Role of lignin in the enzymatic hydrolysis of lignocellulose

Hetti Palonen

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VTT Biotechnology

Dissertation for the degree of Doctor of Technology to be presented with due to permission for public examination and debate in Auditorium Komppa (Ke-2) at Helsinki University of Technology (Espoo, Finland) on the 16th of April, 2004, at 12 o'clock.

**Keywords** enzymatic hydrolysis, lignocellulose, enzymes, cellulases, *Trichoderma reesei*, softwood lignin, pretreatment, wet oxidation, enzymatic modification, laccase

**Abstract**

Characterization, understanding and overcoming barriers of enzymatic hydrolysis of different raw materials is essential for the development of economically competitive processes based on enzymatic treatments. This work focused on factors relevant for the improvement of enzymatic hydrolysis of lignocellulose raw materials derived from softwood. The major interest of the work was in lignin. Specific areas addressed were the role of lignin in the unproductive binding of cellulases, which restricts the hydrolysis of cellulose, and enzymatic modification of lignin in order to improve cellulose hydrolysis. In addition, suitability a new pretreatment method, wet oxidation, was evaluated for softwood.

The binding of *Trichoderma reesei* CBH I and CBH II enzymes on bacterial microcrystalline cellulose (BMCC) was shown to be determined by a cooperative effect of the two domains, the cellulose binding domain (CBD) and the catalytic domain (CD). Binding of the intact CBH I on bacterial microcrystalline cellulose (BMCC) was fully reversible, while the binding of CBH II was only partly reversible. The cellulases CBH I and EG II were adsorbed on steam pretreated softwood (SPS) and lignin. The observation that the presence of CBD clearly enhanced the binding of the enzymes on SPS and especially on lignin, suggests that unspecific adsorption is dominated by the affinity of the CBD.

The wet oxidation pretreatment studies gave information on the importance of substrate structure in the enzymatic hydrolysis. This pretreatment method was applied to softwood for the first time. In the wet oxidation pretreatment studies, the total recovery of carbohydrates was high and the recovery of cellulose even higher than what has been reported for steam pretreated softwood. Lignin fraction of the substrate remained mainly undissolved. No clear correlation between the hydrolysis yield and lignin content could be observed. It was
concluded that the location and chemical/physical structure of lignin affected the enzymatic hydrolysis more than the absolute amount of lignin. It was shown that the hydrolysis result could be improved by optimizing the pretreatment conditions, reducing the hemicellulose content or hydrolysing the residual hemicellulose by selecting a suitable combination of enzymes.

This study showed for the first time that enzymatic modification and/or removal of lignin can be combined with simultaneous cellulose hydrolysis. Both the modification of lignin surfaces by oxidative treatments with laccase alone and delignification treatment with a laccase-mediator system lead to increased hydrolysis of lignocellulose. Oxidation of lignin by laccase was achieved by the three laccases tested, produced by *Trametes hirsuta*, *Melanocarpus albomyces* and *Mauginiella* sp. The new laccase isolated and purified from *Mauginiella* sp. had enzymatic characteristics similar to many basidiomycete laccases. Different adsorption of the three laccases onto SPS did not correlate with the capability of the laccases to oxidize the substrate and consequently, to improve lignocellulose hydrolysis.
Preface

This work was carried out at VTT Biotechnology during the years 1999–2003. My supervisor at the Helsinki University of Technology, professor Simo Laakso is thanked for his support during this work. I would like to thank professor Maija Tenkanen who introduced me to this research field and supervised my work during the years 1999–2000. I wish to express my special gratitude to my second supervisor, professor Liisa Viikari for the inspiring discussions and the encouragement, which helped me to finish this work. My co-authors Dr Kristiina Kruus, Dr Markus Linder and Dr Markku Saloheimo are thanked for their valuable knowledge and help in planning the experiments and preparing the papers. I am grateful to professor Folke Tjemeld, professor Guido Zacchi and Dr Anne Belinda Thomsen for their support and providing me with good working facilities while I visited their laboratories. I also acknowledge my colleagues and all the personnel in the laboratory for the pleasant working atmosphere.

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I wish to thank my family and friends for their support and encouragement outside the laboratory. Finally, I want to warmly thank my husband Janne for all his support and encouragement over the years.
List of publications

This thesis is based on the following publications, which are referred to in the text by Roman numerals I–V. Additional unpublished data is also presented.


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<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABTS</td>
<td>2,2’-azino-bis-(3-ethylbenzothiazoline)-6-sulphonate</td>
</tr>
<tr>
<td>BMCC</td>
<td>bacterial microcrystalline cellulose</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CBD</td>
<td>cellulose binding domain</td>
</tr>
<tr>
<td>CBH I</td>
<td>cellobiohydrolase I</td>
</tr>
<tr>
<td>CBH II</td>
<td>cellobiohydrolase II</td>
</tr>
<tr>
<td>CD</td>
<td>catalytic domain</td>
</tr>
<tr>
<td>DNS</td>
<td>dinitrosalicylic acid</td>
</tr>
<tr>
<td>DP</td>
<td>degree of polymerization</td>
</tr>
<tr>
<td>EG II</td>
<td>endoglucanase II</td>
</tr>
<tr>
<td>ESCA</td>
<td>electron spectroscopy for chemical analysis</td>
</tr>
<tr>
<td>FPU</td>
<td>filter paper unit</td>
</tr>
<tr>
<td>HBT</td>
<td>1-hydroxybenzotrizole</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>IEF</td>
<td>isoelectric focusing</td>
</tr>
<tr>
<td>NHA</td>
<td>N-hydroxy-N-phenylacetamide</td>
</tr>
<tr>
<td>NHA-Ac</td>
<td>N-acetoxy-N-phenylacetamide</td>
</tr>
<tr>
<td>SPS</td>
<td>steam pretreated softwood (spruce)</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>pI</td>
<td>isoelectric point</td>
</tr>
<tr>
<td>WOS</td>
<td>wet oxidation pretreated softwood</td>
</tr>
</tbody>
</table>
1. Introduction

1.1 Lignocellulose – a valuable resource

Cellulosic biomass provides a low cost and abundant resource that has the potential to support large-scale production of fuels and chemicals. Lignocellulosic biomass includes materials such as agricultural residues (e.g. corn stover and wheat straw), forestry residues (e.g. sawdust, thinnings, and mill wastes), portions of municipal solid waste (e.g. waste paper) and various industrial wastes. Herbaceous (e.g. switchgrass) and woody (e.g. poplar trees) crops can also be used.

Lignocellulosic materials are formed from three main components: cellulose, hemicellulose, and lignin. A variety of fungi and bacteria can break down lignocellulose by using a battery of hydrolytic and oxidative enzymes (Eriksson et al., 1990). Cellulose is the major component in plant cell walls and constitutes up to 50% of the dry weight of wood. Cellulose and hemicellulose can be hydrolysed to sugars that can be further converted, either microbiologically or chemically, into energy carriers such as ethanol and butanol, or various other products such as organic acids, acetone, or glycerol (Wyman, 2002). The hexose sugars glucose, galactose, and mannose can be easily metabolized by conventional yeast, *Saccharomyces cerevisiae*. The hydrolysis products of xylans require organisms capable of fermenting both hexoses and pentoses.

Due to the crystalline structure of cellulose as well as the complex structural organisation of cellulose, hemicellulose, and lignin, lignocellulosic materials are more difficult to break down than, for example, starch based materials. Sugar monomers can be produced from cellulose and hemicellulose either by acids or by hydrolytic enzymes. Saccharification of cellulosic biomass by acid has a long history, and both dilute-acid technologies at high temperature and concentrated acids at lower temperatures have been studied (Wyman, 1996). Enzymatic degradation of lignocellulose involves a set of different enzymes. A wide variety of cellulolytic fungi and bacteria have been reported to date and this number is continually increasing. The enzyme system secreted by the filamentous fungus *Trichoderma reesei* has been studied intensively since the 1950s (reviewed in Montenecourt, 1983).
The most abundant source of raw materials available for energy production in Finland consists of forest residues, of which the final cutting site residues and first thinnings comprise the highest potential for bioenergy production. Other interesting sources would be side streams from the pulp and paper industry. Currently, the growing stock volume in Finland is about 2 billion m$^3$, consisting mainly of softwood, namely pine (47%) and spruce (34%), and the hardwood birch (15%) (Sevola, 2002). Bark (8 million m$^3$), sawdust (2.3 million m$^3$) and forest and industrial chips (1.9 million m$^3$), are used for energy generation in Finland, but solely as solid fuel (Ylitalo, 2002).

Softwood, the dominating source of lignocellulose in the Northern hemisphere, has been the subject of interest as a raw material for fuel ethanol production in Sweden, Canada and Western USA (reviewed in Galbe and Zacchi, 2002). Ethanol fuel can reduce greenhouse gas emissions and improve air quality as well as offer strategic or economical advantages (Wyman, 1996). Ethanol is already produced (from sugar cane and maize) in large quantities in Brazil and United States to replace petrol in motor vehicles. Today, all cars equipped with a catalyst can run on a mixture of 90% gasoline and 10% ethanol without engine modification. In Brazil, pure ethanol is used as fuel (Zalin et al., 2000). Ethanol is also used to produce an oxygenated fuel additive, ethyl butyl ether (ETBE), which is formed in the reaction between ethanol and isobutylene. France and Spain, the largest fuel ethanol producers in the European Union, produce ETBE from ethanol (Anonymous, 2002). Several comprehensive reviews on the economics and challenges of ethanol production from biomass, as well as the use of ethanol as fuel are available (Wheels et al., 1999; Kaylen et al., 2000; Zaldivar et al., 2001; Wingren et al., 2003).

Over the past two decades the cost of biological conversion of cellulosic biomass to ethanol has been reduced from about 1.22 USD/l (Wright, 1988) to the point where it is becoming economically viable. The present ethanol production cost, estimated by NREL (National Renewable Energy Laboratory, U.S.), is 0.31 USD/l, and is expected to decrease to about 0.22 USD/l by the year 2010 (Wooley et al., 1999). This is mainly due to advances in biosciences, leading to decreased cost of celulolytic enzymes and development of more efficient organisms for fermentation (Wyman, 2001). Presently, enzymatic hydrolysis is considered the most promising technology for converting biomass
into sugars and to be used as raw material for the production of various other biotechnical bulk chemical products.

1.2 Structural features of lignocellulosic materials

The major components of lignocellulosic materials are cellulose, hemicellulose, lignin and extractives. The wood cells are composed of different layers, which differ from one another with respect to their structure and chemical composition. Basically, cellulose forms a skeleton which is surrounded by other substances functioning as matrix (hemicelluloses) and encrusting (lignin) materials. Cellulose, hemicellulose and lignin are closely associated and covalent cross-linkages have been suggested to occur between lignin and polysaccharides (lignin-carbohydrate complexes, LCC). The side-groups arabinose, galactose and 4-O-methylglucuronic acid are most frequently perceived as connecting links to lignin (Fengel and Wegener, 1983). It is generally agreed that the hemicellulose molecules are oriented parallel to the cellulosic fibrils as shown in Figure 1 (Page, 1976).

![Diagram](image)

*Figure 1. Proposed model for the structure of softwood lignocellulose (Page, 1976).*

The composition of agricultural waste materials or woody biomass depends on the plant species and age, growth conditions and fractionation or processing steps. The compositions of different lignocellulosic materials are given in Table 1. Generally, the lignin content of softwoods is higher than in hardwoods. The differences in the chemical composition of softwood and hardwood are discussed in the following chapters.
Table 1. Chemical composition of various lignocellulosic materials (percent of dry weight).

