping was done. As stated earlier, the chosen substrates were steam-exploded and organosolv ethanol pulp prepared in our lab. The enzymatic hydrolysis for both chosen substrates was carried out at a consistency 2% and 5% (w/w). The enzyme loadings for the cellulosic substrates were 10 FPU/g of cellulose. The obtained results are represented in Figures 5 and 6. After 72 hours of enzymatic hydrolysis, it was observed that the organosolv pretreated samples were hydrolyzed at a higher yield compared to the steam-exploded pretreated samples. Even at different substrate consistencies and using different cellulase complexes, the same pattern of high hydrolyzability was continuously observed. This aspect allows us to consider ethanol pulping as a prospective pretreatment method of softwoods, even though decreases in yields were observed due to change in substrate consistencies.

Detailed examination of the Penicillium hydrolysis of both substrates, a 15% decrease in glucose yield was observed, at both 2% and 5% solid consistencies, between the two different pretreatment methods. The highest decrease in glucose yield was observed between the two substrates when the Fibrilase cellulase complex was used. A 47.5% and a 47.9% decrease in glucose yields were observed at 2% and 5% solid consistencies, respectively. Interestingly, equivalent changes in glucose conversion are observed at both consistencies.

Recent studies have shown that ethanol organosolv pretreatment has a positive prospect as a preceding step to the enzymatic hydrolysis of. This method looks promising for future industrial implementation when compared to the traditionally used steam-exploded pretreatment (Mirochnik et al., 2003). Besides the relatively high hydrolytic
rates and yields derived from the use of pulps pretreated by this method, the quality of the lignin obtained as a by-product of the process is high enough to be used in further applications. This aspect will be compensated partially by the high cost of pretreatment, which is almost 45% of the total costs (Mirochnik et al., 2003). Of course, techno-economical evaluation of both pretreatment processes is required to finally determine which would be better in the final successful softwood-to-ethanol scheme. Our results just show that organosolv (ethanol pulping) pretreatment produced a pulp with good hydrolysis characteristics and lower lignin content.

Figure 5. Hydrolysis yields of organosolv-pretreated and steam-exploded by various cellulase preparations at 10 FPU/g cellulose and 2% solids consistency.
3.4 Effect of lignin on the enzymatic hydrolysis of α-cellulose

Table 5 clearly shows the negative impact of the lignin content on the hydrolysis yield of α-cellulose. α-cellulose was used as a model substrate hydrolysed by T. reesei cellulase complex. An increase of the lignin content to 1 g/L (10% composition of solids) decreased the yield by approximately 7%. The lignin content is usually much higher than that in lignocellulosic substrates; around 13% after organosolv pretreatment and roughly 45% after steam explosion pretreatment. Therefore, the extent of inhibition is expected to be much greater due to higher lignin contents. This experiment provided the bases for further experiments that explore the role of lignin in the enzymatic hydrolysis step.
Table 5. Yields of enzymatic hydrolysis of α-cellulose with different residual lignin contents after three hours hydrolysis at a substrate concentration of 2% (w/w) using *T. reesei* cellulase preparations at an enzyme loading of 10 FPU/g cellulose.

<table>
<thead>
<tr>
<th>α-Cellulose lignin content</th>
<th>Cellulose conversion (%) after 3 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>No lignin</td>
<td>4.98±0.02</td>
</tr>
<tr>
<td>1.00 g/L</td>
<td>4.64±0.04</td>
</tr>
<tr>
<td>1.75 g/L</td>
<td>4.53±0.05</td>
</tr>
</tbody>
</table>

3.5 Quantitative comparison of the inhibition of cellulolytic enzymes by lignin and classical cellulase inhibitors

As part of a systematic study to evaluate the impact of lignin on cellulose hydrolysis, the inhibition of *T. reesei* cellulase complex, purified *T. reesei* CBH I and EG II, and an *A. niger* β-glucosidase was quantified using classical cellulase inhibitors and a softwood residual lignin preparation as a model. Determination of inhibition constants was based on initial rate measurements complicated by the high background level of product initially present in inhibition studies. The relatively low activity of cellulolytic enzymes already at the initial stage, together with the rapid decrease in reaction rates, makes the estimation of true initial rates a difficult and challenging task.

Varying the concentration of the substrate in the reaction mixture permitted the determination of the kinetic parameters of the enzymes as they hydrolyze their substrates. The maximum forward velocity of the reaction is $V_{\text{max}}$. $K_m$ or the Michaelis-Menten constant is an intrinsic parameter, which corresponds to the substrate concentration giving the half-maximal enzymatic velocity. The Michaelis-Menten constant reflects the enzyme binding affinity for the substrate. The results, determined by measuring the initial rate of reducing sugars formation ($v$) as a function of the initial substrate concentration
were linearized in Lineweaver-Burk plots. A simple algebraic conversion of the Michaelis-Menten equation gives the following expression:

\[
\frac{1}{v} = \frac{K_m}{V_{\text{max}}} + \frac{1}{[S]} + \frac{1}{V_{\text{max}}}
\]  

The plot of \(1/v\) versus \(1/[S]\) yields a linear line with a slope of \(K_m/V_{\text{max}}\), x-axis intercept of \(-1/K_m\) and y-axis intercept of \(1/V_{\text{max}}\).

As a first step in the kinetic study, the conditions of the experiment were optimized for each of the enzymes concentrations. This is because under the Michaelis-Menten kinetics assumptions saturation kinetics are obtained at high substrate concentrations when all the enzyme's active sites may be saturated. The optimal level of protein for \(T.\ reesei\) cellulase was 0.09 mg protein/ml for the hydrolysis of \(\alpha\)-cellulose at 15 g/L. Any further increase of the enzyme concentration in the reaction mixture did not significantly influence the concentration of sugars released. Similar enzyme concentration optimization experiments were performed for CBH I, EG II, and \(\beta\)-glucosidase. \(A.\ niger\) \(\beta\)-glucosidase activity was assayed at 0.03 mg enzyme/mL with 20 mM cellobiose as substrate. In addition, \(T.\ reesei\) CBH I activity was assayed using 10 mg/mL \(\alpha\)-cellulose and 0.09 mg protein/mL. \(T.\ reesei\) EG II activity was assayed at 0.45 \(\mu\)g enzyme/mL using 1.0 mg/mL cellopentaose as substrate.

Inhibitors can interact with an enzyme in various ways, and enzyme kinetics studies are essential tools in distinguishing between these mechanisms of inhibition. The experiments were repeated at a number of different inhibitors concentrations. Furthermore, a series of values for \(K_m\) and \(V_{\text{max}}\) were obtained and a number of estimates of the inhibitors constant(s) were calculated using the equation discussed above. Under
these circumstances, a mean value of the results was calculated. The potential inhibitory effect on the hydrolytic activities of all enzyme examined (*A. niger* β-glucosidase, *T. reesei* cellulase complex, *T. reesei* CBH I and *T. reesei* EG II) were assessed by the addition of purified residual lignin. It was apparent that there were differences in the magnitude of inhibition (K_i) and the mechanism of inhibition (Figures 7-18), as summarized in Table 6.

