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17

Improving enzymatic conversion of lignocellulose to platform sugars

Anikó Várnai

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Improving enzymatic conversion of lignocellulose to platform sugars

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Improving enzymatic conversion of lignocellulose to platform sugars

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Abstract

Increasing demand and uncertain availability of fossil fuels urge us to find alternative resources available in large quantities especially for the petrol-based transportation sector. Lignocellulosic biomass, available worldwide in plant cell walls, is a promising alternative feedstock. It can be depolymerised to sugar monomers, which provide potential raw material for sugar platform-based production of fuels and chemicals. However, the enzymatic saccharification of lignocellulose to platform sugars is hindered primarily by the complexity of lignocellulosic substrates as well as by the performance of the hydrolytic enzymes involved. This study focuses on various rate limiting factors such as the decrease in the reactivity and accessibility of the substrates which slow down the hydrolysis, on auxiliary enzymes needed for the efficient solubilisation of cellulose, as well as on the adsorption of enzymes. Consequently, solutions to these limitations were sought to improve the efficiency of biomass conversion processes.

Following the morphological and structural changes in the substrate during hydrolysis revealed that the average crystal size and crystallinity of cellulose remained constant while particle size generally decreased (Paper I). In particular, cellulose microfibrils were proposed to be hydrolysed one-by-one in fibre aggregates by peeling off cellulose chains layer-by-layer from the outer crystals of microfibril aggregates. Microscopic observation showed that almost intact particles remained in the residue even after 60% conversion.

Lignocellulose is a complex network of lignin and polysaccharides. Lignin was found to impede the hydrolysis of cellulose, and its extensive removal doubled the conversion yields of softwood (Paper II). On the other hand, accumulation of lignin during hydrolysis did not affect hydrolysability by commercial cellulase preparations. Residual hemicelluloses, especially glucomannan, were resistant to enzymatic hydrolysis but could be removed together with lignin during delignification. This suggests that especially glucomannans are bound to lignin as lignin-carbohydrate complexes. In addition, cellulose, xylan and glucomannan were shown to be structurally interlinked in softwood (Paper IV). The hydrolysis yield of these polysaccharides remained below 50% without the simultaneous hydrolysis of all polysaccharides. Synergism between the solubilisation of cellulose and hemicelluloses was found, and the release of glucose, xylose and mannose was in linear correlation.

The adsorption and desorption of enzymes were followed during hydrolysis (Paper III). After a quick initial adsorption, slow desorption and re-adsorption of enzymes was observed in alkaline delignified spruce. On the other hand, unproductive adsorption to lignin as well as enzyme inactivation was predicted to play a

primary role in the irreversible adsorption of cellulases in steam pretreated spruce or Avicel during hydrolysis when no desorption of cellulases could be detected.

This study showed for the first time that increasing substrate concentration could compensate for the absence of carbohydrate binding modules (CBMs) in hydrolytic enzymes (Paper V). The performance of cellulases lacking CBMs was comparable to that of cellulases comprising CBM at 20% substrate concentration. At the same time, over 60% of the enzymes without CBMs could be recovered at the end of the hydrolysis. Thus, the major part of hydrolytic enzymes without CBMs could potentially be recovered in industrial high consistency processes.

Keywords lignocellulose, accessibility, enzymatic hydrolysis, *Trichoderma reesei*, glycoside hydrolases, cellulases, xylanases, mannanases, enzyme adsorption, CBM

Academic dissertation

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List of papers

This thesis is based on the following original research papers which are referred to in the text as I–V. The publications are reproduced with kind permission from the publishers. Additional unpublished material is also presented.

I Penttilä, P., Várnai, A., Leppänen, K., Peura, M., Kallonen, A., Jääskeläinen, P., Lucenius, J., Ruokolainen, J., Siika-aho, M., Viikari, L., Serimaa, R. 2010. Changes in submicrometer structure of enzymatically hydrolyzed microcrystalline cellulose. *Biomacromol.* 11(4), pp. 1111–1117.

II Várnai, A., Siika-aho, M., Viikari, L. 2010. Restriction of the enzymatic hydrolysis of steam-pretreated spruce by lignin and hemicellulose. *Enzyme Microb. Technol.* 46(34), pp. 185–193.

III Várnai, A., Viikari, L., Marjamaa, K., Siika-aho, M. 2011. Adsorption of monocomponent enzymes in enzyme mixture analyzed quantitatively during hydrolysis of lignocellulose substrates. *Bioresour. Technol.* 102(2), pp. 1220–1227.

IV Várnai, A., Huikko, L., Pere, J., Siika-aho, M., Viikari, L. 2011. Synergistic action of xylanase and mannanase improves the total hydrolysis of softwood. *Bioresour. Technol.* 102(19), pp. 9096–9104.

V Várnai, A., Siika-aho, M., Viikari, L. 2012. Carbohydrate-binding modules (CBMs) revisited. Reduced amount of water counterbalances the need for CBMs. *Submitted to Proc. Natl. Acad. Sci. U.S.A.*

Author’s contribution

Anikó Várnai planned the investigation leading to Paper I together with the other authors and carried out the enzymatic hydrolysis and the chemical characterization of the samples. She actively participated in the writing of the manuscript.

Anikó Várnai planned the investigation leading to Papers II-IV together with the other authors and performed the experimental work. She had the main responsibility of interpreting the results and was the main and corresponding author of the Papers.

Anikó Várnai designed the experiments of Paper V together with the other authors. She performed the experiments and analysed the data. All authors discussed the results and implications and commented on the manuscript at all stages.

Contents

Appendice V of this publication is not included in the PDF version. Please order the printed version to get the complete publication (http://www.vtt.fi/publications/index.jsp).