<table>
<thead>
<tr>
<th>Lignocellulose</th>
<th>Cellulose</th>
<th>Xylan</th>
<th>Mannan</th>
<th>Galactan</th>
<th>Arabinan</th>
<th>Lignin</th>
<th>Extractives</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spruce wood</td>
<td>41.9</td>
<td>6.1</td>
<td>14.3</td>
<td>na</td>
<td>1.2</td>
<td>27.1</td>
<td>9.6</td>
<td>Hayn et al., 1993</td>
</tr>
<tr>
<td>Pine wood</td>
<td>37.7</td>
<td>4.6</td>
<td>7.0</td>
<td>na</td>
<td>na</td>
<td>27.5</td>
<td>10.8</td>
<td>Hayn et al., 1993</td>
</tr>
<tr>
<td>Birch wood</td>
<td>38.2</td>
<td>18.5</td>
<td>1.2</td>
<td>na</td>
<td>na</td>
<td>22.8</td>
<td>4.8</td>
<td>Wiselogel et al., 1996</td>
</tr>
<tr>
<td>Poplar wood</td>
<td>49.9</td>
<td>17.4</td>
<td>4.7</td>
<td>1.2</td>
<td>1.8</td>
<td>18.1</td>
<td>na</td>
<td>Wiselogel et al., 1996</td>
</tr>
<tr>
<td>Corn stover</td>
<td>36.4</td>
<td>18.0</td>
<td>0.6</td>
<td>1.0</td>
<td>3.0</td>
<td>16.6</td>
<td>7.3</td>
<td>Wiselogel et al., 1996</td>
</tr>
<tr>
<td>Wheat straw</td>
<td>38.2</td>
<td>21.2</td>
<td>0.3</td>
<td>0.7</td>
<td>2.5</td>
<td>23.4</td>
<td>13.0</td>
<td>Wiselogel et al., 1996</td>
</tr>
<tr>
<td>Switchgrass</td>
<td>31.0</td>
<td>20.4</td>
<td>0.3</td>
<td>0.9</td>
<td>2.8</td>
<td>17.6</td>
<td>17.0</td>
<td>Wiselogel et al., 1996</td>
</tr>
</tbody>
</table>

na not available
1.2.1 Cellulose

Cellulose is the main constituent of plant cell walls comprising about 50% of wood as seen in Table 1. Cellulose is closely associated with hemicelluloses and lignin, and the isolation of cellulose requires intensive chemical treatments. Cellulose consists of D-glucopyranose monomer units bound by β-1-4-glycosidic linkages. The successive glucose residues are rotated by 180° relative to each other, and thus the repeating unit of the cellulose chain is the cellobiose unit. The average degree of polymerization (DP) of plant cellulose varies between 7000 and 15 000 glucose units, depending on the source (Fengel and Wegener, 1983).

The functional groups in the cellulose chain are the hydroxyl groups. These OH-groups are able to interact with each other or with O-, N-, and S-groups, forming hydrogen bonds. H-bonds also exist between OH-groups of cellulose and water molecules. These hydroxyl groups make the surface of cellulose largely hydrophilic. The cellulose chain has OH-groups at both ends. The C1 -end has reducing properties. The cellulose chain is stabilized by strong hydrogen bonds along the direction of the chain. In native cellulose found in plant sources, cellulose chains are packed together to form highly crystalline microfibrils in which the individual cellulose chains are held together by hydrogen bonds. An individual cellulose crystal contains tens of glucan chains in a parallel orientation. Seven crystal polymorphs have been identified for cellulose, which are designated as Iα, Iβ, II, IIIα, IIIβ, IVα, and IVβ (O’Sullivan, 1997). In nature, cellulose Iα and Iβ are the most abundant crystal forms (Atalla and Vanderhalt, 1984). In addition to highly crystalline regions, native cellulose contains less-ordered amorphous regions. In wood fibers, the winding direction of cellulose microfibrils varies in different cell wall layers. Several reviews have surveyed cellulose structure and it is still the subject of intense study (Hon 1994; O’Sullivan 1997; Brown, 1999; Kadla and Gilbert, 2000).

1.2.2 Hemicelluloses

Hemicelluloses are generally classified according to the main sugar residue in the backbone, e.g. xylans, mannans, galactans and glucans, with xylans and
mannans being the main groups of hemicelluloses. Hemicelluloses are often reported to be chemically associated with or cross-linked to other polysaccharides, proteins or lignin. Xylans appear to be the major interface between lignin and other carbohydrates (Jeffries, 1990). Hemicelluloses are more soluble than cellulose, and they can be isolated from wood by extraction. However, alkali extractions deacetylate the hemicelluloses completely (Sjöström, 1981). The average degree of polymerization of hemicelluloses varies between 70 and 200 depending on the wood species (Fengel and Wegener, 1983).

Hemicellulose in hardwoods and annual plants is mainly xylan (15–30%), whereas softwood hemicelluloses consist of galactoglucomannans (15–20%) and xylans (7–10%). Hardwood xylan is composed of β-D-xylopyranosyl units, which contain 4-O-methyl-α-D-glucuronic acid and acetyl side groups. The 4-O-methylglucuronic acid is linked to the xylan backbone by O-(1→2) glycosidic bonds and acetic acid is esterified at the carbon 2 and/or 3 hydroxyl group. The molar ratio of xylose: glucuronic acid:acetyl residues is about 10:1:7. Softwood xylans are arabino-4-O-methylglucuronoxylans, which are not acetylated, but the xylan backbone is substituted at carbon 2 and 3 with 4-O-methyl-α-D-glucuronic acid and α-L-arabinofuranosyl residues, respectively (Fengel and Wegener, 1983).

Softwood galactoglucomannan has a backbone of β-1-4-linked β-D-glucopyranosyl and β-D-mannopyranosyl units, which are partially substituted by α-D-galactopyranosyl and acetyl groups (Sjöström, 1981). Two types of galactoglucomannans can be separated; water and alkali soluble fractions, with ratios of mannose:glucose:galactose:acetyl residues 3 : 1 : 1 : 0.24 for the water soluble fraction, and 3 : 1 : 0.1 : 0.24 for the alkali soluble fraction (Timell, 1967).

### 1.2.3 Lignin

Lignin is a complex, hydrophobic, cross-linked aromatic polymer. In nature, lignin is mostly found as an integral part of the plant cell wall, embedded in a carbohydrate polymer matrix of cellulose and hemicellulose. Isolation of native lignin is complicated, when at all possible (Fengel and Wegener, 1983). Lignins
are polymers of phenylpropene units: guaiacyl (G) units from the precursor *trans*-coniferyl-alcohol, syringyl (S) units from *trans*-sinapyl-alcohol, and p-hydroxyphenyl (H) units from the precursor *trans*-p-coumaryl alcohol. The exact composition of lignin varies widely with species. In addition to classification as softwood, hardwood and grass lignins, lignins can be divided into two major groups: guaiacyl lignins and guaiacyl-syringyl lignins (Gibbs, 1958). Guaiacyl lignins are predominantly polymerization products of coniferyl alcohol while guaiacyl-syringyl lignins are composed of varying parts of the aromatic nuclei guaiacyl and syringyl, together with small amounts of p-hydroxyphenyl units (Fengel and Wegener, 1983).

Softwood contains mainly guaiacyl units while hard wood contains also syringyl units. For spruce (*Picea abies*) a ratio G:S:H = 94:1:5 has been reported (Erickson *et al*., 1973), and for pine (*Pinus taeda*) G:S:H = 86:2:13 (Glasser and Glasser, 1981). It has been found that softwood is more resistant to lignin removal by alkaline extraction than hardwood (Ramos *et al*., 1992). It has been suggested that guaiacyl lignin restricts fibre swelling and thus the enzymatic accessibility more than syringyl lignin. It was observed that the residual substrate remained after extensive hydrolysis of steam pretreated aspen and eucalyptus was mainly composed of vessel elements. Vessel elements are known to have a greater guaiacyl to syringyl ratio than other cells found in hardwood (Ramos *et al*., 1992). The 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).

Recent observations have given indications that all lignin is not homogeneous in structure. Lignin seems to consist of amorphous regions and structured forms such as oblong particles and globules (Novikova *et al*., 2002). Lignin in higher plant cell walls is not amorphous. Phenyl rings of softwood lignin have been shown to be aligned preferentially in the plane of the cell wall (Agarwal and Atalla, 1986; Atalla and Agarwal, 1985). There are also indications that both the chemical and three-dimensional structure of lignin is strongly influenced by the polysaccharide matrix (Houtman and Atalla, 1995). Molecular dynamic simulations have suggested that the hydroxyl and methoxyl groups in lignin precursors and oligomers may interact with cellulose microfibrils despite the fact that lignin is hydrophobic in character (Houtman and Atalla, 1995).
The major type of linkage in spruce lignin is ether linkage, of which arylglycerol-β-aryl ether linkage is the most common. In addition, the phenyl propene units are linked by carbon-to-carbon linkages (Sjöström, 1981). The functional groups affecting the reactivity of lignin include free phenolic hydroxyl, methoxyl, benzylic hydroxyl, benzyl alcohol, noncyclic benzyl ether and carbonyl groups. Guaiacyl lignin contains more phenolic hydroxyl groups than syringyl (Sjöström, 1981). The structural scheme for softwood lignin, including new dibenzodiaxocin structures, constructed by Brunow (1998) is presented in Figure 2.

The chemical structure of native lignin is essentially changed under high temperature and acidic conditions, such as the conditions during steam pretreatment. At reaction temperatures higher than 200°C, lignin has shown to be agglomerated into smaller particles and separated from cellulose (Tanahashi et al., 1983). Early studies on hardwood lignin have shown that the β-O-4 aryl ether linkages are cleaved in steam-explosion causing a decrease in molecular weight and an increase in phenolic content (Marchessault et al., 1981). A study on steam exploded softwood has shown that lignin becomes more condensed and the reactive groups at the α-position, such as hydroxyl groups and ethers, are oxidized to carbonyl groups or generate benzylic cations, which form C-C bonds, leading to loss of reactivity at the α-positions (Shevchenko et al., 1999).

Soluble lignin derivatives (lignosulfonates) are formed in the sulfite pulping process at elevated temperatures with sulfur dioxide and hydrogen sulfite ions as pulping chemicals. Lignin dissolved in sodium hydroxide or in a mixture of sodium hydroxide and sodium sulfide is termed sulfate (kraft) lignin (Sjöström, 1981).
Figure 2. The structure of softwood lignin (Brunow, 1998). The most common structures and functional groups in the lignin molecule are pointed out.

1.3 Hydrolytic enzymes of *Trichoderma reesei*

*Trichoderma reesei* is a filamentous fungus, which degrades plant material in the soil in its natural environment. The hydrolytic enzymes of *T. reesei* can efficiently degrade cellulose and hemicellulose to the basic sugar components.
1.3.1 Cellulases

The enzymatic hydrolysis of cellulose represents a special case of enzymology since the substrate is solid and thus the hydrolysis occurs in the solid phase. Efficient hydrolysis of cellulose requires a mixture of different cellulases. Cellulases secreted by *T. reesei* are listed in Table 2. Cellulases have been traditionally categorized into two types. Endoglucanases (EC 3.2.1.4) hydrolyze internal bonds in the cellulose chain and act mainly on the amorphous parts of the cellulose. Cellobiohydrolases (exoglucanases; EC 3.2.1.91) hydrolyze from the chain ends and produce predominantly cellobiose, and are able to degrade crystalline cellulose (reviewed in Teeri, 1997). The different glycosyl hydrolases have recently been grouped according to the structures of their catalytic domains (Coutinho and Henrissat, 1999). Presently, 92 glycosyl hydrolase families have been identified. This classification, however, requires information on the amino acid sequence which is not always available. Presently, there are cellulases in 14 glycosyl hydrolase families.

All *T. reesei* cellulases, except EG III, as well as many other cellulases from other micro-organisms, have a two domain structure consisting of a catalytic domain (CD) and a cellulose binding domain (CBD), which are bound together by a flexible linker (Gilkes *et al.*, 1991). So 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 CBDs are also available and their structure-function interactions with cellulose have been studied extensively. Determination of the crystal structure for nine catalytic domains has confirmed that the members of the same family have similar three-dimensional folds and probably share a similar reaction mechanism (Davies and Henrissat, 1995; Tomme *et al.*, 1995). The structural differences of the catalytic domains of cellobiohydrolases and endoglucanases suggest that their action on polymeric substrates is dictated by the shape of their active site. Endoglucanases have an open active site, which enables action in the middle of the glucan chain, while exoglucanases have a tunnel-shaped active site which can hydrolyse only chain ends (Teeri, 1997).

The two cellobiohydrolases of *T. reesei* exhibit synergy and have been shown to act at different ends of the cellulose chain; CBH I acts at the reducing end and CBH II at the nonreducing end (Barr *et al.*, 1996). CBH I is the main cellulase of
T. reesei comprising about 60% of the total secreted protein and it is generally recognized as the key enzyme in the degradation of crystalline cellulose. It is regarded as a strict exoenzyme. CBH II accounts for about 15% of the total secreted protein and it has some endo-character. CBH I contains an active-site tunnel which is almost twice as long as that observed for CBH II (Rouvinen et al., 1990; Divne et al., 1994).