The crude β-glucosidase hydrolysis activity was assayed toward cellobiose. The K_m of the enzyme for cellobiose was 0.53 g/L. Glucose and γ-gluconolactone inhibited β-glucosidase in a competitive manner, as evidenced from the Lineweaver-Burk plots (Figure 7 and 8). The apparent K_m values from the Lineweaver-Burk plot were plotted versus glucose concentration and this replot can be used to determine K_i (slope =Km/Ki). The K_i values were 0.55 g/L and 0.23 g/L respectively. γ-Gluconolactone appears to be a more powerful inhibitor of β-glucosidase than glucose because it has a lower K_i (0.23 g/L). Palladium chloride inhibited the enzyme activity noncompetitively (Figure 9) because each PdCl_2 inhibitor concentration gave a different value for the maximal velocity. These apparent maximal velocities were read from the graph as their reciprocals (1/V_{apparent}) and then they were plotted against the inhibitor concentration in the secondary plot. The intercept on the inhibitor axis gave the −K_i value, the K_i value being 0.24 g/L. Further, lignin was identified to be a mixed uncompetitive inhibitor of β-glucosidase due to the hyperbolic nature of the replot of 1/v axis intercepts versus [I] (Figure 10). The following equation was used to determine the K_i constant for mixed uncompetitive inhibition system (Segel, 1993).
The kinetic and inhibition properties of Novozym β-glucosidase derived from A. niger assayed against cellobiose as substrate are summarized in Table 6.

For T. reesei complex, the inhibitor binding constants for all classical inhibitors examined were close in order of magnitude (0.28-0.73 g/L). Glucose and γ-gluconolactone were competitive inhibitors (Figure 11 and 12), with γ-gluconolactone having a higher enzyme binding constant (Table 6). PdCl₂ was a non-competitive inhibitor, with a Kᵢ value of 0.73 g/L (Figure 13). Meanwhile, the results from a series of kinetic assays of the T. reesei enzyme carried out at a variety of different lignin concentrations indicated that lignin was a competitive (mixed) type inhibitor because the replots of the v vs. [I] at a constant substrate concentration was hyperbolic in shape. Lignin had an inhibition constant of 8.20 g/L which was close to the binding constant of the enzyme complex substrate α-cellulose (Kᵢ=6.5 g/L). The data shows that the magnitude of inhibition by lignin, on a concentration basis, is comparable to that for glucose, γ-gluconolactone and PdCl₂. In fact, T. reesei cellulases seemed to bind to lignin almost as well as its insoluble cellulose substrate.

Cellobiose appeared to be an end product competitive inhibitor of purified T. reesei CBH I (Figure 15). It also inhibited the purified T. reesei EG II competitively using cellopentaose as a substrate (Figure 17). By comparing the cellulose binding constant

\[
1 = \frac{[I]}{\alpha K_i} + \frac{1}{K_m} \left(1 + \frac{[I]}{K_i}\right)
\]  

[2]

The kinetic and inhibition properties of Novozym β-glucosidase derived from A. niger assayed against cellobiose as substrate are summarized in Table 6.
(K_m) with cellobiose binding constant, it is evident that cellobiose binds 10 times better and therefore is very effective as a competitive inhibitor. CBH I appeared to bind with similar affinities to both its substrate, cellulose, and to lignin.

Figure 16 shows the mixed-competitive inhibition pattern observed from inhibition of CBH I by lignin. The hyperbolic plot of \( v \) vs. [I] clearly indicated mix inhibition. Figure 18 illustrates the lignin uncompetitive inhibition pattern of \( T. reesei \) EG II enzyme. Equation 2 above was used again to determine the lignin inhibition constant. The lignin binding constant (K_i=0.9 g/L) was very similar to that of \( \beta \)-glucosidase lignin inhibition constant (K_i=1.2 g/L) and the exhibited pattern of inhibition was also synonymous. Lignin seems to bind to the enzyme regardless of the substrate being bound or not. This leads to the formation of a triple intermediate complex.

It is concluded that lignin was a relatively good inhibitor of the \( T. reesei \) cellulase complex (K_i = 8.2 g/L) and of \( T. reesei \) CBH I (K_i = 9 g/L) when compared to other classical inhibitors. Both \( T. reesei \) complex and CBH I exhibited similar patterns of inhibition mechanism and the inhibition constants were in the same order of magnitude. Inhibition by glucose and \( \gamma \)-gluconolactone was mainly competitive while inhibition by PdCl_2 was non-competitive. However, in all cases, inhibition by lignin was mixed-type; mixed uncompetitive for \( \beta \)-glucosidase and \( T. reesei \) EG II (Figures 10 and 18); mixed competitive for \( T. reesei \) cellulase complex and CBH I (Figures 14 and 16).
Figure 7. Determination of kinetic parameters of cellobiose hydrolysis by β-glucosidase. The effect of glucose as a competitive inhibitor is shown in (A) Lineweaver-Burk plot and (B) Replot of $K_m$ vs. $[I]$, slope = $K_m/K_i$. 
Figure 8. Plots of β-glucosidase inhibition data by γ-gluconolactone. (A) Double reciprocal plot, (B) Replot of $K_m$ vs. $[I]$, slope = $K_m/K_i$. 
Figure 9. Palladium inhibition of β-glucosidase. (A) Double reciprocal plot at different inhibitor concentrations (B) Replot of $1/V_{max}$ vs. $[I]$, slope $= 1/V_{max}.K_i$
Figure 10. The apparent inhibition of $\beta$-glucosidase by purified organosolv lignin. (A) Double reciprocal plot (B) Secondary plot of $1/v$-axis intercept vs. lignin concentration.
Figure 11. Kinetic parameters determination of α-cellulose hydrolysis by \textit{T. reesei}. The effect of glucose as a competitive inhibitor is shown in (A) Lineweaver-Burk plot and (B) Replot of $K_m$ vs. $[I]$, slope = $K_m/K_i$. 
Figure 12. α-cellulose hydrolysis by *T. reesei* Celluclast® and inhibitory effect by γ-gluconolactone. (B) Replot of $K_m$ vs. [I], slope = $K_m/K_i$. 
Figure 13. Palladium inhibition of *T. reesei* Celluclast® hydrolysis of α-cellulose.

Figure 14. The apparent inhibition of *T. reesei* Celluclast® by purified organosolv lignin.
Figure 15. Cellobiose inhibition of CBH I using α-cellulose as substrate. (B) Replot of $K_m$ vs. [I].
Figure 16. The apparent inhibition effect of purified organosolv lignin on CBH I hydrolyzing α-cellulose. (A) Lineweaver Burk plot (B) the $v$ vs. $[I]$. 
Figure 17. The apparent inhibition of cellobiose on purified EG II (from *T. reesei*) hydrolyzing cellopentaose. (B) Replot of $K_m$ vs. [I], slope $= K_m/K_i$. 