List of abbreviations

AFEX	ammonium fibre explosion
AnCel3A	*Aspergillus niger* β-glucosidase
C	cellulase mix in hydrolysis (Paper IV)
C-2,-3,-5	2^{nd}, 3^{rd} and 5^{th} carbon atoms of sugars numbered from the aldehyde group
CBH	cellobiohydrolase
CBM	carbohydrate binding module
COS	catalytically oxidised spruce
d.w.	dry weight
DNS	dinitrosalicylic acid
e.g.	exempli gratia (for example)
EC	enzyme classification
EG	endoglucanase
FPU	filter paper unit
GGM	galactoglucomannan
GH	glycoside hydrolase
i.e.	id est (which means)
LCC	lignin-carbohydrate complex
M	mannanase in hydrolysis (Paper IV)
MALDI	matrix assisted laser desorption/ionisation
MUG3	4-methylumbelliferyl-β-D-cellotrioside
MUL	4-methylumbelliferyl-β-D-lactoside
NFC	nanofibrillated cellulose

PAGE	polyacrylamide gel electrophoresis
*p*NPG	*p*-nitrophenyl-β-glucopyranoside
PS	polysaccharide
SDS	sodium-dodecyl-sulphate
SPS	steam pretreated spruce
SPS-DL	steam pretreated spruce after delignification
SPS-RES	steam pretreated spruce hydrolysis residue
SPS-RES-DL	steam pretreated spruce hydrolysis residue after delignification
T. reesei	*Trichoderma reesei* (anamorph of *Hypocrea jecorina*)
t.m.y.	theoretical maximum yield
TEM	transmission electron microscopy
ToF	time of flight
TrCel5A	*Trichoderma reesei* endoglucanase II
TrCel6A	*Trichoderma reesei* cellobiohydrolase II
TrCel7A	*Trichoderma reesei* cellobiohydrolase I
TrCel7B	*Trichoderma reesei* endoglucanase I
TrMan5A	*Trichoderma reesei* endomannanase
TrXyn11A	*Trichoderma reesei* endoxylanase II (pI 9)
w/V	weight per volume
w/w	weight per weight
X	xylanase in hydrolysis (Paper IV)

1. Introduction

1.1 Drivers for biomass conversion to platform sugars

Increasing demand for energy and depletion of fossil fuel reservoirs urge us to find alternative resources available in large quantities for the petrol-based chemical industry and transportation sectors. Along with increasing energy consumption, environmental aspects such as green house gas emission should be considered to obtain a sustainable environment (Solomon, 2010). Renewable energy sources such as solar energy, wind and water power, or biomass, when exploited in combination, have a good potential to complement fossil fuels in the growing energy market (de Vries *et al.*, 2007) and to fulfil the requirements to reduce green house gas emissions set by the Kyoto protocol in 1998. In particular, biomass is the most promising alternative feedstock among the renewables for the production of liquid fuels such as bioethanol, which are currently essential for transportation, and could provide alternative raw materials for the chemical industry (Figure 1).

At present, bioethanol and biodiesel are produced and blended with gasoline and diesel worldwide: bioethanol is produced in North America mainly from cornstarch and in Brazil from sugarcane, while biodiesel is produced in Europe from rapeseed oil. However, fuels produced from these raw materials are not considered sustainable for several reasons: because starch, sugar and vegetable oils can be consumed as food or feed (the “food versus fuel” debate), and because the net green house gas and energy savings of these fuels are modest. Thus, recent efforts have been directed towards the use of sustainable raw materials, such as residues from forestry and agriculture as well as municipal wastes (EPA, 2009; Solomon, 2010; Bonin and Lal, 2012).

Cellulose is the most abundant biopolymer on earth, and is available worldwide. It is composed of glucose monomers produced by plants absorbing carbon dioxide from the atmosphere. Cellulose can be depolymerised to glucose monomers, providing raw materials for sugar platform based chemicals and energy carriers. Hence, lignocellulose based biofuels and chemicals have a high potential to replace starch based bioethanol and to fulfil the requirements set to reduce green house gas emissions (Kyoto protocol, 1998; EPA, 2009). On the other hand, even wood or plant based biomass sources, although available worldwide, could only

partially contribute to energy independence due to limited land availability and fairly low biomass production yields (Solomon, 2010; Bonin and Lal, 2012).

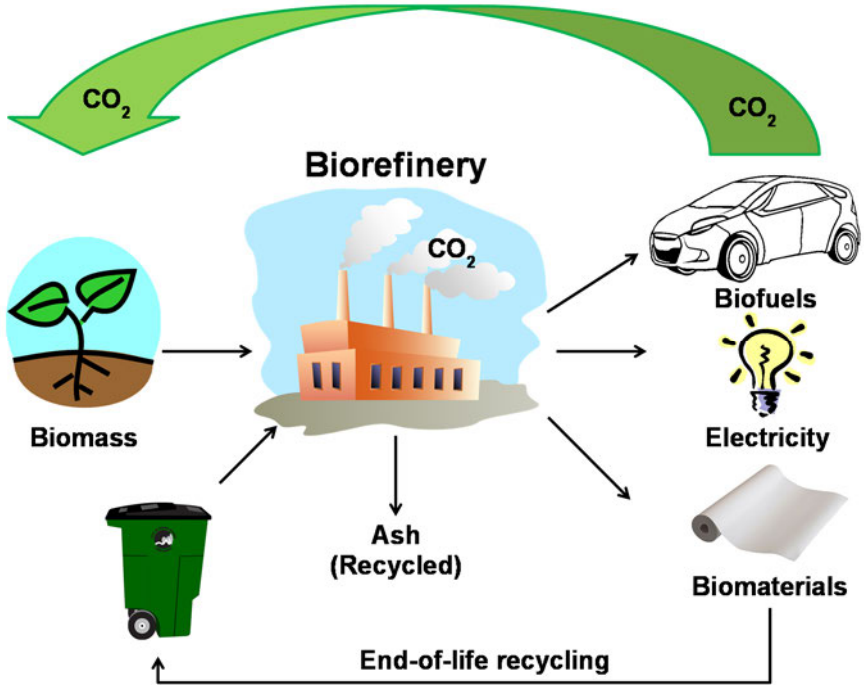

Figure 1. The fully integrated biomass-biofuel-biomaterial-biopower cycle for sustainable technologies.