Table 2. Properties of T. reesei cellulases.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>New name</th>
<th>Molecular mass (kDa)</th>
<th>pI</th>
<th>Conc. (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBH I</td>
<td>Cel7A</td>
<td>59–68</td>
<td>3.5–4.2</td>
<td>50–60</td>
<td>Shoemaker et al., 1983</td>
</tr>
<tr>
<td>CBH II</td>
<td>Cel6A</td>
<td>50–58</td>
<td>5.1–6.3</td>
<td>15–18</td>
<td>Chen et al., 1987</td>
</tr>
<tr>
<td>EG I</td>
<td>Cel7B</td>
<td>50–55</td>
<td>4.6</td>
<td>12–15</td>
<td>Penttilä et al., 1986</td>
</tr>
<tr>
<td>EG II</td>
<td>Cel5A</td>
<td>48</td>
<td>5.5</td>
<td>9–11</td>
<td>Saloheimo et al., 1988</td>
</tr>
<tr>
<td>EG III</td>
<td>Cel12A</td>
<td>25</td>
<td>7.4</td>
<td>0–3</td>
<td>Ward et al., 1993</td>
</tr>
<tr>
<td>EG IV</td>
<td>Cel45A</td>
<td>37 d</td>
<td>na</td>
<td>na</td>
<td>Saloheimo et al., 1997</td>
</tr>
<tr>
<td>EG V</td>
<td>Cel61A</td>
<td>23 d</td>
<td>2.8–3.0</td>
<td>0–3</td>
<td>Saloheimo et al., 1994</td>
</tr>
<tr>
<td>BGL I</td>
<td>Cel3A</td>
<td>71</td>
<td>8.7</td>
<td>na</td>
<td>Chen et al., 1992</td>
</tr>
<tr>
<td>BGL II</td>
<td>Cel1A</td>
<td>114</td>
<td>4.8</td>
<td>na</td>
<td>Takashima et al., 1999</td>
</tr>
</tbody>
</table>

aCBH, cellobiohydrolase; EG, endoglucanase; BGL, β-glucosidase  
bDesignation according to the classification of glycosyl hydrolase families  
cfrom Tolan (2002)  
dcalculated according to the amino acid sequence deduced from gene sequence  
na not available
It has been shown that the ability of cellobiohydrolases to degrade crystalline cellulose clearly decreases when the CBD is absent (Linder and Teeri, 1997). The removal of CBD has little effect on the activity towards soluble substrates or amorphous cellulose. It has been recognized that CBD increases the enzyme concentration on the surface of a solid substrate (Ståhlberg et al., 1991), and it may promote solubilization of individual glucan chains from the cellulose surface (Knowles et al. 1987; Teeri, 1997). The CBD may also promote non-productive binding to the substrate at higher enzyme concentrations (reviewed in Linder and Teeri, 1997).

The third major enzyme type needed for total cellulose hydrolysis, β-glucosidase (EC 3.2.1.21), hydrolyses short cello-oligosaccharides to glucose. The β-glucosidase levels of T. reesei are low, and addition of this enzyme is needed in order to prevent end-product inhibition by cellobiose.

1.3.2 Hemicellulases

In wood, the two most common hemicelluloses are xylans and glucomannans. (discussed in chapter 1.2.2). For the total enzymatic hydrolysis of hemicellulosic polysaccharides, several synergistically acting enzymes are needed (reviewed in e.g. Viikari et al., 2001). Endoxylanases (1,4-β-D-xylan xylanohydrolases, EC 3.2.1.8) and endomannanases (1,4-β-D-mannan mannanohydrolase, EC 3.2.1.78) attack the main chain of xylan and glucomannan, respectively. Xylanases belong to the glycosyl hydrolase families 10 and 11 and differ from each other with respect to their catalytic properties (Biely et al., 1997). Mannanases are a more heterogenous group of enzymes regarding their biochemical properties, and they have not been classified into obvious categories. A number of enzymes that degrade hemicellulose have been identified in T. reesei. These enzymes include four xylanases and a mannanase (Foreman et al., 2003). The mannanase of T. reesei has been found to have a similar multidomain structure as several cellulosytic enzymes (Stålbrand et al., 1995).

Enzymes needed for further hydrolysis of the short oligomeric compounds produced by endo-enzymes from hemicelluloses are β-xylosidase (1,4-β-D-xyloside xylohydrolase, EC 3.2.1.37), β-mannosidase (1,4-β-D-mannoside...
mannohydrolase, EC 3.1.1.25) and β-glucosidase (EC 3.2.1.21). Side groups which are still attached to oligosaccharides after the hydrolysis of xylans and mannans by xylanase or mannanase, respectively, restrict the action of β-xylosidase and β-mannosidase. The side groups are removed by α-glucuronidase (EC 3.2.1.139), α-arabinosidase (α-L-arabinofuranoside arabinofuranohydrolase, EC 3.2.1.55) and α-D-galactosidase (α-D-galactoside galactohydrolase, EC 3.2.1.22). Acetyl substituents bound to hemicellulose are removed by esterases (3.1.1.72) (Viikari et al., 2001).

1.4 Oxidative enzymatic modification of lignocellulose

Reactions catalyzed by oxidative enzymes play a significant role in the complete degradation of lignocellulosic biomass. However, only a few organisms in nature, belonging to white rot fungi, are capable of degrading lignin efficiently (Hatakka, 1994; Hatakka, 2001). White-rot fungi are also able to degrade other compounds such as phenolic pollutants with a chemical structure resembling that of lignin (Fernando and Aust, 1994). The presently recognized enzyme systems anticipated to participate in delignification include lignin peroxidase, LiP (EC 1.11.1.14.), manganese-dependent peroxidase, MnP (EC 1.11.1.13) and laccase (EC 1.10.3.2). Oxidative transformations of various organic molecules in nature require co-operation of a number of enzymes and coenzymes, which transfer hydrogen atoms or electrons from an initial donor to a final acceptor. Oxidases, the enzymes using molecular oxygen as the electron acceptor, are currently regarded to have the highest industrial potential. Addition of an electron-transferring mediator which is able to oxidize several key linkages in lignin (Bourbonnais and Paice, 1990) increases the number and types of substrates to be oxidized. The most intensively studied applications of oxidative enzymes, especially laccases, include pulp delignification, textile dye bleaching, effluent detoxification, biopolymer modification and use of the enzymes as detergent components (Call and Mücke, 1997; Gianfreda et al., 1999; Grönqvist et al., 2003b).
1.4.1 The function of laccases

Laccases (EC 1.10.3.2.) are multi-copper enzymes which catalyze the oxidation of a broad range of substrates such as polyphenols, substituted phenols, diamines and some inorganic compounds (Reinhammar, 1984; Thurston, 1994). The oxidation reaction is coupled to the reduction of molecular oxygen to water with a one-electron oxidation mechanism. Laccases contain four copper atoms in one molecule. The coppers belong to three different types, which can be distinguished using UV/visible spectroscopy (Reinhammar, 1984).

The majority of the isolated and characterized laccases are of fungal origin. The most extensively studied laccases include those from *Agaricus*, *Trametes* (syn. *Polyporus*, syn. *Coriolus*), *Pleurotus*, *Podospora*, *Rhizonia*, *Neurospora*, *Aspergillus*, *Phlebia*, *Botrytis*, *Cerrena* and *Myceliophthora*. Laccases from different organisms show considerable diversity in substrate specificity, molecular weight, pH optimum and other properties. In many cases, special growth conditions, such as including inducers of laccase synthesis in the cultivation medium, are needed to obtain considerable yields of laccase (Gianfreda *et al*., 1999).

Laccases are involved in several physiological functions. Laccases of plant origin are reported to play an important role in wound response and also lignin biosynthesis, since laccase activity was found in lignifying cells (Bao *et al*., 1993; Liu *et al*., 1994). In fungi, laccases are involved in lignin degradation (Hatakka, 1994) as well as in several other functions including pigmentation, fruiting body formation, sporulation, and pathogenesis (Thurston, 1994). It is well recognized that laccases play a role in both the polymerization and depolymerization processes of lignin. *In vitro*, the oxidative reactions of laccase lead mainly into polymerization of lignin, but depolymerization has also been observed (Bourbonnais and Paice, 1992). The substrate is considered to form a reactive phenoxy radical, which can undergo further enzymatic oxidation or non-enzymatic reactions like hydration, and/or polymerization (Thurston, 1994; Yaropolov *et al*., 1994).
1.4.2 Laccase-mediator system

The mechanism of laccase-mediator system involves oxidation of the mediator by laccase, followed by the oxidation of substrate (e.g. lignin) by the oxidized mediator (Figure 3). The low-molecular weight mediator can diffuse to a susceptible structure of the lignin molecule to perform oxidation. Studies with lignin model compounds have shown that in the presence of 1-hydroxybenzotrizole (HBT), the laccase from *Trametes* catalyzed breaking of the Cα-Cβ linkage, oxidation of Cα-carbon, and the cleavage of β-ether bond (Kawai *et al.*, 2002). The oxidative degradation products of lignin are typically aromatic carbonyl compounds and carboxylic acids, however, special functional groups can also be involved; for example, free phenolic hydroxyl groups can be converted to quinones (Fengel and Wegener, 1983). Formation of quinones by laccase (alone) has been observed for some model compounds (Lundquist and Kristersson, 1985).

![Figure 3. Diagram of the mediated oxidation of substrate (lignin) by laccase.](image)

In delignification, the properties of an efficient mediator include a low molecular weight, solubility in water, appropriate redox potential, formation of radicals, and good biodegradation. In addition, the mediator should not inhibit laccase activity. The most effective mediators in delignification usually contain N-OH functional groups (Amann, 1997; Freudenreich *et al.*, 1998). Mediators containing an N-OH group include HBT, N-hydroxy-N-phenylacetamide (NHA), violuric acid (VIO) and 2-nitroso-1-naphtol-4-sulfonic acid (HNNS). In the initial study of the laccase-mediator system, ABTS, the common substrate of laccases, was used as the mediator (Bourbonnais and Paice, 1992). Other
potential candidates as mediators are the natural compounds present or secreted in the environment of white rot fungi (Eggert et al., 1996; Niku-Paavola et al., 2003).

High degrees of delignification, up to 40%, have been reported using HBT as mediator (Poppius-Levlin et al., 1999). Laccase alone did not delignify the pulp, nor did it depolymerize or polymerize the lignin, but minor chemical modifications occurred in the fibre (Poppius-Levlin et al., 1999). The laccase-mediated system has been shown to be able to replace either the oxygen delignification or ozone stage in bleaching of chemical pulp (Chakar and Ragauskas, 1999). In the oxygen delignification process, lignin is activated by alkali and degraded by the oxygen derived radicals; superoxide radical, hydroperoxide radical and hydroxyl radical.

Combination of xylanase and laccase-mediator bleaching systems in sequential treatments has been shown to result in further enhancement of pulp bleachability. For example, the degree of delignification was 44% with NHA as a mediator (Viikari et al., 1999; Oksanen et al., 2002). Simultaneous application of a laccase-mediator system using HBT as mediator with xylanase treatment was found to be relatively ineffective, apparently due to the inactivation of xylanase by the mediator HBT. The latest attempts to improve the laccase-mediator system has involved the synthesis of a mediator derivative, a precursor of NHA, aiming at slow release of active mediators to avoid unproductive side reactions (Paice et al., 2002). The release of NHA from an acetylated precursor (Na-Ac) can be achieved by increasing the reaction pH to 7-8 or with a suitable esterase (Amann et al., 2000).

1.5 Enzymatic hydrolysis of lignocellulose

1.5.1 Factors limiting hydrolysis

In nature, a variety of micro-organisms and enzymatic mechanisms are involved in the complete degradation of lignocellulose. The structure of lignocellulose sets barriers for chemical and enzymatic degradation. Even in the enzymatic hydrolysis of pure cellulose, a gradual drop in the reaction rate is generally observed. Reasons for this decrease in the rate of hydrolysis of pure cellulose are
generally placed in the following categories: end-product inhibition (reviewed by Holtzapple \textit{et al.}, 1990), depletion of easily degradable parts (\textit{e.g.} Wald \textit{et al.}, 1984, suggesting depletion of amorphous parts of cellulose), enzyme inactivation and unproductive binding or entrapment of cellulases in the small pores of cellulose (Converse \textit{et al.}, 1988; Tanaka \textit{et al.}, 1988; Väljamäe, 2002).

Yields obtained in the hydrolysis of lignocellulose are most significantly affected by the type of raw material. Generally, softwoods are recognized as being more refractory than hardwoods. Despite extensive research, there is no general agreement on what factors have the most significant effect on the hydrolysis rate of different lignocellulosic materials. The limiting factors have been traditionally divided into two groups: those related to the structure of the substrate (shown in Table 3) and those related to the mechanism and interactions of the cellulolytic enzymes (Converse, 1993; Mansfield \textit{et al.}, 1999). Specific effects of the removal of an individual component such as hemicellulose, lignin or acetyl groups on the enzymatic hydrolysis of cellulose have been difficult to study because the removal techniques influence the composition of the residual material. For example, hemicellulose is altered by many lignin removal procedures.