$$y = 0.4945x + 0.1059$$ 

$R^2 = 0.9611$
Figure 18. The apparent inhibition of purified organosolv lignin on purified EG II (from *T. reesei*) hydrolyzing cellopentaose.
Table 6. Kinetic parameters, inhibition constants and type of inhibition of cellulases and β-glucosidase by classical inhibitors and lignin.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>$K_m$ (g/L)</th>
<th>Inhibitor</th>
<th>$K_i$ (g/L)</th>
<th>Type of Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. niger</em> β-glucosidase</td>
<td>Cellobiose</td>
<td>0.53</td>
<td>Glucose</td>
<td>0.55</td>
<td>Competitive</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>γ-Gluconolactone</td>
<td>0.23</td>
<td>Competitive</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Palladium chloride</td>
<td>0.24</td>
<td>Non-competitive</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lignin</td>
<td>1.20</td>
<td>Mixed-Uncompetitive</td>
</tr>
<tr>
<td><em>T. reesei</em> Cellulase Complex (TR 2)</td>
<td>α-Cellulose</td>
<td>7.70</td>
<td>Glucose</td>
<td>0.28</td>
<td>Competitive</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>γ-Gluconolactone</td>
<td>0.19</td>
<td>Competitive</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Palladium chloride</td>
<td>0.73</td>
<td>Non-competitive</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lignin</td>
<td>8.20</td>
<td>Mixed-competitive</td>
</tr>
<tr>
<td><em>T. reesei</em> CBH I</td>
<td>α-Cellulose</td>
<td>6.50</td>
<td>Cellobiose</td>
<td>0.70</td>
<td>Competitive</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>γ-Gluconolactone</td>
<td>n/d</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Palladium chloride</td>
<td>n/d</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lignin</td>
<td>9.00</td>
<td>Mixed-competitive</td>
</tr>
<tr>
<td><em>T. reesei</em> EG II</td>
<td>Cellopentaose</td>
<td>0.12</td>
<td>Cellobiose</td>
<td>0.19</td>
<td>Competitive</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>γ-Gluconolactone</td>
<td>n/d</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Palladium chloride</td>
<td>n/d</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lignin</td>
<td>0.91</td>
<td>Mixed-Uncompetitive</td>
</tr>
</tbody>
</table>

ND - not determined
3.6 *Comparison between the *T. reesei* cellulase system with other cellulase complexes.*

In order to compare the relative performances of the various preparations, the percent conversion of cellulose after 72 hours and the initial hydrolytic rates (g/L*h) were determined. Data collected for the hydrolysis of pretreated softwood substrates, by the four different cellulase preparations, are shown in Figures 19-22. Visual inspection of the data suggests that *Penicillium* MSUBC1 performed the best on both tested softwood substrates. The superior performance of *Penicillium* MSUBC1 on both softwood substrates was also reflected by the relatively high values of conversion and initial hydrolytic rates of reaction (Table 7-8). MSUBC1 achieved roughly 76% conversion of steam-exploded containing approximately 44% lignin, without delignification, at low enzyme loading (10 FPU/g cellulose) in 72 hours, and without β-glucosidase supplementation and at 2% (20 g/L) solids consistency. This yield was 2 folds higher than that of the best performing *T. reesei* complex. It was then followed by *T. reesei* MSUBC2. For the first 30 hours of hydrolysis, the commercial cellulase preparation TR1 (Fibrilase) out preformed MSUBC 2. However, later in the hydrolysis, TR1 hydrolysis rate began to plateau meanwhile MSUBC2 hydrolysis rate kept on increasing. It was hypothesized that the TR2, as a complex, was more affected by end product inhibition as the reaction progressed. It could also be suggested that TR1 hydrolyzed the easily accessible and degradable substrate regions faster but was less efficient at hydrolyzing the crystalline structure. Nevertheless, it was apparent that the initial hydrolysis rate determined over the first hours of reaction was not indicative of the final hydrolysis yields. The commercial cellulase TR2 occupied the last position. Further, similar patterns
of hydrolysis yields were observed for the hydrolysis of steam-exploded pulp at higher substrate consistency (5% w/w).

The best result of hydrolysis for organosolv pretreated pulp was obtained by *Penicillium* MSUBC1 cellulase. At 2% (20 g/L) substrate consistency, 10 FPU/g of this preparation were able to converge 91% glucose in 72 hours, compared to 68% yield for the best performing *T. reesei* complex. Interestingly, the commercial Fibrilase (TR1) out preformed *T. reesei* (MSUBC 2) and Celluclast (TR 2), which was unlike what was observed for the steam-exploded substrate. This latest observation led to the conclusion that external factors, other than end product inhibition took a role in hindering Fibrilase (TR 1) hydrolysis of steam-exploded substrate after 24 hours.

A strong correlation between the ability of the enzyme preparations to hydrolyze pretreated softwood samples and the levels of intrinsic β-glucosidase and xylanase activity was also observed. Hemicellulases can make a significant contribution to the effectiveness of cellulase preparations on softwood and could be appropriate targets in attempts to further improve the performance of cellulase preparations on lignocellulosic feedstocks. It would seem that elevated β-glucosidase activity improves cellulose hydrolysis by reducing end-product inhibition caused by accumulation of cellobiose. While the xylan content of pretreated softwood samples is low (Table 4), xylanases may significantly increase the accessibility of cellulose to cellulases by removal of residual hemicellulose, including material re-deposited on fibers during pretreatment.

Furthermore, increasing the substrate loading significantly increased the initial rate of hydrolysis, but the final hydrolysis yields dropped overall. Also, increasing the substrate concentration results in increasing reaction rates because the reaction equilibrium shifts
towards products. However, the enzyme becomes saturated when substrate concentration is very high. Looking at *Penicillium* hydrolysis of organosolv substrate, it is evident that the increase in consistency caused a decrease of about 17% in glucose yield (Table 8). For steam-exploded samples, the increase in consistency resulted in 15% decrease in hydrolysis yield (Table 7).

![Graph](image)

**Figure 19.** Yields of enzymatic hydrolysis of steam-exploded chips (not delignified) at a substrate concentration of 2% (w/w) using different cellulase preparations at an enzyme loading of 10FPU/g cellulose.
**Figure 20.** Enzymatic hydrolysis of steam-exploded chips (not delignified) at a substrate concentration of 5% (w/w) using different cellulase preparations at an enzyme loading of 10FPU/g cellulose.

**Table 7.** Steam-exploded conversion (%) and initial rate (g/L*h) of hydrolysis with four different cellulase complexes at enzyme loadings of 10 FPU/g cellulose and substrate consistencies of 2% and 5%.

<table>
<thead>
<tr>
<th>Cellulase Preparation</th>
<th>2 % SE D. fir</th>
<th>5% SE D. fir</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose Yield in 72 h (%)</td>
<td>$V_o$ (g/L.h)</td>
</tr>
<tr>
<td><strong>TR 1</strong> Fibrilase</td>
<td>35.7±0.02</td>
<td>0.39±0.01</td>
</tr>
<tr>
<td><strong>TR 2</strong> Celluclast® 1.5 L</td>
<td>29.6±0.5</td>
<td>0.25±0.00</td>
</tr>
<tr>
<td><strong>MSUBC 2</strong> T.reesei sp.</td>
<td>41.3±0.05</td>
<td>0.27±0.06</td>
</tr>
<tr>
<td><strong>MSUBC 1</strong> Penicillium sp.</td>
<td>76.4±0.9</td>
<td>0.48±0.01</td>
</tr>
</tbody>
</table>
Figure 21. Enzymatic Hydrolysis of ethanol pulp with different cellulase complexes, at substrate consistency of 2% and cellulase enzyme loadings of 10 FPU/g cellulose.

Table 8. Ethanol pulp D. fir hydrolysis summary at 2% and 5% substrate consistencies with four different cellulase complexes at enzyme loadings of 10 FPU/g cellulose.