Despite much effort, saccharification of lignocellulose is still hampered by technical and economic obstacles. The conversion of biomass to platform sugars is hindered primarily by the complexity of lignocellulosic substrates as well as by the performance of the hydrolytic enzymes. Therefore, efficient, optimised and economically competitive pretreatment and hydrolysis technologies are the most commonly studied approaches aimed at overcoming the challenges in biomass conversion (Himmel *et al.*, 2007). Accordingly, the major cost factors in the biomass conversion process are due to the feedstock (including feed handling), pretreatment and the enzymes (Humbird *et al.*, 2011).

1.2 Lignocellulosic biomass, a renewable raw material

Lignocellulosic plant cell walls are complex materials consisting of three major components: cellulose, hemicellulose and lignin. The ratio and detailed composition of these components vary significantly between plant species. Various biomasses may also contain small amounts of pectin, starch, minerals (ash) and various

extractives. As an example, Norway spruce (*Picea abies*), one of the most widespread woody feedstocks in Scandinavia, consists of 45–47% cellulose, 24–26% hemicellulose, 27–28% lignin and 0.4–0.9% extractives (Bertaud and Holmbom, 2004). Pectin and starch are water-soluble and are largely removed during most of the pretreatment methods of biomass prior to enzymatic saccharification, and are thus not addressed in this work.

Cell walls are built up from several layers: the middle lamella, the primary and secondary cell walls, and the warty layer (Sjöström, 1993). The middle lamella, which is highly lignified, is located between cells and binds the cells together. The outmost layer of the cell, the primary cell wall, consists of an irregular network of cellulose microfibrils combined with hemicellulose, lignin, pectin and proteins. The primary cell wall is supported by a thicker secondary cell wall, which can be divided into three distinct sublayers consisting of highly organised cellulosic microfibrils with various orientations. The middle layer of the secondary cell wall comprises most of the lignin in the cell wall because of its thickness, even though the concentration of lignin is higher in the middle lamella and the primary wall. In the cell wall, hemicelluloses and cellulose have been reported to be associated to each other and cross-linked with lignin (Eriksson *et al.*, 1980; Tenkanen *et al.*, 1999).

1.2.1 Cellulose

Cellulose, making up approximately 35–50% of the biomass, is a linear homopolymer, built up of glucose monomers via β-1,4-linkages (Timell, 1967; Vogel, 2008). In nature, linear cellulose chains line up parallel to each other shortly after biosynthesis (Mueller and Brown, 1980) and form elementary fibrils that are held together via intermolecular hydrogen bonds (Figure 2) (Nishiyama *et al.*, 2002). Elementary fibrils form cellulose crystals which associate into highly ordered structures, microfibrils, containing both ordered (crystalline) and less ordered (amorphous) regions (Ramos, 2003). The organisation of cellulose microfibrils (and hence the crystallinity) changes from layer to layer in the primary and secondary cell walls along with the composition of the layers (Sjöström, 1993). Cellulose crystals vary in size depending on their origin, the crystal width (cross-section) being 3–4 nm in wood (Hult *et al.*, 2001; Andersson *et al.*, 2004).

The shape, exact size and number of participating cellulose chains in a single elementary fibril in higher plants are still uncertain. Based on the rosette-shape of cellulose synthase complexes, it is suspected that the elementary fibrils consist of 36 cellulose chains (Mueller and Brown, 1980). Recently, Ding and Himmel proposed that elementary fibrils are not heterogeneous in their structure: the middle chains surrounded by other cellulose chains form a crystalline core, while cellulose chains located on the surface (or between these two) are non-crystalline or subcrystalline (Figure 2) (Ding and Himmel, 2006). The crystallinity of the surfacial cellulose chains also depends on the assembly of elementary fibrils. In particular, if elementary fibrils are attached to each other, the cellulose chains which are located on the matching edges of the fibres are not exposed to water and hence

might be more crystalline than those located on the outer edges. This could also influence the measured size of the cellulose crystals.

Several methods have been developed to define crystallinity indices (Park *et al.*, 2010). The most common methods are X-ray diffraction and nuclear magnetic resonance spectroscopy. In addition, several calculation methods have been implemented to calculate cellulose crystallinity and crystal size from X-ray measurements (Bansal *et al.*, 2010).

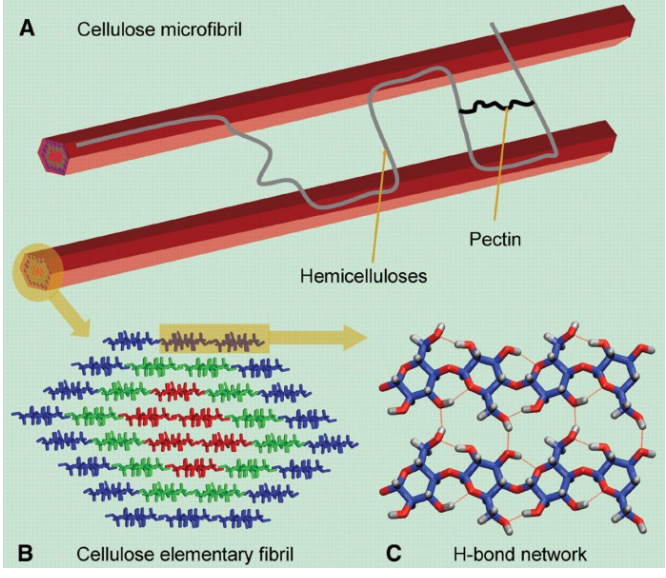

Figure 2. (A) A simplified model showing the interaction of the major polysaccharides in the cell wall. **(B)** The 36-chain model of the cellulose elementary fibril. **(C)** The intra- and interchain hydrogen-bond network in cellulose Iβ. (Himmel *et al.*, 2007, Figure 1, p. 806. Reproduced with kind permission from The American Association for the Advancement of Science).