\textit{Table 3. Structural properties potentially limiting enzymatic hydrolysis of cellulose fibers at different structural levels (Mansfield \textit{et al.}, 1999).}

<table>
<thead>
<tr>
<th>Structural level</th>
<th>Substrate factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microfibril</td>
<td>Degree of polymerization</td>
</tr>
<tr>
<td></td>
<td>Crystallinity</td>
</tr>
<tr>
<td></td>
<td>Cellulose lattice structure (I, II, III, V or X)</td>
</tr>
<tr>
<td>Fibril</td>
<td>Structural composition (lignin content and distribution)</td>
</tr>
<tr>
<td></td>
<td>Particle size (fibril dimension)</td>
</tr>
<tr>
<td>Fiber</td>
<td>Available surface area</td>
</tr>
<tr>
<td></td>
<td>Degree of fiber swelling</td>
</tr>
<tr>
<td></td>
<td>Pore structure and distribution</td>
</tr>
</tbody>
</table>
Recently, it was hypothesized that in enzyme-catalyzed hydrolysis, the highly structured layers of water might present a substantial barrier to cellulase enzymes in their approach of the cellulose crystalline surface (Skopeč et al., 2003). However, both increased and decreased hydrolysis yields have been observed with increasing crystallinity of different lignocellulosic substrates (Sasaki et al. 1979; Sinitsyn et al., 1991).

The accessibility of lignocellulosic materials has been shown to play a crucial role in the improvement of enzymatic hydrolysis (Stone and Scallan, 1969; Grethlein 1985; Burns et al., 1989; Thomson and Chen, 1992; Esteghlalian et al., 2001). Cellulose has both external (shape and size of the particles) and internal (capillary structure of the fibers) surfaces. In untreated cellulose, only a minor fraction of the pores are accessible to cellulases. Grohmann et al. (1985) related the improvement in enzyme digestibility of dilute acid pretreated hardwood (aspen) to the removal of hemicellulose. Lignin removal was reported not necessary. Grethlein (1985) has shown that the removal of hemicellulose results in an increase in both the accessible pore volume and the specific surface area. He found that the hydrolysis yield correlate with the pore volume accessible to a solute of 51 Å in size. The pore size has been found to be strongly dependent on the degree of swelling (Stone and Scallan, 1969). Several studies indicate that drying of the lignocellulosic substrate and the consequent collapse of the cell wall capillaries and decrease in pore size decrease the effectiveness of enzymatic hydrolysis (e.g. Wong et al., 1988; Esteghlalian et al., 2001). Hemicellulose deacetylation has been shown to have a relatively minor role in enzymatic hydrolysis of pretreated poplar wood (Chang and Holzapple, 2000), however, the methods used were not very selective.

It is well recognized in the literature that lignin content and distribution have an impact on enzymatic hydrolysis (Vinzant et al., 1997; Mooney et al., 1998). High enzymatic conversions of cellulose have been obtained from extensively delignified softwood (Douglas fir), such as kraft pulp, containing 4% lignin or delignified refiner mechanical pulp, containing 8% lignin (Mooney et al., 1998). Delignification of steam-exploded Douglas fir with hot alkali peroxide (when residual lignin content was 8.2%) increased the yield of hydrolysis as well. In addition, the recovery of enzymes after enzymatic hydrolysis was increased (Lu et al., 2002). On the other hand, partial lignin removal (with a final lignin content of 32–36%) from steam pretreated softwood by alkaline NaOH
treatment has resulted in decreased hydrolysis yields (Wong et al., 1988; Schell et al., 1998). Also, partial removal of lignin by the oxygen delignification process using NaOH as pulping chemical has been shown to decrease the hydrolysis rate and yield in the case of steam pretreated softwood. In the case of softwood kraft pulp, the increase in the hydrolysis yield correlated with the increasing degree of delignification (Draude et al., 2001). Thus, alkaline partial removal of lignin seems to make the steam pretreated wood difficult to hydrolyze. A reason for the decreased hydrolysis yield after alkaline treatment of steam pretreated Pinus radiata is suggested to be the redeposition of unextracted lignin to the accessible pores and cellulose surfaces (Wong et al., 1988).

Unspecific adsorption of cellulases to lignin, limiting the hydrolysis, is discussed further in chapter 1.5.4.

1.5.2 Pretreatments of lignocellulose for enzymatic hydrolysis

The major steps in biomass-to-sugars process include raw material preparation (cleaning and size reduction), pretreatment and hydrolysis. Most pretreatment methods for lignocellulose conversions are aimed at the removal of hemicellulose or lignin. Several physical or chemical (or their combination) pretreatment methods are possible including ball milling, dilute acid pretreatment, steam pretreatment, ammonia fibre explosion, alkaline pretreatment and organosolv processes. Detailed descriptions of various pretreatment technologies are reviewed by Hsu (1996).

There are several desirable goals for pretreatment processes, however, in practice not all of them are achieved with any current treatment. Generally, the pretreatment should promote high product yields in a subsequent enzymatic hydrolysis and fermentation operations 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 facilitate the recovery of lignin and other nonfermentable constituents for their conversion to co-products. Downstream processing of pretreated raw material, including filtering, should be simple.
Acid-catalyzed steam pretreatment is the most extensively studied pretreatment of softwoods. Other tested pretreatments for softwood include supercritical CO₂ treatment (Kim and Hong, 2001), oxygen delignification of kraft pulp or reject knots (Charles et al., 2003) and two-stage dilute-acid pretreatment (Nguyen et al., 2000). The most investigated acid catalysts for steam pretreatment are SO₂ and H₂SO₄ (Galbe and Zacchi, 2002). Usual pretreatment conditions for softwood are: temperature 190–230°C, SO₂ content 2–5 % (w/w) and residence time 2–10 minutes. After steam pretreatment, the lignocellulosic material contains mainly cellulose and lignin, while most of the hemicellulose is dissolved. A typical cellulose conversion obtained in a 48-h hydrolysis of steam pretreated softwood with a cellulase dosage of 10 FPU per gram of dry weight is about 60% (Tengborg et al., 2001). Higher conversions have been reported (Stenberg et al., 1998; Szengyel et al., 2000), but they involve either a longer hydrolysis time or an increased amount of cellulases. In the steam pretreatment of softwood, the recovery of a maximal amount of sugars from both hemicellulose and cellulose has been shown to require a two-step pretreatment procedure (Tengborg et al., 1998; Söderström et al., 2002). The highest overall yields of hemicellulose and cellulose derived sugars in a one-step pretreatment have been 66% and 67%, respectively (Tengborg et al., 1998).

The wet oxidation method operates with water and oxygen or air at an elevated temperature and pressure (McGinnis et al., 1983). The wet oxidation process has been found to convert many organic polymers to oxidized compounds, such as low-molecular weight carboxylic acids, or even to CO₂ and H₂O (Taylor and Weygandt, 1974). Wet oxidation has been applied for the treatment of polluted soil and wastewater, and recently also for the pretreatment of wheat straw and hardwood (Schmidt et al., 1996; Schmidt and Thomsen, 1998). The wet oxidation process has been reported to produce less furfural and 5-hydroxymethylfurfural, which are potential inhibitors in the fermentation step, when compared to steam pretreatment (Bjerre et al., 1996). Wet oxidation of wood material has been shown to dissolve mainly hemicellulose (McGinnis et al., 1983; Schmidt et al., 1996).
1.5.3 Surface interactions in enzyme adsorption

There are several basic surface and intermolecular forces which determine the power of adsorption for proteins onto solid/liquid interfaces. These interactions are usually noncovalent, *i.e.* mediated by hydrogen bonding, electrostatic or hydrophobic interactions (Brash and Horbett, 1995). Other adsorbed proteins as well as low-molecular weight ions in the interfacial region may affect the adsorption. Electrostatic forces contribute to the binding, but they do not dominate protein adsorption in all conditions (Norde and Haynes, 1995).

Proteins are polyampholytes containing both positive and negative charges, which makes them intrinsically surface-active molecules. The most hydrophobic residues in proteins are those containing large aromatic groups like tryptophane (Trp), phenylalanine (Phe) and tyrosine (Tyr) (Parker *et al*., 1986). As a rule, when the protein is folded, the non-polar hydrophobic amino acids are hidden inside the protein. However, some of the non-polar amino acid residues are preferentially located on the surface of the protein molecule, therefore forming binding sites for hydrophobic substances through hydrophobic interaction and hydrogen bonding. The affinity of proteins increases as the hydrophobicity of the surface increases although electrostatic interactions may interfere with this trend. Generally, adsorption of protein onto hydrophobic surfaces is more irreversible than adsorption on hydrophilic surfaces. This is due to the fact that many proteins undergo changes upon adsorption onto hydrophobic surfaces (van Oss, 1995).

Cellulose surface is often considered highly hydrophobic because of hydrogen bonding of the cellulose interchains. Native cellulose does not contain any charged groups whereas pulp fibres carry a negative charge, mainly due to oxidation of hemicelluloses and lignin. In practice, a surface charge of any substance is easily formed by the dissociation of ions from surfaces. Generally, surfaces in contact with water are more often negatively charged (Shaw, 1980). Studies with steam-exploded softwood have shown that the degree of hydrophobicity of wood, determined by the water drop penetration time (WDTP), increased after the pretreatment (Negro *et al*., 2003).
Relative importance of charge-to-charge interactions and the hydrophobic effects on the adsorption behavior of proteins vary depending on the specific properties of the protein and the surface. In a study with the protease subtilisin where protein engineering was applied, the change of a single hydrophilic positively charged lysine at the surface of the enzyme to a hydrophobic neutral phenylalanine decrease enzyme adsorption on both hydrophilic and hydrophobic model surfaces. It was concluded that in this case, the electrostatic interaction is a more important driving force for adsorption (Brode et al., 1994).

1.5.4 Binding of cellulases

Cellulases interact with the cellulose surface with the cellulose binding domain, CBD, and the catalytic domain, CD. Cellulases can bind to solid surfaces specifically or non-specifically. The overall binding efficiency of cellulases is markedly enhanced by the presence of CBDs. All T. reesei cellulose binding domains are small molecules, consisting of 36 amino acid residues. Sequence comparison of T. reesei CBDs have shown that the three conserved tyrosines (Y5, Y31 and Y32) are involved in the binding to cellulose. In CBH I, one of the tyrosines is replaced with tryptophane (Linder, 1996).

Structural differences, for example, in the hydrophobicity of the surfaces of these enzymes may have an effect on the general adsorption affinity. Gusakov et al. (2000, 2001) have compared the hydrophobicity of the surface amino acids in catalytic domains of several T. reesei cellulases and related the high degree of hydrophobicity to the high denim-washing performance. The number of the most hydrophobic surface residues (Tyr, Phe, Trp) in the catalytic domains of CBH I and CBH II is quite similar, 5 and 6, respectively. However, the total amount of other non-polar amino acids (Val, Leu, Ile, Pro, Met) on the surface of the catalytic domain is higher for CBH II (Gusakov et al., 2000). The charge of the surfaces of different enzymes also varies. For example, a charge-charge interaction is possible for CBH II CBD but not for CBH I CBD or EG II CBD, since CBH II CBD contains an aspartic acid at position 30, whereas the corresponding amino acid in CBH I CBD and EG II CBD is a proline (Linder, 1996).
The adsorption of CBH I has been shown to be influenced by nonelectrostatic interactions between the surface and the enzyme. Baker et al. (2001) showed that CBH I from T. reesei adsorb on silanized silica, which is a hydrophobic substance exposing a uniform field of methyl groups to the solution. CBH I did not adsorb on hydrophilic silica. Adsorption to silanized silica was largely irreversible to dilution. It has been found that the binding of CBH I was improved along with growing ionic strength which indicates the presence of hydrophobic interaction in the adsorption (Reinikainen et al., 1995; Kim and Hong, 2000). In contrast, the binding of CBH II has found to be decreasing by ionic strength (Kim and Hong, 2000).

The charge on the cellulases and/or the presence of hydrophobic groups can lead to general adsorption and non-productive binding to cellulose or lignin. Several authors have suggested that cellulases adsorb to the lignin fraction of lignocellulose (Sutcliffe and Saddler, 1986; Chernoglazov et al., 1988; Converse et al., 1990; Hogan and Mes-Hartree, 1990; Ooshima et al., 1990). Chernoglazov et al. (1988) found a correlation between the carbohydrate content of native T. reesei endoglucanases and their adsorptivity on larch and birch lignin, which were prepared by Bjorkman method (extraction with dioxane). In addition to cellulases, β-glucosidase, which does not have a cellulose binding domain, appears to adsorb strongly on isolated aspenwood lignin (Sutcliffe and Saddler, 1986; Tatsumoto et al., 1988).