<table>
<thead>
<tr>
<th>Cellulase Preparation</th>
<th>2% EtOH pulp D. fir</th>
<th>5% EtOH pulp D. fir</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose Yield in 72 h (%)</td>
<td>V₀ (g/L.h)</td>
</tr>
<tr>
<td>TR 1 Fibrilase</td>
<td>68.0±2.7</td>
<td>0.52±0.09</td>
</tr>
<tr>
<td>TR 2 Celluclast® 1.5 L</td>
<td>53.7±1.1</td>
<td>0.255±0.02</td>
</tr>
<tr>
<td>MSUBC 2 T. reesei sp.</td>
<td>60.6±3.0</td>
<td>0.31±0.01</td>
</tr>
<tr>
<td>MSUBC 1 Penicillium sp.</td>
<td>91.4±2.1</td>
<td>0.66±0.07</td>
</tr>
</tbody>
</table>
Figure 22. Rate of hydrolysis of ethanol pulp with different cellulase complexes at substrate consistency of 5% and cellulase enzyme loadings of 10 FPU/g cellulose.

3.7 Influence of softwoods and hardwood lignins on the enzymatic hydrolysis of cellulose

This series of experiments examined the influence of softwood and hardwood lignin preparations on the enzymatic hydrolysis of α-cellulose by *T. reesei* cellulase complex (0.91 FPU/mg protein, 14.1 CMCase U/mg protein, 2.18 Avicelase U/mg protein) and *Penicillium* sp. (0.88 FPU/mg protein, 16.9 CMCase U/mg protein, 2.03 Avicelase U/mg protein). Reaction mixtures contained 20 g/L (2 %) α-cellulose, 3 g/L (0.3%) lignin (w/w) and 0.7 mg/mL cellulose.

Table 9 and Table 10 summarize the results of initial rates of hydrolysis of α-cellulose at 20 g/L (2%) consistencies by *T. reesei* (TR2) and *Penicillium* sp. (MSUBC1)
cellulase complex. The effects of various purified residual lignin preparations on the initial hydrolysis yields were investigated. Whole cellulase complexes from *T. reesei* and *Penicillium* sp. demonstrated significant differences in their susceptibility to inhibition by lignin derived from both softwoods and hardwoods (Fig. 23). The addition of up to 3.0 g/L lignins to the final substrate concentration reduced the hydrolysis glucose yield by 2-9%. Lignins were more detrimental to cellulose hydrolysis than hardwood poplar lignin. Interestingly, it was also apparent that the *Penicillium* cellulase complex was less susceptible to inhibition by lignin, as compared to the *T. reesei* cellulase. Further, it should be mentioned that *Penicillium* sp. cellulase preparation demonstrated relatively high hydrolysis yields and initial rates of reaction for its substrate.

Figure 23 illustrates the impact of the lignin source on the binding ability of cellulases. Only the source of lignin was changed; the same pretreatment and isolation methods were used for both lignins. It is clear that organosolv softwood lignin had a greater impact on initial hydrolysis yields, in comparison to hardwood lignin. It could be assumed that the difference observed in binding ability of cellulase to lignin was due to the differences in the chemical nature, since the same isolation and pretreatment methods were used to render the physical nature invariable.
Table 9. Initial rates of α-cellulose hydrolysis (2% consistency) by *T. reesei* cellulase complex and the inhibitory effects of two different residual lignins, all extracted from ethanol pulp.

<table>
<thead>
<tr>
<th>Inhibitor type</th>
<th>Glucose Yield in 0.5 h (g/L)</th>
<th>$V_0$ (g/L.h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No lignin</td>
<td>0.85</td>
<td>1.70</td>
</tr>
<tr>
<td>Lignin</td>
<td>0.77</td>
<td>1.54</td>
</tr>
<tr>
<td>Poplar lignin</td>
<td>0.84</td>
<td>1.68</td>
</tr>
</tbody>
</table>

Table 10. Initial rates of α-cellulose (2% consistency) hydrolysis with *Penicillium* sp. cellulase complex and the inhibitory effects of two different residual lignins, all extracted from ethanol pulp.

<table>
<thead>
<tr>
<th>Inhibitor type</th>
<th>Glucose Yield in 0.5 h (g/L)</th>
<th>$V_0$ (g/L.h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No lignin</td>
<td>1.48</td>
<td>2.96</td>
</tr>
<tr>
<td>Lignin</td>
<td>1.38</td>
<td>2.76</td>
</tr>
<tr>
<td>Poplar lignin</td>
<td>1.46</td>
<td>2.95</td>
</tr>
</tbody>
</table>
Figure 23. Influence of a softwood (D. fir) and a hardwood (yellow poplar) residual lignin on the cellulose hydrolytic ability of two cellulase complexes. $\Delta V$ – difference of initial hydrolytic rates with and without lignin.
4. DISCUSSION

A softwood-to-ethanol bioconversion process might be achieved if the obstacles limiting the enzymatic hydrolysis process are identified, characterized, understood, and overcome. The focus of this dissertation was to help elucidate the role of cellulase-lignin interactions in the enzymatic hydrolysis softwood bioconversion step. Numerous scientists consider this role to be a significant limiting factor in softwood enzymatic hydrolysis (Palonen et al., 2003; Converse et al., 1990; Ooshima et al., 1990; Chernaglazov et al., 1988; Sutcliffe and Saddler, 1986). This aspect can be also considered as a novel strategy for improvements in cellulase specific activities towards lignocellulosic substrates based on a reduction of these types of interactions. Chapter two of this dissertation depicted some of the methods developed and used to study the lignin inhibitory role. The results obtained were shown in chapter three.

4.1 The inhibitory role of lignin in the enzymatic hydrolysis of cellulose

The major interest of this work was focused on lignin, which was shown to limit the hydrolysis of cellulose by binding unproductively to cellulases. It is believed, and in some cases, it has been suggested and indirectly demonstrated, that a reduction of this limiting factor leads to a significant enhancement of the enzyme effective activity (Alkasrawi et al., 2003; Eriksson et al., 2002b, McCarter et al., 2002). Non-ionic surfactants, added to the reaction mixture have been recently observed to bind to lignin molecules, resulting in an increase in enzymatic hydrolysis (Helle et al., 1993; Park et al., 1992). One study has showed that the addition of a non-ionic surfactant significantly improved the simultaneous saccharification and fermentation (SSF) of steam-exploded
spruce. One probable reason for that improvement was a reduction of the unproductive binding of the cellulases to lignin (Alkasrawi et al., 2003). In another study done, Eriksson also attributed the reduction in unspecific binding by addition of non-ionic surfactant to be most probably due to coating of the lignin surface by the added surfactants (Eriksson et al., 2002a).

Using initial-rate kinetic studies, it was found that the enzymatic hydrolysis was significantly restricted by the substrate lignin content and type. Even though, lignin is an insoluble inhibitor, it was possible to simulate a homogeneous solution using a constant uniform stirring regime. α-Cellulose, being a substrate, which combines in itself both amorphous and crystalline cellulose, was hydrolysed using \textit{T. reesei} Celluclast 1.5L (TR2) enzyme complex at a variety of lignin contents. The use of initial velocity simplifies the interpretation of kinetic data and avoids complications that may arise as the reaction progresses, such as product inhibition, substrate depletion, and slow denaturation of the enzyme. This simplification was mainly intended to aid in extracting more objective conclusions about the behavior of a complex system with defined properties.