Cellulose (nano)crystals (often denoted as nano-whiskers because of their needle-like shape in microscopic images) can be separated by acid hydrolysis from the agglomerates, cleaving microfibrils at the amorphous regions (Bondeson *et al.*, 2006). Cellulose fibrils can also be separated from each other by shear forces without significant fragmentation by applying mechanical pressure on chemical pulp in microfluidizers, resulting in nanofibrillated cellulose (NFC) consisting of longer fibrils. As NFC is produced from chemical pulp without weight loss, it is rich in hemicellulose, which contributes to the stabilisation of the separated cellulose fibrils in NFC. NFC has several potential future uses, but today it is mostly studied to reinforce composites (Klemm *et al.*, 2011).

1.2.2 Hemicellulose

Hemicelluloses – xylans and glucomannans (Figure 3) – are branched polysaccharides consisting of pentoses D-xylose and L-arabinose, hexoses D-mannose, D-glucose and D-galactose, and uronic acids (Saka, 1991). Hemicelluloses in softwoods, hardwoods and agricultural plant materials differ in composition and structure. Softwood consists of 15–20% galactoglucomannan (GGM), the dominant hemicelluloses in softwood, whereas hardwood and agricultural plants are rich in xylans (Timell, 1967; Willför *et al.*, 2005a; Willför *et al.*, 2005b; Vogel, 2008). Xylan-type polysaccharides constitute about 20–30% of the biomass of dicotyl plants (hardwoods and herbaceous plants) and 50% of monocotyl plants (grasses and cereals), while only 10–15% of softwood cell walls (Timell, 1967). Hardwoods also contain 3–5% glucomannan.

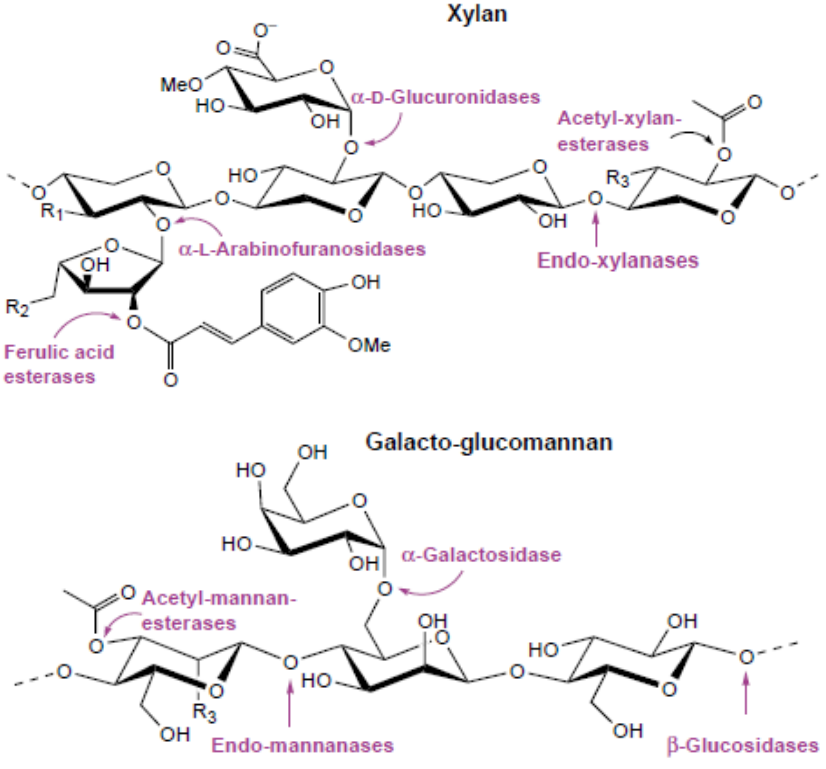

Figure 3. The basic structure of xylan and galactoglucomannan and the hemicellulases responsible for their degradation. (The figure is based on Shallom and Shoham, 2003, Figure 1, p. 220).

Xylans consist of a homopolymer backbone of β-D-xylopyranose units with β-1,4 linkages (Figure 3). The β-D-xylopyranose units may be substituted at C-2 with

(4-O-methyl-)α-D-glucopyranuronic acid residues, which can be esterified in hardwood or agricultural plants by an *O*-acetyl group at C-2 or more frequently at C-3 (Timell, 1967; Kabel *et al.*, 2002; Willför *et al.*, 2005a; Willför *et al.*, 2005b; Ebringerová and Heinze, 2000). Xylose units can also be acetylated. Unlike xylans in hardwood or annual plants, softwood xylans are not esterified by acetyl groups. In softwood and agricultural plants, β-D-xylopyranose units may be substituted at the positions C-2 and C-3 with α-L-arabinofuranose units. Occasionally, the L-arabinofuranose side chains are esterified with ferulic and *p*-coumaric acid in annual plants (Iiyama *et al.*, 1994; Hatfield *et al.*, 1999).

Glucomannans, the second most important hemicellulose in hardwood, consist of a linear backbone which is a copolymer of β-1,4 linked β-D-glucopyranose and β-D-mannopyranose units (Figure 3). In softwood galactoglucomannans, β-D-mannopyranose units are partially substituted by α-D-galactopyranose units at the position C-6 and acetylated at C-2 or C-3 (Lundqvist *et al.*, 2002). In softwood, two types of glucomannans have been identified which differ in the degree of substitution by galactose and hence in solubility: the low galactose containing GGM has a molar ratio of galactose:glucose:mannose 0.1:1:4 and is less soluble, whereas the high galactose containing GGM has a corresponding ratio of 1:1:3 (Sjöström, 1993). Grasses contain only insignificant amounts of glucomannan (Vogel, 2008).

1.2.3 Lignin and phenolic acids

Several aromatic compounds, such as lignin and phenolic acids, have been recognised to take part in the organisation of cell walls. In biomass, lignin is considered to play an important role in the structural integrity of cell walls by keeping the polysaccharides together in the cell walls. Lignins are heterogeneous hydrophobic polymers formed of phenylpropane units: guaiacyl units (from the *trans*-coniferyl-alcohol precursor), syringyl units (from the *trans*-sinapyl-alcohol precursor) and *p*-hydroxyphenyl units (from the *trans*-*p*-coumaryl-alcohol precursor), which differ in the extent of methoxylation (Campbell and Sederoff, 1996). The amount, composition and structure of lignin vary widely within plant species, within a single plant, and even within a given cell wall. In general, softwood lignin contains predominantly guaiacyl units with a minor proportion of unmethoxylated *p*-hydroxyphenyl units (Obst and Landucci, 1986); hardwood lignin is richer in syringyl units, however, with guaiacyl units dominating (Fergus and Goring, 1970); and annual gramineous plants contain all three units in fairly equal amounts (Buranov and Mazza, 2008). In addition, lignins in annual plants are also etherified and esterified by phenolic acids, *i.e.* by ferulic and *p*-coumaric acids (Scalbert *et al.*, 1985; Lam *et al.*, 2001; Buranov and Mazza, 2008).