It has been observed in several studies, that different pretreatment conditions affect the adsorption of cellulases. The adsorption capacity of the pretreated (by partial acid hydrolysis with explosive decompression) hardwood for the unpurified cellulases has been found to depend on the temperature of the pretreatment. In this case, the adsorption capacity of enzymatically isolated lignin decreased, but the adsorption capacity of lignocellulose increased, when the treatment temperature was increased (Ooshima et al., 1990). It has been suggested that the adsorption capacity of lignin decreases due to increased melting and agglomeration (Ooshima et al., 1990).

It would be beneficial to be able to decrease the nonspecific adsorption of cellulases to lignin in the commercial hydrolysis applications. In theory, the cellulase enzymes could also be modified using protein engineering in such a way that they do not bind non-specifically. However, the ability of the enzyme
to adsorb onto crystalline cellulose is crucial for the hydrolysis and therefore should not be disturbed. Currently, there are no reports of cellulases that would have been improved by engineering the surface amino acid residues in order to make them more effective in the enzymatic hydrolysis of lignocellulose.

1.6 The aim of the present study

This work focuses on factors relevant for improvement of enzymatic hydrolysis of lignocellulose raw materials derived from softwood. The major interest of the work was in lignin. Specific areas addressed were evaluating the role of lignin in the unproductive binding of cellulases, which restricts the hydrolysis of cellulose, and enzymatic modification of lignin in order to improve cellulose hydrolysis. In addition, suitability a new pretreatment method, wet oxidation, was evaluated for softwood.

The specific aims were:

- To study the adsorption of individual cellulases on pure cellulose and different lignocellulosic materials.
- To evaluate the impact of the composition of lignocellulosic material in enzymatic hydrolysis by exploring different pretreated raw materials.
- To purify and characterize a new fungal laccase for lignin modification.
- To gain new information on the role of laccase-mediator systems in lignin modification in order to improve enzymatic hydrolysis.
2. Materials and methods

A summary of the materials and methods used in this work is presented in this section. More detailed information can be found in the original publications I–VI. Detailed information is given for the methods used for the unpublished results, presented in this work.

2.1 Enzymes

2.1.1 Cellulases

The T. reesei cellulases CBH I, CBH II and EG II were purified as described by Rahkamo et al. (1996) and their CDs as described by Suurnäkki et al. (2000). The CBH I CBD and CBH II CBD used in publication I were provided by G. Carrard (VTT Biotechnology). Tritium labelling of CBH I, CBH II, EG II and their catalytic domains is described in I and II. In this labelling, tritium-containing methyl groups were introduced into the free amino groups of the proteins.

The commercial cellulase mixtures used were Celluclast 1.5 L (Novozyme) and Multifect (Genencor). The β-glucosidase preparation used was Novozym 188 (Novozone).

The enzymes were administered according to the filter paper unit (FPU) -activity, which was assayed using Whatman No. 1 filter paper as substrate (IUPAC, 1987). Cellulase and hemicellulase activities were determined as described in publications I, II and III.

2.1.2 Laccases

The laccase produced by Trametes hirsuta (Poppius-Levlin et al., 1999) was purified as described by Rittstieg et al. (2002). The laccase from Melanocarpus albomyces was purified as described in Kiiskinen et al. (2002). The laccase from the ascomycete Mauginiella sp. (VTT D-84228; originally identified as
Galactomyces geotrichum) was purified to electrophoretic homogeneity and biochemically characterized as described in publication IV.

Laccase activity was routinely determined according to Niku-Paavola et al. (1988) using ABTS [2,2’-azino-bis-(3-ethylbenzothiazoline)-6-sulphonate] as a substrate. The laccase activity was also measured by following the consumption of oxygen in the reaction mixture. This measurement was carried out in order to determine the capability of laccases to oxidize solid substrates (SPS), to determine the effect of potential inhibitors (in publication IV) or to determine the activity of bound laccase, when the routinely used ABTS method was not applicable.

### 2.2 Cellulosic and lignocellulosic materials

#### 2.2.1 Preparation of materials

Bacterial microcrystalline cellulose, BMCC, from Acetobacter xylinum used in I was prepared as described earlier (Gilkes et al., 1992). Steam pretreated softwood (SPS) was prepared by steam explosion with SO2-impregnation from spruce (Picea abies) chips at the Lund University using methods described by Stenberg et al. (1998). Conditions used for the SPS treatment in publication II were: temperature 215°C, SO2 content 2.8% (w/w) and residence time 5 minutes. For the SPS used in publications III and V, the treatment conditions were as above except that the SO2 concentration was 2% (w/w). SPS was briefly washed with distilled water and stored at -20°C.

Softwood pretreated by wet oxidation (WOS) was used in publication III. The wet oxidation pretreatment was preliminarily optimized for softwood in order to enhance enzymatic hydrolysis as described in III. Six different combinations of reaction time, temperature and pH were applied and the compositions of solid and liquid fractions were analyzed. The WOS-materials were stored at -20°C.

Three different isolated lignin materials were used in publication II. The lignin fraction, designated as alkali-lignin, was extracted from SPS by NaOH. The lignin-rich hydrolysis residue, designated as CEL-lignin, was prepared using commercial cellulose and hemicellulose degrading enzyme preparations.
All concentrations of lignocellulosic materials are expressed as dry weights. The dry weight determination was carried out by drying the material at 105°C overnight.

### 2.2.2 Analytical procedures

The composition of lignocellulosic materials was determined by gravimetric and acid hydrolysis methods as described in publication III. Lignin contents were also determined as Klason-lignin, after removing the polysaccharides by hydrolysis with 72% (v/v) sulfuric acid.

### 2.3 Adsorption experiments

#### 2.3.1 Adsorption of cellulases

Binding studies were performed generally at 4°C using 50 mM sodium acetate buffer (pH 4.8 or 5) and tritium-labeled enzyme preparations. In publication I, where the temperature dependency of the adsorption was studied, the adsorption studies were performed also at 22°C and 40°C. The concentrations of cellulase stock protein solutions were determined by UV adsorption at 280 nm using the molar extinction coefficients determined by amino acid analysis. In publication I, BMCC was used, and the samples contained 1% bovine serum albumin (BSA) to prevent nonspecific adsorption of protein to glass tubes and filters. In publication II, SPS and isolated lignins were used.

The applied enzyme concentrations varied between 0.02 µM and 6 µM. The enzyme and the substrate (1 g l⁻¹ in publication I and 10 g l⁻¹ in publication II) were incubated with mixing on a magnetic stirrer or on a rotating shaker for 30 or 90 minutes. After the incubation, samples were centrifuged and/or filtered. Protein concentration in the filtrate was quantified by scintillation counting. The portion of adsorbed enzyme was calculated by subtracting the amount of free enzyme from the initial enzyme concentration.

The adsorption of tritium-labelled CBH I or EG II was monitored during a 72-h hydrolysis of SPS (60 g l⁻¹) in Na-acetate buffer (pH 4.8; 200 ml) at 40°C.
(unpublished results). The solution contained small amounts of tritiated CBH I or EG II, Celluclast with cellulase activity of 27 FPU g⁻¹ and Novozyme 188 with β-glucosidase activity of 68 IU g⁻¹. Hydrolysis residue was separated by centrifugation every 24 hours. The content of tritiated enzyme in solution was measured and fresh buffer and new (non-tritiated) enzymes were added at the same concentration as initially.

2.3.2 Adsorption of laccases

The adsorption of different laccases on SPS was determined. A solution containing 1% SPS and laccase (1000 nkat g⁻¹ SPS) in 50 mM citrate buffer was incubated for 1 h at room temperature. The solution was centrifuged and residual laccase activity determined using ABTS as substrate according to Niku-Paavola et al. (1988). Three laccase preparations used, two of these were from the ascomycetes Mauginiella sp and Melanocarpus albomyces, and one came from the basidiomycete Trametes hirsuta.

Enzymatic activity of the M. albomyces laccase bound to SPS was measured using the oxygen consumption method. Laccase (30 nkat) and SPS (1%) were incubated in 50 mM acetate buffer (pH 5) for 60 min, the solution was centrifuged and the liquid and solid phases separated. SPS was mixed with fresh buffer. The oxygen consumption was measured in the SPS solution in the presence of 0.3 g l⁻¹ ABTS using an oxygen electrode (Orion Research 081010) at room temperature under atmospheric air saturation in sealed flasks.

2.4 Enzymatic hydrolysis of cellulose

2.4.1 Hydrolysis conditions

Hydrolysis experiments were carried out in 50 mM citrate buffer (pH 5) in 5 ml volume using 0.1% BMCC or 2% lignocellulose (steam pretreated or wet oxidized softwood) at 30–45°C for various time periods (I, III, V). Solutions were mixed with magnetic stirrers. The cellulase concentration used was 10–30 FPU g⁻¹ lignocellulose, with an addition of β-glucosidase, 500–1000 nkat g⁻¹.
After hydrolysis, the samples were cooled on ice, centrifuged and the hydrolysate was boiled for 10 minutes.

### 2.4.2 Analysis of hydrolysates

The release of soluble, reducing sugars in the hydrolysates was monitored by the DNS-method (Bernfeld, 1955) with glucose as a standard (III, V) or by high performance liquid chromatography to detect the amounts of glucose and cellobiose in the hydrolysates (I).

### 2.4.3 Laccase treatments

In publication V, the effect of the laccase and laccase-mediator treatment on the enzymatic hydrolysis of SPS was tested. Laccases were purified from *Trametes hirsuta* (ThL), *Melanocarpus albomyces* (MaL) and *Mauginiella* sp. (MsL), and the mediators NHA and NHA-acetate were obtained from Wacker GmbH. A commercial lipase, Novozyme 252L (Novozymes) was used to liberate NHA from NHA-Ac. The mixture containing 2% SPS, laccase 1000 nkat g\(^{-1}\) (and NHA or NHA-Ac) was oxygenated for the first 3 hours of the 24-h treatment as described in V. The three different laccases were compared in a 6-h hydrolysis of SPS in a mixture contained 2% SPS and 1000 nkat g\(^{-1}\) laccase. The laccases were administered according to their activity on ABTS at pH 5.

The release of soluble, reducing sugars in the hydrolysates was monitored by the DNS-method (Bernfeld, 1955) with glucose as a standard. Electron spectroscopy for chemical analysis (ESCA) was used to analyze the changes in the surface of the hydrolysis residue after the treatment with laccase and cellulases as described in V.

The oxidation of SPS by *T. hirsuta* laccase (described in V) was studied by measuring the oxygen consumption using an oxygen electrode (Orion Research 081010) at room temperature under atmospheric air saturation in sealed flasks.
3. Results and discussion

Improvement of the hydrolysis of lignocellulosics is a challenging task because of the recalcitrance of this material. A number of parameters in the lignocellulosics-to-glucose process, including the selection of raw material, type of a pretreatment method as well as the type and dosage of the cellulolytic enzymes, have an effect on the hydrolysis of lignocellulose. In this work, the focus was on different aspects of enzyme binding and the role of lignin in the hydrolysis.

3.1 Role of pretreatment in enzymatic hydrolysis

The wet oxidation pretreatment studies \((\text{III})\) gave information on the importance of substrate structure in the enzymatic hydrolysis. In addition, the studies resulted in new data on this pretreatment for comparison with the more frequently used steam pretreatment. The wet oxidation pretreatment method was applied to softwood for the first time.

3.1.1 Effect of pretreatment on the composition of softwood

The parameters chosen for the wet oxidation pretreatment resulted in very different compositions of fractions. The yields of substrate components (cellulose, hemicellulose, lignin) varied after the pretreatments. After wet oxidation, the solid fraction contained 58–64% cellulose, 2–16% hemicellulose and 24–30% lignin (Table 4). Lignin fraction of the substrate remained mainly undissolved (24–42% was dissolved), and would thus still be available for energy production by combustion. The addition of \(\text{Na}_2\text{CO}_3\) resulted in slightly increased solubilization of lignin but higher retention of hemicellulose. Without the addition of \(\text{Na}_2\text{CO}_3\), the pH decreased to about 2.8 obviously due to liberation of acetic acid (Table 1/\(\text{III}\)). The recoveries of hemicellulose and cellulose were lowest when higher temperature and longer treatment time were applied. In contrast, the recoveries were highest when lower temperature and shorter times were applied (Table 4/\(\text{III}\)). The steam pretreated softwood used as reference material had a considerably higher concentration of lignin together with compounds classified as dissolved lignin and extractives. The recovery of
cellulose derived sugars in both liquid and solid fractions was, however, higher when wet oxidation pretreatment was applied (Table 4/III).