From the results, it was apparent that lignin content can significantly affect the hydrolysis rate (Table 5). It is evident that the increase in lignin content had a negative impact on hydrolysis rates and yields. In various publications, it has been suggested that lignin could be acting as a physical barrier surrounding the cellulose microfibrils, and reducing the accessibility of cellulases to substrates. Previous work done by Mooney et al. (1998) has shown that lignin restricts enzyme accessibility by preventing fiber swelling as well as chemically binding to the enzymes themselves. In turn, the removal of lignin leads to the increase in porosity and specific surface area. Sewalt et al. (1997)
found a 70% reduction, meanwhile Excoffier et al. (1991) found a 24% reduction in the extent of hydrolysis after 24 hours by adding an additional 15% and 25% residual lignin respectively to the hydrolysis vessel. Our results indicate that in addition to lignin distribution, lignin content of 1.0g/L (10% of solids in solution) can decrease the hydrolysis rate by more than 7% in the first hour. It seems that lignin could actually bind to cellulases in a non-specific way, effectively removing the enzymes from active hydrolysis. This unproductive binding of cellulases to lignin reduces the effective concentration of enzyme in solution; and since the rate of enzyme reaction is directly proportional to the enzyme concentration, a decrease in reaction rate was observed.

The non-productive adsorption of enzymes to lignin is significantly affected by the chemical and physical nature of the lignin isolated and used, making the comparison between different studies challenging. Some studies suggest that the method of pretreatment and manner of storage of the lignocellulosic substrate had a considerably significant effect on the rate and extent of cellulase adsorption (Palonen et al., 2003; Glasser et al., 1983). Purified lignin morphology is inevitably modified and may contain some degradation products and other impurities, such as residual polysaccharides. However, despite these reservations, lignin isolation is needed so that researchers can study the inhibitory effect of lignin on cellulase systems and individual enzymes. Numerous researchers have shown that the enzymatic isolation method allows for the unification in the nature of lignin over the course of experiments (Chang et al., 1979).

Hence, after having confirmed the fact that lignin did inhibit cellulases action, it was interesting to understand and determine the degree of this interaction and if some mechanistic information could be obtained. β-glucosidase from A. niger and cellulases
from the complex secreted by *T. reesei* were used for this section of the study. The substrates chosen were cellobiose for β-glucosidase and α-cellulose for the *T. reesei* cellulase complex.

### 4.2 Significance and mechanism of the lignin inhibition of β-glucosidase, cellobiohydrolase I (CBH I) and endoglucanase II (EGII)

Previous studies have looked at the adsorption of cellulase mixtures and purified individual cellulolytic enzymes on lignocellulosic materials, such as pulps and steam pretreated wood substrates and even isolated lignins (Boussaid and Saddler, 1999; Galbe et al., 1990; Ooshima et al., 1990; Sutcliffe and Saddler, 1986; Palonen et al., 2003). However, the literature fails to reliably illustrate the mechanism lignin follows to exercise its inhibitory role during the enzymatic hydrolysis of lignocellulosics. Moreover, contrary to literature available about other known cellulase inhibitors, information about the type and extent of lignin inhibiting effect is neither quantitative nor precise. Therefore, a quantitative evaluation of the inhibition of different types of lignin, hardwood and softwood lignins, on different enzymes, *T. reesei* cellulase complex, purified *T. reesei* CBH I and EG II, and an *A. niger* β-glucosidase, was conducted. The significance of the inhibitory role of lignin in the enzymatic hydrolysis of lignocellulosic substrates and the mechanism of lignin inhibition, in comparison to classical cellulase inhibitors, were also investigated.

Although the conventional Michaelis-Menten formalism is not directly applicable to insoluble cellulose hydrolysis, the experimental data were found to fit the Michaelis-Menten equation, the use of the data as an empirical approximation. Based on the
aforementioned equation, the initial reaction rates could be determined. The cellulose-cellulase system displays dual saturation character as in both the substrate and enzyme can be saturated with each other (Lynd et al., 2002). Therefore, at a cellulose concentration that is much higher than the total concentration of the enzyme, the hydrolysis kinetics would be consistent with the conventional Michaelis-Menten model. A micro-method was developed based on obtained initial rate measurements to avoid problems with measuring low hydrolysis rates and difficulties with product convergence. The Michaelis constant, $K_m$, and the maximal rate, $V_{\text{max}}$, were readily derived from the rates of catalysis measured at different substrate concentrations. The Michaelis constant, $K_m$ has two meanings. It can be interpreted as the concentration of substrate at which half the active sites are filled. Furthermore, $K_m$ can be related to the rate constants of the individual steps in the catalytic mechanism. When being significantly smaller than $k_2$ (Figure 24), $K_m$ can be treated as the dissociation constant of the Enzyme Substrate (ES) complex, and it relates inversely to the binding strength. Since any kind of inhibitor slows down an enzymatic reaction, it must clearly have an effect on the kinetics. Therefore, the nature of this effect may be used to distinguish between different inhibitor types (Figure 24).
4.2.1 The inhibition of β-glucosidase by classical inhibitors and lignin

The enzyme β-glucosidase (EC 3.2.1.21) occurs widely in prokaryotes and eukaryotes. It catalyzes the hydrolysis of aryl- and alkyl-β-D-glucosides as well as glucosides with only a carbohydrate moiety (e.g. cellobiose). Cellulases break down cellulose to cellobiose and β-glucosidases hydrolyze cellobiose to two glucose molecules. β-glucosidase is inhibited by its end product, glucose; the substrate cellobiose accumulates and in turn inhibits the cellulase complex. If the rate limiting step catalyzed by β-glucosidase in cellulose hydrolysis can be overcome, glucose production from cellulose by enzymatic means should become economically feasible (Dekker, 1985).

The apparent Michaelis constant ($K_m$) of β-glucosidase for cellobiose was 0.53 g/L. Glucose inhibited β-glucosidase in a competitive manner with cellobiose as the substrate, as evidenced by the Lineweaver-Burk Plot, illustrated as an increase in the slope of the Lineweaver-Burk plot (Figure 7-A) with a $K_i$ value of 0.55 g/L (3.06 mM). Our kinetic
studies data have also shown that \( \gamma \)-gluconolactone is a high affinity competitive inhibitor of the enzyme. \( \gamma \)-gluconolactone binds to \( \beta \)-glucosidase 4 orders of magnitude more tightly than does D-glucose. Dekker (1985) also observed that glucose and \( \gamma \)-gluconolactone were competitive inhibitors of \( \beta \)-glucosidase. Furthermore, he also showed that \( \gamma \)-gluconolactone was a more powerful inhibitor of \( \beta \)-glucosidase than glucose.

In most cases, competitive inhibitors are substrate or product analogs that bind the enzyme in a similar manner as a substrate, but do not undergo catalysis. Furthermore, a competitive inhibitor binds to the enzyme forming an enzyme inhibitor complex (EI). \( V_{\text{max}} \) is the velocity at a very high substrate concentration. In a competitive inhibitor system, the apparent \( K_{\text{m}} \) increases but \( V_{\text{max}} \) remains the same. This is because the inhibitor competes with the substrate for the enzyme reducing initial rates. However, at sufficiently high substrate concentrations, the enzyme is saturated resulting in catalysis at the maximum velocity. Product molecules are capable of binding to free enzyme to form enzyme-product complex (Segel, 1993). However, transition state analogs are the most potent competitive inhibitors, as was observed with \( \gamma \)-gluconolactone.