In grass arabinoxylans, phenolic acids have been found to esterify hydroxyl groups in α-L-arabinofuranosyl residues, mostly at the position C-5 (Iiyama *et al.*, 1994; Hatfield *et al.*, 1999; Vogel, 2008). Ferulic acids can form various dimers with free-radical condensation reactions, similar to the polymerisation reactions of

lignin, and hence contribute to cross-linking between polysaccharides or between polysaccharides with lignin (Iiyama *et al.*, 1990; Ralph *et al.*, 1995).

1.2.4 Lignin-carbohydrate complexes

Covalent linkages between cellulose, hemicellulose and lignin have been reported in wood and other plant materials (Jeffries, 1990; Iiyama *et al.*, 1994; Vogel, 2008). Covalent linkages have been characterised by studying lignin-carbohydrate complexes (LCCs), which are structures containing oligosaccharides linked to lignin oligomers. Different LCCs isolated from kraft pulp differ in their solubility, providing possibilities for separation and characterisation of various linkages (Lawoko *et al.*, 2003). Three types of cross-links between lignin and polysaccharides have been described: direct ester and ether linkages between the phenolic hydroxyl groups of lignin and the carboxylic acids and free hydroxyl groups of the carbohydrates, respectively; and indirect hydroxycinnamic acid ester-ether bridges between phenolic hydroxyl groups of lignin and hydroxyl groups of the carbohydrates (Eriksson *et al.*, 1980; Jeffries, 1990; Iiyama *et al.*, 1994).

In wood, lignin can be linked covalently through the free hydroxyl groups of its phenylpropane subunits to the major hemicellulose components, *i.e.* arabinoglucuronoxylans or (galacto)glucomannans (Lawoko *et al.*, 2006). Galactose and arabinose have been proposed to be the sugar units with the most potential to be linked to lignin because of their predominant presence as side groups (Choi *et al.*, 2007). Nevertheless, glucose, mannose and xylose have also been detected in linkages between lignin and carbohydrates (Lawoko *et al.*, 2006). Apart from the direct lignin-carbohydrate linkages, bifunctional ferulic acids form ester-ether bridges between lignin and primarily arabinoxylan in grasses; the phenolic groups may be ether linked to the hydroxyl groups of lignin (Scalbert *et al.*, 1985), and simultaneously, the carboxyl group esterified by the arabinose substituents in arabinoglucuronoxylan (Iiyama *et al.*, 1990).

1.3 Enzymes degrading lignocellulose

A vast array of enzymes have been discovered to participate in the degradation of lignocellulose. Cellulose, the major component of lignocellulose, is hydrolysed by a complex cellulase system generally consisting of cellobiohydrolases, endoglucanases and β-glucosidase (see Chapter 1.3.1). For the efficient hydrolysis of lignocellulose, additional helper, *i.e.* auxiliary, enzymes are required to increase the accessibility of cellulases to cellulose. Auxiliary enzymes may participate in the hydrolysis of other polysaccharide components such as hemicellulose (hemicellulases in general, see Chapter 1.3.2) or pectin (pectinases), in the hydrolysis of lignin-carbohydrate linkages (primarily feruloyl- and *p*-coumaroyl-esterases, see Chapter 1.3.3), or in the disruption of fibre structure (swollenins). The hydrolytic enzymes were first classified based on enzymatic activities (EC classification). The classification of glycoside hydrolases (GHs) into families is based on their

amino acid sequence and their three-dimensional structure organisation (Henrissat, 1991; Davies and Henrissat, 1995; Henrissat and Davies, 1997; Cantarel *et al.*, 2009). Cellulolytic systems seem to differ between aerobic and anaerobic microorganisms (Lynd *et al.*, 2002). In aerobes such as saprophytic fungi, various cellulases and other plant cell wall degrading enzymes are mostly secreted extracellularly and they exhibit extensive biochemical synergy. In contrast, the hydrolytic enzymes of most anaerobic microorganisms such as cellulose-degrading rumen bacteria are associated to an integrating subunit (scaffoldin) which contains a carbohydrate binding module (CBM), forming a supramolecular complex called cellulosome (Gilbert, 2007). This thesis focuses on noncomplexed cellulase systems, in particular on the cellulase system of the mesophilic fungus *Trichoderma reesei* (anamorph of *Hypocrea jecorina*), and this chapter discusses only the noncomplexed cellulase systems, including enzymes acting on cellulose and hemicelluloses.

Due to historical reasons and their high GH production capacity, *T. reesei* strain variants today are still the preferred industrial microorganisms in the production of cellulases and hemicellulases. The cellulolytic enzyme system of *T. reesei* has served as a model for basic cellulose degradation studies, and strains have been tailored to produce specific mixtures of these enzymes for various industrial applications. In addition, *T. reesei* strains serve as production hosts for efficient heterologuous expression of proteins of other organisms. Surprisingly, the genome of the well-studied fungus *T. reesei* carries about only 200 glycoside hydrolase (GH) genes encoding plant cell wall polysaccharide degrading enzymes (Martinez *et al.*, 2008). Despite its high hydrolytic efficiency, the genome of *T. reesei* encodes only 10 cellulolytic and 16 hemicellulolytic enzymes, which is actually less than in many other sequenced fungi. The major cellulolytic and hemicellulolytic enzymes are discussed as follows.