Table 4. Pretreatment conditions, yields, and compositions of pretreated softwood assessed by gravimetric analysis following wet oxidation and steam pretreatment (values from III).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Pretreatment conditions</th>
<th>Yield of insol. material (%)</th>
<th>Composition of pretreated softwood (% of d.w.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Temp (°C)</td>
<td>Time (min)</td>
<td>pH</td>
</tr>
<tr>
<td>Untreated wood</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WOS-1 a</td>
<td>185</td>
<td>10</td>
<td>3.5</td>
</tr>
<tr>
<td>WOS-2</td>
<td>185</td>
<td>20</td>
<td>7</td>
</tr>
<tr>
<td>WOS-3</td>
<td>185</td>
<td>10</td>
<td>10.5</td>
</tr>
<tr>
<td>WOS-4</td>
<td>200</td>
<td>20</td>
<td>3.5</td>
</tr>
<tr>
<td>WOS-5</td>
<td>200</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>WOS-6</td>
<td>200</td>
<td>20</td>
<td>10.5</td>
</tr>
<tr>
<td>SPS b</td>
<td>215</td>
<td>5</td>
<td>nd</td>
</tr>
</tbody>
</table>

a WOS, wet oxidized softwood  
b SPS, steam pretreated softwood  
nd not determined
3.1.2 Relationship of substrate composition and yield in enzymatic hydrolysis

The wet oxidation method showed potential for enhancing enzymatic hydrolysis, although further optimizations are still needed. A high residual hemicellulose content was shown to correlate with a low yield in the enzymatic hydrolysis, as seen in Fig. 2/III. No clear correlation between the hydrolysis yield and lignin content could be observed, but variations in the lignin content were very small. The optimal wet oxidation pretreatment conditions for enzymatic hydrolysis were 10 min at 200°C with addition of 12 bar oxygen. These conditions ensured a high conversion of polysaccharides, 79% of theoretical, in the enzymatic hydrolysis (72 h) by a commercial cellulase preparation Celluclast (Table 6/III), as well as rapid filtration after the pretreatment. The samples with sulfuric acid or sodium carbonate additions were slow to filter after the wet oxidation reaction. The best yield of sugars in the 24-h hydrolysis (with 30 FPU g⁻¹ cellulase) of WOS was 26% of the original wood, which corresponds to a 55% conversion of polysaccharides. The best sugar yield from steam pretreatment (SPS) was 28% of the original wood (Table 7/III). Thus, the sugar yields from the solid fraction were in the same range. However, part of the cellulose fraction in the SPS was dissolved during the pretreatment procedure (Table 4/III). Stemberg et al. (1998) obtained a similar glucose yield (29%) of the original wood after a 96-h enzymatic hydrolysis (with 15 FPU g⁻¹ cellulase) of steam pretreated softwood.

High recovery of hemicellulose-derived sugars and high conversion of cellulose after the pretreatment has proven to be difficult to obtain at the same time. In the steam pretreatment of softwood, the recovery of a maximal amount of sugars from both hemicellulose and cellulose has been shown to require a two-step pretreatment procedure (Tengborg et al., 1998). In this study, more than a half of the hemicellulose was degraded to products other than sugars under conditions where the hydrolysis was most efficient (WOS-5). On the other hand, the hemicellulose-containing WOS-3 was hydrolyzed as efficiently as the two other substrates, SPS and WOS-5, by the other commercial cellulase used, Multifect. According to the sugar composition analysis (Table 3/III), the residual hemicellulose in WOS-3 composed mainly of mannan but some xylan was also present. The hydrolysis of softwood galactoglucomannan requires action of endomannanases to degrade the backbone, acetylglucomannan esterases to
remove the acetyl groups and α-galactosidases to remove galactose residues. In addition, β-mannosidases and β-glucosidases are required. It can be expected that the somewhat higher mannanase and xylanase activities were one reason for the better performance of Multifect when compared to that of Celluclast (Table 5/III). Compared to Multifect, Cellulast had a 10% lower endoglucanase activity against hydroxyethyl cellulose (HEC), 20% lower mannanase activity and 40% lower xylanase activity standardized to 10 FPU g\(^{-1}\) of substrate.

Previously, it has been proposed that the wet oxidation method was especially suited for the removal of lignin during the pretreatment (Schmidt and Thomsen, 1998), however, according to this study, the improvement of the hydrolysis was mainly due to the removal of hemicellulose. The residual hemicellulose played an important role in the enzymatic hydrolysis. It should be noted that most commercial cellulase preparations are not designed for efficient hydrolysis of lignocellulose, therefore their performance could be enhanced by adding hemicellulases and other accessory enzymes to break down the residual hemicellulose more efficiently. Consequently, enzymatic hydrolysis of the 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 of cellulose.

### 3.2 Adsorption of cellulases

Understanding the interactions between cellulases and the lignocellulosic substrate is crucial for the development of an efficient system for conversion of lignocellulose to glucose. The dynamic process of adsorption and desorption of both cellulase enzyme domains (CD and CBD) on cellulose allows processive hydrolysis of the cellulose chain and relocation of enzymes at a new site on the cellulose.

#### 3.2.1 Tritium labelling of enzymes

The tritium labelling method of enzymes gave a practical detection limit of about 0.01 µM. The labelling method did not affect enzymatic activity (I, II).
The measured specific radioactivities of the enzymes (Table 5) were dependent on the activity of the labeling reagent and the amount of free amino groups.

**Table 5. Enzymes used in the binding studies.**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific radioactivities of the labeled enzymes (Ci/mmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBH I</td>
<td>3.3 (II), 7.7 (I)</td>
</tr>
<tr>
<td>CBH I CD</td>
<td>4.1 (II), 8.3 (I)</td>
</tr>
<tr>
<td>CBH II</td>
<td>6.6</td>
</tr>
<tr>
<td>CBH II CD</td>
<td>6.1</td>
</tr>
<tr>
<td>EG II</td>
<td>5.5</td>
</tr>
<tr>
<td>EG II CD</td>
<td>5.3</td>
</tr>
</tbody>
</table>

**3.2.2 Adsorption on crystalline cellulose**

Binding of the cellulose binding domain of CBH I on BMCC has been shown to be reversible (Linder and Teeri, 1996), although earlier studies have suggested that it may bind irreversibly (Ong et al., 1989; Nidetzky et al., 1994). In publication I, it was shown that the binding of the intact CBH I on BMCC is also fully reversible (Fig. 2/I). However, while the CBH II CBD bound irreversibly to BMCC (Carrard and Linder, 1999), the intact CBH II was only partly irreversibly bound as shown in publication I.

Another important conclusion reached in I was that the intact CBH I and CBH II exhibited significantly higher affinity to BMCC than either domain alone. Relative affinities or partition coefficients of the studied enzymes are presented in Table 6. The coefficients were calculated from the initial slopes of the binding isotherms. Higher value corresponds to higher affinity of binding. These results indicate that binding is determined by a co-operative effect of the two domains, CBD and CD and that binding can occur through both of the domains.
The affinity of adsorption to BMCC was clearly temperature dependent for both CBH I and CBH II and their isolated domains, and better binding occurred at low temperature (Table 1/I). In this work, the adsorption time was short (30 minutes) and the enzyme concentration low, and it is unlikely that the surface of the cellulose was significantly altered by hydrolysis. The relative order of adsorption of the enzymes did not change at different temperatures. A similar temperature dependency for *T. reesei* cellulases to that detected in this work, has been observed previously by several authors. The temperature dependency for adsorption has been reported for CBH I CBD on BMCC (Linder and Teeri, 1996), for CBH I and it's CD on BMCC (Srisodsuk, 1994) and for CBH I and CBH II on partially crystalline cellulose, Avicel (Kim and Hong, 2000; Medve et al., 1994).

Cellobiose was found to increase binding of the catalytic domains of CBH I and CBH II even at concentrations lower than 1 mM (I). By contrast, the binding of intact CBH I was unaffected, whereas the binding of CBH II was slightly reduced. Previously, it has been noted that at very high concentrations, cellobiose can cause an increase in the binding of the catalytic domain of CBH I but not of the intact enzyme (Ståhlberg et al., 1991).

### 3.2.3 Adsorption on lignocellulose

The role of the cellulose binding domain was further clarified in publication II, where it was shown to play an important role in the unspecific adsorption of CBH I and EG II on lignin. The intact proteins exhibited significantly higher affinity to SPS and lignin than the catalytic domain alone as seen in Table 6.

There was a significant difference between the adsorption affinities of CBH I and EG II on SPS. The removal of CBD from CBH I had a more notable decreasing effect on the adsorption of the enzyme to SPS when compared to EG II. It has been shown previously that the binding affinity of the catalytic domain of EG II to steam pretreated willow was nearly as high as the affinity of the intact EG II (Kotiranta et al., 1999). Interestingly, in this work it was observed that EG II CD had a considerably higher affinity to the alkaline isolated lignin than the CBH I CD (Table 6). One possible explanation for the high affinity of EG II to SPS and lignin could be the more open active site of endoglucanases,
when compared to the tunnel shaped active site of CBH I. The more open nature of the active site may reveal the aromatic residues to lignin, which may lead to adsorption through hydrophobic interaction. Nevertheless, more studies are needed to draw conclusions on the role of the structure of the active site in the unspecific adsorption. The catalytic domains may also have other surface properties, e.g. clusters of surface amino acids, which contribute to unspecific adsorption. Unfortunately, the three-dimensional structure of EG II has not been resolved yet.

Comparison of the relative binding affinities to pure cellulose (BMCC) and SPS (Table 6) showed that the adsorption affinity of CBH I to SPS was significantly lower than its affinity to BMCC. This implies that BMCC contains significantly more available cellulose surfaces for enzyme binding than SPS. The adsorption affinity of the catalytic domain of CBH I was quite similar for both SPS and BMCC. The catalytic domain of CBH II bound with a very low affinity to BMCC and SPS (Table 6).

*Table 6. Partition coefficients (l g⁻¹) of CBH I, CBH II and EG II and their catalytic domains (from publications I and II). The values were estimated from the initial slopes of the adsorption isotherms.*

<table>
<thead>
<tr>
<th>Protein</th>
<th>BMCC</th>
<th>SPS</th>
<th>Alkali-lignin</th>
<th>CEL-lignin</th>
<th>AH-lignin</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBH I</td>
<td>43</td>
<td>5.5</td>
<td>1.7</td>
<td>0.6</td>
<td>0</td>
</tr>
<tr>
<td>CBH I CD</td>
<td>2.0</td>
<td>1.1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CBH II</td>
<td>5.8</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>CBH II CD</td>
<td>0.2</td>
<td>0.3ᵃ</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>EG II</td>
<td>nd</td>
<td>1.0</td>
<td>0.6</td>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td>EG II CD</td>
<td>nd</td>
<td>0.05</td>
<td>0.2</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

ᵃ not published elsewhere; CEL cellulase hydrolysis; AH acid hydrolysis; nd not determined
A difference in the adsorption behaviour of CBH I and EG II was also observed in the competition studies (publication II), where EG II influenced the binding of CBH I but not vice versa (Fig. 3/II). The synergism of CBH I and EG II in the hydrolysis of cellulose has been extensively studied and optimal ratios have been suggested by several authors. It is generally recognized that enzymes compete for common binding sites during the hydrolysis process. It has been reported that the presence of EG II does not affect the binding affinity of CBH I when Avicel is used as substrate (Medve et al., 1998), but a minor decrease is observed when steam pretreated willow is used as substrate (Karlsson et al., 1999).

It has been observed that the method applied for lignin isolation has a strong influence on the surface properties of the substrate, and consequently on the adsorption of enzymes. In addition, isolated lignins suffer from various impurities and they may contain residual polysaccharides and proteins. In this work, the enzymes did not adsorb on lignin, which was prepared by acid hydrolysis of carbohydrates (Table 6). This implies that the lignin isolation procedure or eventually the drying, had dramatically changed the surface properties of the acid hydrolysis-lignin. The affinity of CBH I and EG II to CEL-lignin, which was prepared by hydrolysing carbohydrates by cellulases, was low most probably because the available binding sites were already blocked by irreversibly bound cellulase enzymes. The protein content of CEL-lignin was 5.5% (Table 1/II).

The frequently observed increase of enzymatic hydrolysis by addition of a non-ionic surfactant (Helle et al., 1993; Ooshima et al., 1986; Park et al., 1992) has been recently attributed to a decreased unspecific binding of cellulases on lignin (Eriksson et al., 2002a). Eriksson et al. (2002a) found that addition of bovine serum albumin (BSA) decreases the adsorption of CBH I and consequently increases conversion of SPS to the same extent as the addition of Tween 20. BSA, added in excess, decreased the binding of both CBH I and EG II onto CEL-lignin in this study. The relative amount of free enzyme was 21% (of the initially added) without BSA, while the addition of 2.5 g l⁻¹ BSA (ca. 60 times higher concentration of BSA than the CBH I content) increased the amount of free CBH I to 39%. BSA at a concentration of 25 g l⁻¹ increased the amount of free CBH I further to 47% (publication II). BSA (2.5 g l⁻¹) decreased the adsorption of CBH I on CEL-lignin even when added after 30 min preincubation of CBH I with lignin (Figure 4).
Figure 4. The effect of BSA (2.5 g l\(^{-1}\)) on the binding of CBH I to CEL-lignin. BSA was added either simultaneously with CBH I, or after a 30 min preincubation.