The kinetic study of the inhibition of \( \beta \)-glucosidase by lignin suggested that lignin is a high-affinity uncompetitive inhibitor of the enzyme. Uncompetitive inhibitors bind only to ES, and not to the free enzyme. Therefore, the inhibitor, probably, binds at an inhibitory site other than the active site. Experimentally, the lines on a double-reciprocal plot, representing the varying concentrations of lignin, all have the same slope, indicating proportionally decreased value for \( K_{\text{m}} \) and \( V_{\text{max}} \). In uncompetitive inhibition, the \( V_{\text{max}} \) is decreased by the conversion of some molecules of free enzyme to the inactive ternary
form Enzyme-Substrate-Inhibitor (ESI) complex. Since it is the ES complex that binds the inhibitor, the decrease in $V_{max}$ is not reversed by the addition of more substrate. Uncompetitive inhibitors also decrease the $K_m$ because the equilibria for the formation of both ES and ESI are shifted toward the complexes by the binding of the inhibitor. The system observed in Figure 10 yields the same reciprocal plot pattern as pure uncompetitive inhibition. However, in pure uncompetitive inhibition the curves continue to move apart as the concentration of inhibitor increases. In the mixed system, the displacement reaches a limit at high inhibitor concentrations (Segel, 1993). Therefore, lignin binds to the $\beta$-glucosidase two orders of magnitude less tightly than does the competitive inhibitor D-glucose. Thus, lignin inhibition appears to be significant and comparable to the effect of end product inhibition. Also, it was found that lignin forms a ESI triple complex in the mixed uncompetitive inhibition, that still can form products but at a slower rate. In mixed inhibition, the inhibitor binds to the enzyme or the ES with different affinities and ES or ESI, maybe active but with different $k_{cat}$.

4.2.2 The inhibition of *T. reesei* enzyme complex, CBH I and EG II by lignin and various classical inhibitors

The inhibitory mechanism of lignin studied with various carbohydrases seemed to depend both on the enzyme and, in particular, on the type of substrate. Some of the results obtained suggest that an uncompetitive inhibition pattern can be observed for soluble substrates (cellobiose and cellopentaose), while in the case of insoluble substrates ($\alpha$-cellulose) a competitive mechanism is observed. Further studies will have to be performed to support this observation. It was also found that the inhibitory ability of
lignin is slightly lower but it is still comparable in magnitude to the common inhibitors of cellulases.

Inhibition mechanism of carbohydrases by glucose, cellobiose and \( \gamma \)-gluconolactone appeared to be competitive. Competitive inhibitors may work by direct competition with the substrate by binding to the active site, or by binding to a remote site and causing a conformational change in the enzyme. Both mechanisms give identical kinetic results evidenced by the increase in slopes of the Lineweaver-Burk plots.

Meanwhile, inhibition by \( \text{PdCl}_2 \) was shown to be non-competitive. The study done by Lassig et al. (1995) determined that palladium cations are noncompetitive inhibitors of CBH I. Our results confirm these findings based on thorough examination of the Lineweaver-Burk plot. A non-competitive inhibitor binds to the enzyme, or the enzyme substrate complex at a distinct site other than the active site. The inhibitor does not prevent the substrate from binding but rather prevents the enzyme from carrying on the catalysis process. A classical noncompetitive inhibitor has no effect whatsoever on substrate binding or the enzyme-substrate affinity, and hence the \( K_m \) is unchanged.

Inhibition by lignin followed a mixed-type pattern (competitive or uncompetitive) depending on the enzyme and substrate assayed. A variant of competitive inhibition is non-productive binding. This arises when a substrate molecule can fit into the enzyme's binding site in such a way that the normal catalytic event cannot occur. In this situation, the ES is the enzyme-substrate complex that cannot lead to the product. A partial competitive inhibitor can be distinguished from pure competitive inhibition by simply plotting initial rates versus \([I]\) at a fixed substrate concentration. A partial competitive inhibitor cannot drive the velocity to zero at very high inhibitor concentrations. This is
because the high concentrations of inhibitor will drive the entire enzyme to the Enzyme-Inhibitor EI and ESI forms. However, ESI complex can produce products.

An uncompetitive inhibitor binds only to the enzyme substrate complex and not to the free enzyme. The inhibitor only works in the presence of the substrate. The inhibitor reduces $V_{\text{max}}$ as the substrate cannot compete to saturate the active site of the enzyme. Surprisingly, the inhibitor also reduces $K_m$ due to the fact that the inhibitor reduces the concentration of the ES complex by converting it to the inactive the ESI complex. By the Law of Mass Action, this has the effect of pulling the equilibrium of the substrate binding reaction to the right. In effect, then, the inhibitor has increased the amount of substrate which binds to the enzyme, giving an apparent increase in enzyme-substrate affinity and a decrease in $K_m$. Moreover, uncompetitive inhibition is often seen in multiple substrate reactions.

Both studies by Vonhoff et al. (2002) and Väljamäe (2002) showed that the inhibition of cellulases by the hydrolysis product, cellobiose was competitive in nature. The hydrolysis of a low molecular weight model substrate, such as $p$-nitrophenyl cellobioside, by CBH I, was found to be strongly inhibited by cellobiose with an inhibition constant of 20 $\mu$M. Whereas the hydrolysis of cellulose was more resistant to inhibition with an apparent inhibition constant roughly 1.5 mM (0.52 g/L) for cellobiose (Väljamäe et al., 1998). Using $\alpha$-cellulose as a substrate, an inhibition constant of 0.7 g/L for cellobiose was observed in the present study. It is interesting to note that a competitive inhibitor constant is independent of the type of substrate used because competitive inhibitors bind to the enzyme exclusively, thus preventing the substrate from binding. In fact, the inhibitor only needs to have a strong affinity for the binding site.
Ryu and Lee (1986) also studied a cellulase preparation from *T. reesei* and found a competitive inhibition constant of 3.75 mg/mL (11 mM) for cellobiose. Therefore, our results indicate that product inhibition can cause the decrease in hydrolysis rate, especially during the late stages of hydrolysis when the concentration of cellobiose accumulates in the reaction vessel.

Examining Table 6, it is evident that the enzyme affinity for its substrate \( \alpha \)-cellulose (6.5 g/L) is of the same order as its affinity for lignin (9.0 g/L). Both *T. reesei* CBH I and the whole *T. reesei* complex shared similar inhibition patterns when lignin was present. CBH I constituted more than 60% of the whole *T. reesei* cellulase complex (Table 3). However, the minor monocomponents EG II and \( \beta \)-glucosidase displayed similar mechanisms of inhibition by lignin but different from that of the whole complex.