1.3.1 Cellulolytic enzymes

Despite the simple, linear structure of cellulose, a wide spectrum of enzymes have been found necessary to give efficient and complete hydrolysis (Figure 4): cellobiohydrolases (CBHs, exo-1,4-β-glucanases) release cellobiose units from the nonreducing (EC 3.2.1.91) or reducing end (EC 3.2.1.176) of the cellulose chains; endoglucanases (EGs, endo-1,4-β-glucanases) cleave cellulose chains in amorphous regions; the recently discovered oxidoreductases (GH 61 enzymes, no EC number assigned at the moment) contribute to the hydrolysis with oxidative cleavage of cellulose (Quinlan *et al.*, 2011); and finally, β-glucosidases (EC 3.2.1.21) hydrolyse the solubilised cello-oligomers and dimers into glucose (Medie *et al.*, 2012).

Cellulolytic systems without CBHs have also been described in brown-rot species within wood-rotting basidiomycetes. Thus, *e.g.* in *Postia placenta*, cellulose is degraded through oxidative depolymerisation by free hydroxyl radicals that are generated extracellularly by secreted, structurally divergent oxidases, analogous to the oxidative Fenton chemistry (Martinez *et al.*, 2009).

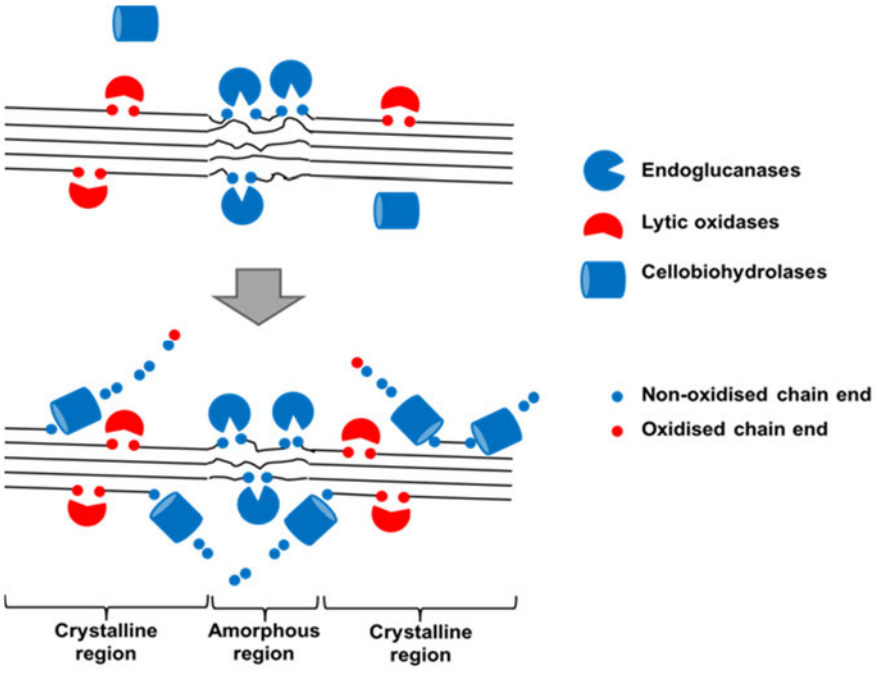

Figure 4. Schematic representation of enzymatic cellulose breakdown in the presence of lytic oxidative enzymes. Non-oxidised chain ends are marked with blue circles, oxidised chain ends with red circles. (The figure is based on Medie *et al.*, 2012, Figure 1, p. 228).

Cellobiohydrolases (EC 3.2.1.176 and 3.2.1.91) hydrolyse the glycosidic linkages mainly in the crystalline regions of cellulose, while endoglucanases (EC 3.2.1.4) produce new starting ends for CBHs by cleaving cellulose chains in the amorphous regions (Teeri, 1997). Fungal CBHs belong to the glycoside hydrolase (GH) families 6 and 7, bacterial CBHs to the GH families 5, 6, 9 and 48, fungal EGs to the families 5, 6, 7, 9, 12, 18, 19, 44, 45 and 74, and bacterial EGs to the families 5, 6, 8, 9, 10, 12, 18, 26, 44, 45, 48, 51, 74 and 124. Some cellobiohydrolases hydrolyse cellulose in a processive manner due to the shape of their active site (Teeri *et al.*, 1998; von Ossowski *et al.*, 2003). Processivity increases the performance of CBHs, however, the action may be restricted by obstacles on the cellulosic substrates which block the CBHs from gliding along cellulose chains (Kurasin and Väljamäe, 2011). Consequently, when a CBH molecule is being retarded by an obstacle, it can prevent the processive movement of other CBHs along the cellulose chain (Igarashi *et al.*, 2011). EGs, on the other hand, have been found to contribute to the termination of the processive action of CBHs. The degree of processivity was noticeably decreased after the treatment of the substrate by EGs (Kipper *et al.*, 2005; Fox *et al.*, 2012). In addition, enzymes capable of faster de-

sorption would be needed to overcome the retardation of hydrolysis due to cellulases being blocked by obstacles (Kurasin and Väljamäe, 2011).

1.3.2 Hemicellulolytic enzymes

Because of the complex structures composed of versatile building blocks, a vast array of enzymes is needed for the complete depolymerisation of hemicelluloses (Figure 3). Hemicellulases participating in the hydrolysis of xylan and glucomannan are discussed separately in the following.

1.3.2.1 Xylanases

Xylan is solubilised by endo-1,4-β-xylanases (EC 3.2.1.8) cleaving the xylan backbone to oligomers. Endo-1,4-β-xylanases have been classified into GH families 5, 8, 10, 11 and 43, among which xylanases belonging to the two major families 10 and 11 have been studied most extensively (Kolenová *et al.*, 2006; Pollet *et al.*, 2010). Xylan side-groups hinder the action of endo-1,4-β-xylanases sterically (Kolenová *et al.*, 2006; Collins *et al.*, 2005). Thus, for the complete hydrolysis of the polymers, several debranching enzymes including α-glucuronidase (EC 3.2.1.139), α-L-arabinofuranosidase (EC 3.2.1.55), acetylxylan esterase (EC 3.1.1.72), feruloyl esterase (EC 3.1.1.73) and *p*-coumaroyl esterase (EC 3.1.1.-) are essential to further hydrolyse the xylo-oligomers to monomers (Biely, 2003). The debranching enzymes act in a co-operative manner with the endo-1,4-β-xylanases in the degradation of xylan (Biely, 1985; de Vries and Visser, 2001). Xylo-oligomers are hydrolysed by β-xylosidases (EC 3.2.1.37) to xylose monomers.