BSA has been used to prevent unspecific adsorption of cellulases on glass tubes and filters (Linder 1996; publication I). It has been generally observed that one protein may have higher affinity for the surface and may even displace other proteins from the coated surface. BSA obviously has a high affinity for lignocellulose and lignin. The reduction of unspecific binding of CBH I after addition of BSA or a non-ionic surfactant is most probably due to coating of the lignin surface by the added substance (Eriksson et al., 2002a).

### 3.2.4 Adsorption during hydrolysis

Cellulose substrate obviously changes during hydrolysis. Reduction in the particle size should increase the available surface. During hydrolysis of lignocellulosics, more lignin is exposed on the surface as seen by the ESCA studies (Table 5/V).

In publication I, it was found that the density of bound enzymes increased with both CBH I and CBH II (and their catalytic domains) as the hydrolysis of crystalline cellulose, BMCC, proceeded (Fig. 3/I). In this experiment, the level of hydrolysis was about 75% and the increase in adsorption was clear when a conversion of 20–30% was reached. The increase in adsorption of CBH I and
CBH II during hydrolysis of filter paper have been observed previously (Nidetzky and Claeyssens, 1994). The reason for this effect is not easy to pinpoint, but observations so far would support the "surface erosion model" for enzymatic hydrolysis of cellulose (Väljamäe et al., 1998; Väljamäe 2002). This model has recently been suggested for enzymatic hydrolysis of softwood as well (Eriksson et al., 2002b). The surface erosion model identifies both the nonproductive binding and erosion of cellulose surface as the major contributors to the decrease in the rate of hydrolysis. According to the model, CBH I would bind unproductively on the cellulose surface because of the obstacles formed, and these obstacles could be removed by the action of endoglucanases. In contrast to the results presented here, Ståhlberg et al. (1991) and Medve et al. (1994) observed desorption of intact CBH I or CBH II during hydrolysis of Avicel, however, the hydrolysis level was considerably lower (6.5–11%). In the studies of Jung et al. (2002), desorption of the bound catalytic domains of Thermobifida fusca exoglucanases was observed during hydrolysis of BMCC, and attributed to loss of binding sites of the easily hydrolysable BMCC (Jung et al., 2002).

### 3.2.5 Desorption from hydrolysis residue

Recycling of cellulases after the enzymatic hydrolysis of lignocellulose has proved to be difficult due to low enzyme recoveries. Adsorption of tritium labeled CBH I in the commercial cellulase preparation (Celluclast) was monitored during a step-wise enzymatic hydrolysis of SPS, in which the hydrolysis residue was separated every 24 hours, the content of tritiated enzyme in solution was measured, and fresh buffer and new unlabelled enzymes were added. In Fig. 5, it can be seen that more than 60% of initially added CBH I or EG II remained adsorbed to the hydrolysis residue. The amount of adsorbed CBH I and EG II decreased only slightly after each hydrolysis step.
Figure 5. Adsorption of initially added CBH I or EG II on SPS in a mixture with Celluclast and Novozyme 188 after hydrolysis and washing of the residue.

In several studies, the recovery of cellulases from hydrolysis residue has been more effective at an increased pH (Sinitsyn et al., 1983; Otter et al., 1989) indicating that electrostatic forces may be important in adsorption. At elevated pH, the cellulases are generally more negatively charged, and thus nonspecific binding to the negatively charged lignocellulose would be weaker. Washing of the SPS hydrolysis residue was tested using buffers at pH values from 4.5 to 10. Borax buffer at pH 10 was found to be the most effective in releasing adsorbed enzymes (publication II). However, only a minor fraction of the enzymes were desorbed by the washing procedure as the final protein content of the hydrolysis residues was as high as 5.5%. The desorption of EG II was somewhat less effective when compared to CBH I (Figure 5).

3.3 Modification of lignocellulose by laccase treatments

Partial removal of lignin by a laccase-mediator treatment during the enzymatic hydrolysis of cellulose would offer means to overcome the negative effects of lignin on hydrolysis. Laccases, together with small molecular weight mediators are capable of oxidizing phenolic compounds and lignin. It would be beneficial to perform these two enzymatic treatments simultaneously, because as the hydrolysis proceeds, more lignin is exposed.
3.3.1 Laccase from the fungus *Mauginiella* sp.

The fungal strain *Mauginiella* sp. used in this work was originally identified as *Galactomyces geotrichum* (anamorph *Geotrichum candidum*). During the course of this work, it was re-identified as *Mauginiella* sp., based on the cell wall and hyphal septa characteristic of this anamorphic ascomycete (von Arx *et al.*, 1981).

The fungus *Mauginiella* produced multiple extracellular laccase isoforms that could be separated into two fractions by anion exchange chromatography (Fig. 2/IV). About 30 nkat/ml of laccase activity was measured in the liquid culture after 8 days of growth. The main protein with pI-values ranging from 4.8 to 6.4 was purified and characterized. A three step purification procedure was developed (Table 1/IV) involving ammonium sulphate precipitation, anion exchange and hydrophobic interaction chromatography. The purified *Mauginiella* laccase had characteristics typical of fungal laccases, e.g. an intense blue color. The ultraviolet-visible adsorption spectrum showed two peaks at 279 and 610 nm and a shoulder at 330 nm. The molecular mass of the laccase was 63 kDa as determined by mass spectrometry and it existed as six isoforms with isoelectric points of 4.8, 5.0, 5.2, 5.6, 6.0 and 6.4. Deglycosylation of the laccase did not change the pI pattern (Fig. 1/IV). Thus, it was likely that the different pI forms were caused by post-translational modifications of a single polypeptide chain and not by heterogeneity of the N-glycans.

The N-terminal and internal amino acid sequences from *Mauginiella* laccase, altogether 52 amino acid residues, displayed 100% identity to the peptides corresponding to *Trametes versicolor lcc1* and *Trametes villosa lcc2* genes. Also, the genomic laccase gene fragment isolated, representing about 52% from the total *Mauginiella* laccase gene, was nearly identical to the corresponding regions of the *Trametes* genes. The gene fragment showed the highest identity, 97 %, at the nucleotide level, to the *T. versicolor lcc1* gene (Fig. 5/IV). This was unexpected, since the laccase sequences of *Ascomycetes* usually have identities of 20–30 % with the basidiomycete laccases. One possible explanation for our findings could be that *Mauginiella* could have obtained the laccase gene from a basidiomycete strain by horizontal gene transfer at a much later time than that of the separation between *Ascomycetes* and *Basidiomycetes*. Horizontal gene transfer is occurs widely with prokaryotes (Doolittle, 1999) and has been
recently suggested to play a role in the evolution of fungi as well (Walton, 2000; Garcia-Vallvé et al., 2000).

The *Mauginiella* laccase showed activity towards typical substrates of laccases such as various phenolic compounds and ABTS. The specific activity was highest with ABTS, 2870 nkat/mg. Also guaiacol, 2,6-DMP, syringaldazine and DOPA were oxidized (Table 2/IV). The laccase was inhibited by typical inhibitors of laccase activity (Table 3/IV). Inhibition of laccase activity was tested by monitoring the oxygen consumption in the reaction because it has been shown that the observed inhibition effect of many sulphhydryl-containing compounds (e.g. L-cysteine) were actually caused by reduction of the oxidized substrate and not by true inhibition of the enzyme (Johannes and Majcherczyk, 2000).

The pH optima of *Mauginiella* laccase towards various substrates were similar to those of other fungal laccases, which typically prefer an acidic environment. This laccase had optimal activity at the lowest limit of the pH range, pH 2.4, when ABTS was used as a substrate. Oxidation of 2,6-DMP showed a pH optimum around pH 3.5. The pH optimum for guaiacol was also in the acidic pH range, at pH 4.0. (Fig. 3/IV). The enzyme was sensitive to high temperatures. Although the enzyme retained almost full activity at 40°C after a 2-h incubation, the half-life at 60°C was only 40 min (Fig. 4/IV).

### 3.3.2 Adsorption of laccase on SPS

Currently, there are no published reports on the adsorption of laccase to lignocellulose. Therefore, it was of interest to analyze whether the laccases adsorb on lignocellulose, such as steam pretreated softwood, and whether they remain active after adsorption.

The adsorption of laccases from the ascomycetes *Mauginiella* sp. and *Melanocarpus albomyces*, and the basidiomycete *Trametes hirsuta* on steam pretreated softwood (SPS) was measured in a dilute solution containing only 10 nkat/ml of laccase activity. As shown in Figure 6, the laccases from *T. hirsuta* and *Mauginiella* sp. did not adsorb on SPS and a high free activity was measured in solution with SPS. In contrast, the laccase activity from *M. albomyces* was...
significantly reduced in the presence of SPS suggesting that the laccase was bound to SPS with a high affinity. The presence of SPS seemed to enhance the stability of laccases from *T. hirsuta* and *Mauginiella* sp. Previously, enhanced stability of laccase has been detected in the presence of phenolic compounds (Mai *et al.*, 2000).

**Figure 6. Free laccase activity after 1 h incubation with SPS. Laccases: ThL *T. hirsuta*, MsL *Mauginiella* sp., MaL *M. albomyces*.**

Enzymatic activity of the adsorbed *M. albomyces* laccase was further studied by oxygen consumption methods in order to confirm whether the laccase remained active while adsorbed. The results showed that the laccase was able to oxidize ABTS while adsorbed on SPS as efficiently as a corresponding amount of unbound laccase (Table 7). Thus, it can be concluded that binding to lignocellulosic substrate does not inactivate the *M. albomyces* laccase. Several examples of the laccase immobilized on solid carriers, such as glass beads activated with 3-aminopropytriethoxysilane, are present in the literature. Usually, the activity of laccase is retained after immobilization (reviewed in Durán *et al.*, 2002).
Table 7. Activity of free and SPS-bound laccase from *M. albomyces* measured by the oxygen consumption (between 2 and 8 minutes).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Oxygen consumption (mg/l/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laccase control (without SPS)</td>
<td>0.290</td>
</tr>
<tr>
<td>Laccase bound to SPS (solid phase)</td>
<td>0.297</td>
</tr>
<tr>
<td>Laccase unbound to SPS (liquid phase)</td>
<td>0.062</td>
</tr>
<tr>
<td>SPS control (without laccase)</td>
<td>0.034</td>
</tr>
</tbody>
</table>

The adsorption of laccase to lignocellulose seems to be strongly dependent on the surface properties of the laccase. Because the SPS contains, in addition to cellulose, a considerable amount of lignin, the binding could have occurred onto both of these components. The adsorption behaviour of the laccase from *M. albomyces* was found to be interesting, and further studies have shown that this laccase adsorbs also to bacterial cellulose (Kiiskinen *et al*., 2004).

### 3.3.3 Effect of laccase and mediator compounds on cellulase activities

To be able to perform two enzymatic treatments (such as lignin modification and cellulose hydrolysis) simultaneously, reaction conditions should be made compatible with both types of enzymes. Both cellulases and laccases are catalytically active at the usually applied hydrolysis conditions, pH 5 and temperature of 40–45°C. However, it has been previously shown that mediators oxidized by laccase inhibit the activity of other enzymes. Inactivating effect of the mediator HBT has been observed towards xylanase (Viikari *et al*., 1999) and laccase itself (Freudenreich *et al*., 1998). The mediator NHA oxidized by laccase inhibits the activity of xylanase and mannanase (Oksanen *et al*., 2002). Therefore, effects of laccase and mediator compounds on the activities of cellulases was tested.
In this work, it was found that NHA oxidized by laccase significantly decreased the cellulase activities of *T. reesei* (Table 1/V, Table 2/V). However, the presence of SPS clearly protected the cellulase activity, probably by offering sites for the radical attacks of the activated NHA (Table 3/V). Laccase alone decreased the activity of EG II slightly but did not practically affect the activity of CBH I (Table 2/V). The small difference in the inhibitory effect of laccase on CBH I and EG II may be caused by differences in the surface amino acid composition between the two cellulases. The location and structure of the active site may also play a role. The notable decrease of FPU-activity of Celluclast (in the absence of SPS) by laccase alone and by laccase-oxidized mediator could be explained by combined inhibition of β-glucosidase and hemicellulases and their synergistic action with cellulases. The Celluclast preparation contains also β-glucosidase, hemicellulases and accessory activities (Table 5/III). It is also possible that the enzyme preparation may contain minor amounts of oxidizing substances, which may act as mediators in the reaction.