Eriksson et al. (2002a) suggested that the non-productive binding of CBH I and EG I is the main cause of declining rate in hydrolysis of steam pretreated spruce (SPS). In another study conducted by Palonen et al. (2003), the researchers observed that CBH I and EG II had significantly different adsorption affinities to SPS and isolated lignin. Another conclusion they suggested was that CBD seemed to participate in the unspecific binding to lignin. According to this work, the binding of the enzyme to lignin seemed to slow the catalytic activity. However, the triple complex can still form products. The mechanism of inhibition by lignin was different between CBH I and EG II. Nevertheless, the data shows that the magnitude of inhibition by lignin, on a concentration basis, is comparable to that of other known cellulase inhibitors, such as glucose, cellobiose, \( \gamma \)-gluconolactone and PdCl\(_2\).
Therefore, it has been verified that lignin can reduce the hydrolysis efficiency by physical adsorption of active enzymes, a phenomenon called pseudoinhibition. This phenomenon is likely due to the hydrophobicity of the enzymes and the hydrophobic interactions between the lignin and cellulases during the hydrolysis reaction. Consequently, cellulases seem to differ in their ability to bind to lignin. Hence, it might be possible to find in nature or engineer cellulases with low lignin affinity and, as a result of their use, improve the hydrolytic process of lignocellulosics by reduction of the unproductive binding of cellulases to lignin.

4.3 Comparison of the lignocellulosics hydrolability of T. reesei with other cellulase complexes

Most commercially available cellulase preparations, mainly derived from T. reesei, are not designed for optimal hydrolysis of lignocellulose. They were initially developed for applications in the textile industries, where a mild hydrolytic ability is required. For this reason, they demonstrate some drawbacks when being applied in bioconversion studies, such as relatively low specific activity, low thermostability and high sensitivity to end-product inhibition (Mandels, 1985; Klyosov, 1988). Low cellobiase activity of these enzyme systems leads to incomplete hydrolysis of cellobiose to glucose. Besides, they are poor in hemicellulase content. Therefore, their performance could be enhanced by the addition of hemicellulases, cellobiases and other accessory enzymes to break down residual hemicellulose, a physical barrier for cellulases, and cellobiose more efficiently. It has been found that enzymatic hydrolysis of residual hemicellulose in the solid fraction can offer an alternative to the complete removal of hemicellulose in the pretreatment in order to improve the hydrolysis (Palonen, 2004).
The percent compositions of samples pretreated by steam explosion or ethanol organosolv extraction are shown in Table 4. The organosolv-pretreated sample contained a significantly higher amount of cellulose, and significantly lower amounts of lignin, than the sample pretreated by steam explosion. Differences in composition are largely due to differences in the extent of delignification by the two pretreatment processes. Untreated D. fir contains approximately 28% total lignin (Klason lignin plus acid-soluble lignin), as can be seen from Table 1. As previously observed, there was no delignification as a result of steam explosion. In fact, a substantial increase in lignin content (to approximately 46%) was observed (Table 4), mainly due to the removal of most of the hemicellulose fraction (Robinson et al., 2003). Ethanol organosolv pretreatment also resulted in solubilization of hemicellulose accompanied by extensive delignification. Samples contained similar amounts (2.5-3.0%) of residual hemicellulose-derived sugars (arabinose, galactose, mannose and xylose) even though there were noteworthy differences in the amounts of the individual sugars. The glucose content of the steam-exploded and organosolv-pretreated samples was approximately 47 and 85%, respectively, corresponding to around 42 and 76% cellulose, assuming all glucose is derived from cellulose.

4.3.1 Activities of cellulase preparations on pretreated softwood substrates

Data for the hydrolysis of steam-exploded softwood shows that *Penicillium* MSUBC1 performed significantly better than the other cellulase preparations at both 2% and 5% cellulose consistencies (Figures 19 and 20). Superior performance by MSUBC1 was also seen on organosolv-pretreated substrate, although the difference was less pronounced.
(Figures 21 and 22). A more detailed comparison of enzyme performance is provided in Tables 7 and 8 reflected by the values of conversion and initial hydrolytic rates.

Although filter paper activity is commonly used as an index of cellulase performance, it clearly does not provide a reliable indication of the ability of a preparation to hydrolyze complex lignocellulosic substrates. *Penicillium* MSUBC1 showed a lower specific filter paper activity (0.88 FPU/mg) than the TR1 (1.04 FPU/mg) and approximately the same activity as TR2 and MSUBC2 (Table 2). Similarly, there was no observed correlation between enzyme performance on softwood substrates and CMCase or Avicelase activity. Further examination of the enzyme activities revealed that *Penicillium* MSUBC1 had significantly higher intrinsic β-glucosidase activity than the other three preparations (Table 2). High β-glucosidase activity could be at least partially responsible for the superior performance by *Penicillium* MSUBC1 on softwood substrates, presumably by hydrolyzing cellobiose and relieving product inhibition of cellobiohydrolases and endoglucanases. It was previously illustrated that supplementation of a commercial cellulase complex with β-glucosidase, beyond relatively low levels, did not increase its filter paper activity, but did significantly enhance long term hydrolysis of lignocellulosic substrate (Breuil et al., 1992). Nevertheless, TR1 and TR2, which have similar FPA activity, but greater than 3-fold difference in β-glucosidase activity (Table 2), had similar performance on softwood substrates (Figures 5-8, Tables 6 and 7), suggesting that other factors contribute to the efficiency of lignocellulose hydrolysis.

In a study performed by Castellanos et al. (1995b), the authors indicate that *Penicillium sp.* preparations demonstrated relatively high β-glucanase and xylanase activities. The conversion of cellulose to glucose can be significantly increased by
enzymatically removing the inhibitory cellobiose from the reaction system using β-glucosidase. MSUBC1 also shows significantly higher levels of xylanase activity, which may contribute to improved performance by removing residual hemicellulose that impedes cellulase attack. Alternatively, xylanases may increase accessibility indirectly, by facilitating the removal of lignin. Other possible reasons for the improved activity shown by particular cellulase complexes during long-term incubation with softwoods include better enzyme stability, reduced sensitivity to product inhibition by glucose and lower affinity for residual lignin. Experiments to evaluate these possibilities are currently in progress.

The data for MSUBC1 leads to the conclusion that fungal cellulase preparations with significantly improved performance on lignocellulosic substrates can be obtained by selection and random mutagenesis. It is anticipated that the use of such preparations would result in substantial cost savings, by reducing or eliminating the need to supplement preparations with additional accessory enzymes. It is particularly noteworthy that MSUBC1, unlike the other preparations tested, hydrolyzed steam-exploded (containing approximately 44% lignin to yield 76% cellulose conversion) at relatively low enzyme loading (10 FPU/g of cellulose). Under the same conditions the hydrolysis yield was 36% for *T. reesei* (TR1), 30% for *T. reesei* (TR2), and 41% for *T. reesei* (MSUBC2). Also, it should be noted that all softwood hydrolysis experiments described above were performed at 45°C, the optimum for *Trichoderma* cellulase preparations; however, the optimum for MSUBC 1, is 52-55°C (Castellanos et al., 1995a). Therefore, it is anticipated that greater conversion of cellulose in steam-exploded will occur, without delignification and using longer hydrolysis times or higher enzyme loading at optimum
temperature. Typically, steam-exploded softwood shows poor hydrolysis characteristics without further treatment to remove lignin, as illustrated in Figures 19 and 20. Currently, delignification costs are a major drawback to softwood bioconversion schemes based on steam explosion. One of the main reasons that softwoods are harder to pretreat than hardwoods is the decrease in the amount of acetylated hemicellulose sugars. Breakdown of the acetyl groups leads to the formation of acetic acid, which aids in the hydrolysis of hemicellulose sugars (Hon and Shiaishi, 2001). The use of *Penicillium* sp. cellulases could improve the subsequent hydrolysis step without the further requirement of delignification.