1.3.2.2 Mannanases

The hydrolysis of mannan containing hemicelluloses also require a wide array of enzymes (Stålbrand, 2003; Moreira and Filho, 2008). Endo-1,4-β-mannanase (EC.3.2.1.78) cleaves the (galacto)glucomannan (GGM) main chain to oligosaccharides leaving a mannose residue on the reducing end (Tenkanen *et al.*, 1997). As the glucomannan backbone consists of glucose and mannose monomers, the formed oligosaccharides are further hydrolysed by β-mannosidase (EC 3.2.1.25) and β-glucosidase (EC 3.2.1.21), hydrolysing mannose and glucose residues at the non-reducing-end respectively. Analogously to endo-1,4-β-xylanases, the action of endo-1,4-β-mannanases is restricted by the substitution groups on the backbone, and the galactosyl side groups can be cleaved off by α-galactosidase (EC 3.2.1.22) (McCleary and Matheson, 1983; Tenkanen *et al.*, 1997). Acetylmannan esterase (EC 3.1.1.6) catalyses the deacetylation of the glucomannan backbone in softwood (Tenkanen *et al.*, 1995). Similar to xylanolytic enzymes, also (galacto)glucomannan degrading enzymes co-operate in the solubilisation and complete hydrolysis of GGM (de Vries and Visser, 2001; Moreira and Filho, 2008).

1.3.2.3 Cellulases with hemicellulolytic side activity

Enzymes are not always fully substrate specific, and often catalytic action towards several substrates has been detected. Accordingly, unspecific cellulases, especially endoglucanases, have been identified with xylanolytic and mannanolytic side activities (Vlasenko *et al.*, 2010; Lawoko *et al.*, 2000). Some GH 7 endoglucanases were found to hydrolyse birchwood xylan and wheat arabinoxylan (Vlasenko *et al.*, 2010). Among these EGs, TrCel7B from *Trichoderma reesei* has been investigated most thoroughly (Biely *et al.*, 1991; Lawoko *et al.*, 2000). In addition, GH 5 EGs showed activity on mannan and galactomannan (Vlasenko *et al.*, 2010). Four *T. reesei* EGs, namely EG I, II, III and V belonging to GH families 7, 5, 12 and 45 respectively, were, however, found to be inactive on locust bean mannan (having a mannan backbone without glucose subunits) (Lawoko *et al.*, 2000), whereas these endoglucanases were active towards konjak glucomannan (having a glucomannan backbone consisting of mannose and glucose subunits) (Karlsson *et al.*, 2002). This difference implies that glucose units in the glucomannan backbone play an important role in the substrate specificity of endoglucanases.

1.3.3 Enzymes hydrolysing lignin-carbohydrate complexes

In plant cell walls, covalent linkages between cellulose, hemicellulose and lignin have been reported, as described in Chapter 1.2.4 (Jeffries, 1990; Iiyama *et al.*, 1994; Vogel, 2008). Lignin-carbohydrate linkages include ester and ether linkages involving arabinose, galactose and glucuronic acid linked to free phenolic hydroxyl groups of lignin (Lawoko *et al.*, 2006; Choi *et al.*, 2007). The carbohydrates are released by hemicellulolytic enzymes hydrolysing side-groups of xylan and glucomannan, as listed in Chapters 1.3.2.1 and 1.3.2.2. Direct ester and ether linkages between lignin and carbohydrates, however, are very resilient; no enzymes capable of their enzymatic hydrolysis are currently known: a single sugar residue always remains attached to lignin even after complete digestion of the hemicellulose moiety (Jeffries, 1990). On the other hand, indirect ester-ether bridges formed by bifunctional hydroxycinnamic acids such as ferulic acid or *p*-coumaric acid can be hydrolysed by feruloyl esterase (EC 3.1.1.73) and *p*-coumaroyl esterase (EC 3.1.1.-).

1.3.4 Domain structure of glycoside hydrolases

Cellulolytic enzymes can be divided into two distinct domains: the catalytic or core domain and the carbohydrate binding module (CBM), which are linked together via a flexible linker (Figure 5) (Gilkes *et al.*, 1991). Traditionally, the presence of CBMs has been considered necessary for the action of cellulases on insoluble substrates. Recently, however, the catalytic activity of the catalytic domain has been shown not to depend on the presence of CBMs, both on soluble and insoluble substrates. Previously, several cellulases have been shown to hydrolyse solu-

ble substrates with similar efficiency (van Tilbeurgh *et al.*, 1986; Tomme *et al.*, 1988; Ståhlberg *et al.*, 1988; Reinikainen *et al.*, 1992). Recently, Jalak and Väljamäe developed a method to measure the observed catalytic constant for cellobiohydrolases acting on the reducing end of cellulose (Jalak and Väljamäe, 2010) and found that intact cellobiohydrolases and their catalytic domains had a similar turnover number towards insoluble cellulose, and hence both proceeded along the cellulose chain with a similar speed. This latter phenomenon was also visualised by high-speed atomic force microscopy by Igarashi and co-workers (Igarashi *et al.*, 2009). Thus, contrary to previous expectations, the presence of a CBM does not seem to improve the catalytic activity. Nevertheless, CBMs are still considered necessary for efficient hydrolysis of insoluble lignocellulose.

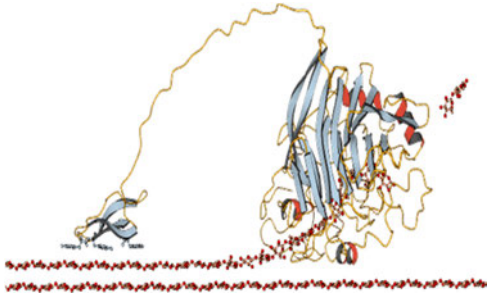

Figure 5. The two-domain structure of the cellobiohydrolase TrCel7A from *Trichoderma reesei* during its action on cellulose. Picture courtesy of Christina Divne.