An approach to overcome the inhibitory effect of the laccase-mediator on hydrolytic enzymes was taken by studying a slow release mediator, the acetylated derivative of NHA, namely NHA-Ac.

### 3.3.4 Enzymatic hydrolysis with laccase treatment

Laccase treatment was combined with the enzymatic hydrolysis of steam pretreated softwood in order to improve the conversion of cellulose. The effects of two mediators, NHA and an acetylated NHA precursor with the *Trametes hirsuta* laccase on enzymatic hydrolysis of steam pretreated softwood and cellulase activities, were examined in publication V. The cellulase dosage used was low (10 FPU/g) and the hydrolysis time was only 24 h, aiming at incomplete hydrolysis in order to enable better detection of differences between the treatments.

Both the simultaneous application of cellulases with the laccase-mediator system and their sequential addition to the substrate resulted in improved enzymatic hydrolysis, as analyzed by release of glucose or reducing sugars (Fig. 2/V). Conversion of polysaccharides was increased by 18–21% when applying NHA together with laccase. The highest conversion, 65% of polysaccharides, was
obtained with the sequential use of the oxidative and hydrolytic enzymatic treatments. The optimum concentration of NHA in the simultaneous treatment was 5 mM, while at higher concentrations NHA was found to be inhibitory (Fig. 3/V). The in situ generation of NHA was examined, but no further improvements could be obtained. The NHA-Ac concentration of 20 mM was also clearly inhibitory (Fig. 4/V). The amount of dissolved aromatic compounds increased when the NHA concentration was increased (Fig. 5/V).

Interestingly, the treatment with laccase alone increased the hydrolysis of cellulose by about 13% (Fig 2/V). Laccase was able to oxidize the SPS substrate as measured by oxygen consumption during the reaction (Fig 1/V). Laccase alone decreased the amount of soluble aromatic compounds, obviously due to polymerization of these compounds.

ESCA analysis revealed a change in the atomic composition and the oxidative status of the carbon atoms on the SPS surface. The chemical shifts for carbon can be classified into four categories: aliphatic carbons (C-C; C1), carbon with one oxygen bond (C-O; C2), carbon with two bonds to oxygen (O-C-O or C=O; C3) and carbons with three bonds to oxygen (O=C-O; C4). The amount of C1 corresponds to the amount of lignin and extractives on the surface of the residue most closely.

The surface analysis by ESCA revealed a strong enrichment of lignin after hydrolysis of cellulose and a slight enrichment of carboxyl acid groups after treatment with T. hirsuta laccase (Table 5/V). Similar results have been obtained in the studies of residual lignin on surfaces, where enrichment of carboxyl acid groups was found after a laccase-HBT treatment (Sealey and Ragauskas, 1998; Poppius-Levlin et al., 1999). It is possible that the SPS solution or the enzyme solutions contain minor amounts of oxidizing substances, which may act as mediators in the reaction. Previously, it has been proposed that solubilized or colloidal lignin may act as a mediator in the oxidation process (Felby et al., 1997; Grönqvist et al., 2003a).

The increase of surface lignin analyzed by ESCA measurements indicates that enzymatic hydrolysis of cellulose had obviously revealed more lignin on the fiber surface. The C1 content was not markedly changed by the laccase and cellulase treatment, indicating no precipitation of laccase polymerized lignins on
the surfaces of the residue. In some studies, repolymerization and adsorption of dissolved, isolated spruce lignans on fibre surfaces by a laccase treatment has been detected (Buchert et al., 2002).

The effects of the three different laccases on the hydrolysis of SPS were compared. The results suggest that the adsorption behaviour of the various laccases onto SPS could not correlate with the capability of the laccases to oxidize the substrate and consequently, to improve the hydrolysis. From the *T. hirsuta*, *Mauginiella* sp. and *M. albomyces* laccases studied, all three had a positive effect on the hydrolysis of lignocellulose (Fig. 7) despite of their different binding behaviour (Fig. 6).

![Figure 7. Hydrolysis of SPS polysaccharides after 6-h hydrolysis of SPS. Conditions: cellulases 10 FPU g⁻¹ SPS, laccase 1000 nkat g⁻¹ SPS, time 6 h, 40 °C). Laccases: ThL *T. hirsuta*, MsL *Mauginiella* sp., MaL *M. albomyces.*](image)

Oxidative treatments of SPS with different laccases reduced the amount of aromatic compounds in the solution (Figure 8). Thus it can be speculated that increase in the enzymatic hydrolysis was at least partly due to the decreased amount of potentially inhibitory compounds present in the solution by the laccase treatment. However, it should be noted that in this study, only washed SPS was used. Inhibitory effects of the prehydrolysate (liquid fraction after pretreatment), which has shown to inhibit the enzymatic hydrolysis of SPS (Tengborg et al., 2001), were not considered critical (discussed in V). However, the sequential treatment, which includes an experimental procedure with more extensive washing of the SPS prior to the hydrolysis, was slightly more efficient than the simultaneous treatment. The main reason for this was most probably
due to avoiding the inhibitory effects of laccase and NHA on the cellulase activities.

Figure 8. Dissolved aromatic compounds in the SPS solution (A$_{280}$) before and after laccase treatment. Laccases: ThL T. hirsuta, MsL Mauginiella sp., MaL M. albomyces.

This study shows for the first time that enzymatic modification and/or removal of lignin can be combined with simultaneous cellulose hydrolysis. Interestingly, modification of lignin surfaces by oxidative treatments with laccase alone and delignification treatment with a laccase-mediator system, both lead to increased hydrolysis of lignocellulose.
4. Conclusions

Characterization, understanding and overcoming the barriers for enzymatic hydrolysis of different raw materials is essential for the development of economically competitive processes based on enzymatic treatments. The synergistic action of different cellulases is crucial for efficient hydrolysis of lignocellulose. In this thesis, the adsorption of purified cellulases was studied in order to obtain deeper understanding of the function of cellulases. The binding of CBH I and CBH II on bacterial microcrystalline cellulose (BMCC) was shown to be determined by a co-operative effect of the two domains, the cellulose binding domain (CBD) and the catalytic domain (CD). It was also shown that binding of the intact CBH I on BMCC was fully reversible, while that of CBH II was only partly reversible. This implies that these two enzymes differ with respect to the reversibility of binding.

Both the CBH I and EG II cellulases from \textit{T. reesei}, adsorbed on steam pretreated softwood (SPS) and lignin, indicating that unspecific adsorption has a role in decreasing the efficiency of enzymatic hydrolysis of lignocellulose. The observation that the presence of CBD clearly enhanced binding of the enzymes on SPS and especially on lignin, suggests that unspecific adsorption is dominated by the affinity of the CBD. The catalytic domains of CBH I and EG II were not bound to lignin isolated by enzymatic hydrolysis. However, since the catalytic domain of EG II showed adsorption to alkaline extracted lignin, interactions other than those related to the CBD were present. It is possible that the open shape of the active site in EG II may increase the adsorption on lignin. Nevertheless, the removal of CBD from CBH I had a more notable effect on the adsorption of the enzyme on SPS, when compared to EG II.

In the wet oxidation pretreatment studies, the total recovery of carbohydrates was high and the recovery of cellulose even higher than what has been reported for steam pretreated softwood. The high residual hemicellulose content was shown to correlate with a low yield in the enzymatic hydrolysis. However, a complete hemicellulose removal during the pretreatment would not be needed in order to obtain high conversion of cellulose, if the cellulase preparation used contains high enough amounts of hemicellulase activities. No clear correlation between the hydrolysis yield and lignin content could be observed. On the other hand, variations in the lignin content of the materials studied were very small.
Obviously, the location and chemical/physical structure of lignin affect the enzymatic hydrolysis more than the absolute amount of lignin. In conclusion, wet oxidation seems to offer an attractive alternative to the more thoroughly investigated methods, such as dilute acid hydrolysis or steam pretreatment.

The work presented here provides novel information on enzymatic modification of lignin in relation to enzymatic hydrolysis of lignocellulose. Oxidation of lignin by laccase was achieved by all three fungal laccases tested, produced by *T. hirsuta*, *M. albomyces* and *Mauginiella* sp. The new laccase isolated and purified from *Mauginiella* had enzymatic characteristics similar to many basidiomycete laccases. This work showed for the first time that enzymatic modification and/or removal of lignin and cellulose hydrolysis can be successfully carried out simultaneously. Both the oxidation of lignin by laccase alone and delignification of the substrate by laccase-mediator-treatments increased the sugar yield in the enzymatic hydrolysis of steam pretreated softwood. The effects of both sequential and simultaneous treatments with a laccase-NHA system were positive. A more detailed analysis of the effects of laccase treatment on the enzymatic hydrolysis of lignocellulose would be beneficial.

The results described in this thesis provide several practical ways to enhance the enzymatic hydrolysis. It was shown that the hydrolysis result could be improved by optimising the pretreatment conditions, reducing the hemicellulose content or hydrolysing the residual hemicellulose by selecting a suitable combination of enzymes. The lignin fraction can be modified by laccase treatment, including oxidation or degradation of lignin to enhance the enzymatic hydrolysis of cellulose.

Control of unspecific adsorption of enzymes to the substrate and optimization of the cellulase mixtures for different raw materials will contribute further to efficient hydrolysis. The future success of (enzyme based) lignocellulosic-to-ethanol process depends mainly on the progress in cellulase enzyme development. For example, protein engineering can be used to design enzymes with optimal binding properties for enzymatic hydrolysis of lignin-containing substrates. Engineering of the production organism can lead to economical production of optimal mixtures of oxidative and hydrolytic enzymes for lignocellulosic substrates.
References


Role of lignin in the enzymatic hydrolysis of lignocellulose

Abstract

Characterization, understanding and overcoming barriers of enzymatic hydrolysis of different raw materials is essential for the development of economically competitive processes based on enzymatic treatments. This work focused on factors relevant for the improvement of enzymatic hydrolysis of lignocellulose raw materials derived from softwood. The major interest of the work was in lignin. Specific areas addressed were the role of lignin in the unproductive binding of cellulases, which restricts the hydrolysis of cellulose, and enzymatic modification of lignin in order to improve cellulose hydrolysis. In addition, suitability a new pretreatment method, wet oxidation, was evaluated for softwood.

The binding of *Trichoderma reesei* CBH I and CBH II enzymes on bacterial microcrystalline cellulose (BMCC) was shown to be determined by a co-operative effect of the two domains, the cellulose binding domain (CBD) and the catalytic domain (CD). Binding of the intact CBH I on bacterial microcrystalline cellulose (BMCC) was fully reversible, while the binding of CBH II was only partly reversible. The cellulases CBH I and EG II were adsorbed on steam pretreated softwood (SPS) and lignin. The observation that the presence of CBD clearly enhanced the binding of the enzymes on SPS and especially on lignin, suggests that unspecific adsorption is dominated by the affinity of the CBD.

The wet oxidation pretreatment studies gave information on the importance of substrate structure in the enzymatic hydrolysis. This pretreatment method was applied to softwood for the first time. In the wet oxidation pretreatment studies, the total recovery of carbohydrates was high and the recovery of cellulose even higher than what has been reported for steam pretreated softwood. Lignin fraction of the substrate remained mainly undissolved. No clear correlation between the hydrolysis yield and lignin content could be observed. It was concluded that the location and chemical/physical structure of lignin affected the enzymatic hydrolysis more than the absolute amount of lignin. It was shown that the hydrolysis result could be improved by optimizing the pretreatment conditions, reducing the hemicellulose content or hydrolysing the residual hemicellulose by selecting a suitable combination of enzymes.

This study showed for the first time that enzymatic modification and/or removal of lignin can be combined with simultaneous cellulose hydrolysis. Both the modification of lignin surfaces by oxidative treatments with laccase alone and delignification treatment with a laccase-mediator system lead to increased hydrolysis of lignocellulose. Oxidation of lignin by laccase was achieved by the three laccases tested, produced by *Trametes hirsuta*, *Melanocarpus albomyces* and *Mauginiella* sp. The new laccase isolated and purified from *Mauginiella* sp. had enzymatic characteristics similar to many basidiomycete laccases. Different adsorption of the three laccases onto SPS did not correlate with the capability of the laccases to oxidize the substrate and consequently, to improve lignocellulose hydrolysis.

Keywords

enzymatic hydrolysis, lignocellulose, enzymes, cellulases, *Trichoderma reesei*, softwood lignin, pretreatment, wet oxidation, enzymatic modification, laccase
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