### 4.3.2 Cellulase improvement based on a weak-lignin binding criterion

This dissertation evaluated the effect of lignin on the hydrolytic ability of a novel mutant fungal preparation from *Penicillium* sp. (MSUBC1) and the commercial cellulase preparations from *T. reesei* (TR2). Hydrolysis rates of α-cellulose, with or without added lignin were measured after 1 hour hydrolysis. It was shown that *T. reesei* cellulases were significantly more affected by lignin content when compared to the *Penicillium* preparation. As can be seen from Figure 23, Tables 9 and 10, lignin caused inhibition of the enzymes and lowered the hydrolysis rates. However, *T. reesei* hydrolysis rate decreased by 25% more than *Penicillium*. A similar pattern of deactivation was observed using hardwood lignins. Hence, cellulases can differ in their susceptibility to inhibition by lignin. Moreover, lignin's source influences the degree of inhibition observed. This may possibly be due to differences in the chemical structures of the different lignins (Sewalt et al., 1997). This aspect needs to be explored further.
It was shown that the superior properties of MSUBC1 cellulase complex are at least due to its better xylanase activity, higher level of cellobiose activity and lower lignin-enzyme interactions. Nonetheless, it was suggested that the higher β-glucosidase activity would reduce the cellobiose end-product inhibition, while the xylanase activity would increase the enzyme accessibility to cellulose by removing the shielding xylan. Weaker lignin-cellulase interactions would then lead to an increase in the free enzyme in solution. If this hypothesis is confirmed in the future, it could be used as a basis for further improvements to the commercially available lignocellulose bioconversion cellulase complexes. This improvement of the effective activity of the enzymes on real substrates could be understood as the enzymatic activity resulting from the action of productively bound enzymes.

A promising strategy to improve the biomass-to-ethanol process is the reduction of the enzyme costs by increasing the hydrolytic performance of cellulases. It was illustrated and documented by the examples cited above that enzymatic hydrolysis is affected by lignin content of the substrate. Thus, control of unspecific adsorption of enzymes to lignin and optimization of the cellulase mixtures for different raw materials will contribute to further efficient hydrolysis. Given the profound influence of lignin on reducing the hydrolytic efficiency, an alternative approach based on protein engineering can be employed to design enzymes with optimal binding properties for enzymatic hydrolysis of the currently available pretreated lignocellulosic substrates. Protein engineering to reduce lignin-enzyme interactions appears to offer a considerably long-term potential. If the cellulase preparation used was less susceptible to lignin binding, then complete lignin removal during the pretreatment would not be necessary in order to
obtain high conversion of cellulose. Therefore, by reduction of the unproductive binding of cellulases to lignin, the presence of residual lignin would not be problematic. Instead, a balance would be reached between lignin content and pretreatment costs. It is also anticipated that this approach would increase the efficiency of enzyme recycling because binding to residual lignin results in low recovery of enzymes following extensive hydrolysis of lignocellulosic substrates. As a consequence, an improvement of the lignocellulose-to-glucose hydrolysis should be achieved.
5.  FUTURE WORK

The enzymatic hydrolysis step in an overall biomass-to-ethanol process is considered and has proven to be one of the major technical bottlenecks of the overall process. The enzyme adsorption staged is of key importance to the fundamental knowledge of enzymatic hydrolysis of cellulose. This project has highlighted the influence that lignin exerts on the enzymatic hydrolysis step. Further future work should examine the particulars of cellulases adsorption to the lignin fraction. The driving forces that lead to an enzyme-lignin complex formation should be characterized and understood. Also, a study based on the cellulase enzymes complete adsorption isotherms must be completed for a final conclusion in terms of lignin-binding affinity of these proteins. A more detailed understanding of these factors would lead to optimization of pretreatment process design.

It is generally accepted that the interaction of proteins with their environment is defined by their surface properties. Protein surface properties can play an important role in the mechanism, rate and extent of adsorption to lignin. During the hydrolysis process, the total exposed surface area of the lignin increases and, as a consequence, the effective concentration of the inhibitor also increases. Accordingly, it is logical to suggest that the degree and type of interaction of cellulases with a specific type of lignin will be defined by the protein surface properties, namely by their surface hydrophobicity (HF), their net surface charge, and by their ability of forming hydrogen bonds with lignin. Also, future research should study and analyze the role played by enzyme surface hydrophobicity and net surface charge in the enzymes ability to bind to lignin. A detailed thermodynamic and
kinetic study looking at the dependence of the adsorption of different glycosyl hydrolases to lignin on pH, temperature, and ionic strength should be performed to complement our current work. This would help in the development of a state-of-the-art model to define the optimal low lignin-binding cellulases.

This work has displayed that cellulases seem to differ in their ability to bind to lignin. We were able to find natural cellulases with high hydrolytic activities and low lignin affinity and, as a result of their use; we could improve the hydrolytic process of lignocellulosics. Moreover, *Penicillium* MSUBC1 had significantly better performance than the other commercially known cellulase preparations on both pretreated softwood substrates tested. Future research may point out why this ubiquitous enzyme complex performed better. This last aspect allows us to suppose that more enzymes with higher hydrolytic activities and different lignin affinity might also be found in nature. In essence, this should make it possible to engineer a tuned cellulase complex with low lignin affinity. However, what we are still unaware of is how and why these enzymes differ in their lignin binding affinity and their hydrolytic activity. This aspect needs to be studied further and in greater detail.

It would be beneficial in the future to be able to decrease the nonspecific adsorption of cellulases on lignin in the commercial hydrolysis applications. In theory, the cellulase enzymes could be modified so that they do not bind so tightly to lignin. Another option would be to modify or block the surface of lignin so that cellulases do not adsorb to it. For instance, surfactants can be added to decrease the unspecific binding to lignin by occupying the binding sites. These results will be of great importance for the studies oriented at enzyme engineering of cellulases.
6. REFERENCES


Eriksson, T.; Karlsson, J.; and Tjerneld, F. 2002b. A model explaining declining rate in hydrolysis of lignocellulose substrates with celllobiohydrolase I (Cel 7A) and endoglucanase I (Cel 7B) of Trichoderma reesei. Applied Biochemistry and Biotechnology 101:41-60.


Hogan, C.M. 1987. Enzymatic and structural factors limiting hydrolysis of cellulose by Trichoderma harzianum E58 cellulases. MSc. thesis


Semenova M.V.; Grishutin S.G.; Gusakov A.V.; Okunev O.N.; and Sinitsyn A.P. 2003 Isolation and properties of pectinases from fungus Aspergillus japonicus. Biochemistry (Moscow), 68: 559-569.


www.cofi.org, 2004
www.greenfuels.org, 2002
www.nrel.gov/clean_energy/bioenergy.html
http://www.eere.energy.gov/biomass/, 2004
http://www.eere.energy.gov/biomass/pdfs/novozymesEsp_review.pdf
http://www.nccp.ca/NCCP/pdf/Canada's_Wood_Residues.pdf, 1999