CBMs have been proposed to have multiple roles in the hydrolysis (Reinikainen *et al.*, 1992; Boraston *et al.*, 2004; Arantes and Saddler, 2010). The main role of CBMs is to concentrate the enzymes onto the surface of the substrate, which increases the apparent catalytic efficiency, leading to a higher degree of hydrolysis compared to enzymes without CBMs (Ståhlberg *et al.*, 1991; Nidetzky *et al.*, 1994; Kotiranta *et al.*, 1999). The removal of CBMs has been shown to dramatically decrease the enzyme efficiency on insoluble substrates, but not on soluble substrates (van Tilbeurgh *et al.*, 1986; Tomme *et al.*, 1988; Ståhlberg *et al.*, 1988; Reinikainen *et al.*, 1992). CBMs have also been proposed to disrupt the polysaccharide structure (Wang *et al.*, 2008), contributing to the amorphogenesis (Arantes and Saddler, 2010). The disruption and loosening of cellulosic fibres are believed to facilitate the initiation of cellulose hydrolysis. CBMs have also been predicted to play a role in the processivity of cellobiohydrolase TrCel7A (Bu *et al.*, 2009). Nevertheless, intact cellobiohydrolases and their catalytic domains have been detected to proceed along the cellulose chains with a similar speed, an observation questioning the role of CBMs in the processivity of cellulases (Igarashi *et al.*, 2009).

1.3.5 Co-operativity between glycoside hydrolases

Synergism between enzyme components of non-complexed cellulase systems is a well documented phenomenon. Certain enzyme components perform more efficiently when acting together compared to the individual action of the components. If the overall quantity of hydrolysis products produced by the simultaneous action of these enzymes is greater than the sum of the hydrolysis products produced by the enzymes acting individually, synergism of two or more enzyme components is said to have occurred.

1.3.5.1 Synergism in the hydrolysis of homopolymers

Synergism between cellulases was already described by the 1980s (Henrissat *et al.*, 1985). In particular, endo-exo and exo-exo synergisms have been distinguished based on the action of the enzyme components contributing to the other's action and have been extensively studied on various cellulosic substrates (Henrissat *et al.*, 1985; Tomme *et al.*, 1988; Nidetzky *et al.*, 1993). In the endo-exo synergism of the well-characterised cellulase system of *T. reesei*, the endoglucanases TrCel7B or TrCel5A produce new chain ends by randomly cleaving the cellulose chains, thus creating new starting-points and end-points for the action of cellobiohydrolases (Henrissat *et al.*, 1985; Medve *et al.*, 1998; Karlsson *et al.*, 1999; Väljamäe *et al.*, 1999; Eriksson *et al.*, 2002). Exo-exo synergism occurs when the cellobiohydrolases TrCel7A and TrCel6A hydrolyse simultaneously cellulose chains from the reducing and non-reducing ends (Henrissat *et al.*, 1985; Tomme *et al.*, 1988; Nidetzky *et al.*, 1993). This has traditionally been attributed to the action of one type of cellobiohydrolase shaving cellulose chains from the surface of the substrate and uncovering new chain ends (*i.e.* action sites) for the other type of cellobiohydrolase.

In addition, β-glucosidases explicitly, if not synergistically, contribute to the hydrolysis of cellulose by hydrolysing cellobiose, which causes significant end-product inhibition of cellobiohydrolases when accumulating at higher degrees of hydrolysis (Holtzapple *et al.*, 1990; Gruno *et al.*, 2004).

1.3.5.2 Synergism in the hydrolysis of heteropolymers

The synergistic action of enzymes in the hydrolysis of heteropolysaccharides such as hemicellulose is more complex than the synergism in homopolymers (de Vries and Visser, 2001). Unlike cellulose, xylan and glucomannan are branched heteropolysaccharides. Thus, the most important type of synergism concerns the co-operativity between the enzymes that attack the backbone (endoxylanases and endomannanases) and enzymes that liberate side groups, such as acetyl, arabinofuranosyl and galactosyl residues (Tenkanen *et al.*, 1995; Suh and Choi, 1996a; Suh and Choi, 1996b). The co-operativity is attributed to the backbone hydrolysing enzymes releasing substituted oligomers, which are more readily hydrolysed by

the side group cleaving enzymes than the substituted polymers (Verbruggen *et al.*, 1998; de Vries *et al.*, 2000). On the other hand, the action of the endo-1,4-β-xylanases and endo-1,4-β-mannanases is sterically hindered by the side groups. Therefore, the removal of substituents creates new sites for the action of endoenzymes (Tenkanen *et al.*, 1997; Verbruggen *et al.*, 1998; de Vries *et al.*, 2000). The release of phenolic acids by esterases is also synergistically enhanced by the action of endo-1,4-β-xylanases (de Vries *et al.*, 2000; Faulds *et al.*, 2006).

1.3.5.3 Synergism in the co-hydrolysis of interlinked polysaccharides and lignin-carbohydrate linkages in plant cell walls

In plant cell walls, cellulose, hemicelluloses and lignin are physically and chemically interlinked with each other. Hence, to enhance the exposure of cellulose fibres for complete hydrolysis, the addition of enzymes is required which degrade either different polysaccharides, especially hemicellulases, or lignin-carbohydrate linkages. Accordingly, the hydrolysis of cellulose has been found to be enhanced by the simultaneous hydrolysis of hemicellulose (Berlin *et al.*, 2005a; Himmel *et al.*, 2007). The role of hemicellulases as auxiliary enzymes has therefore been studied in order to optimise enzyme mixtures for improved biotechnical conversion of biomass to ethanol (Berlin *et al.*, 2007; Banerjee *et al.*, 2010). The range of enzymes that are necessary for the total exploitation of biomass eventually depends on the substrate composition. In addition to synergism between cellulases, xylanases and mannanases, synergism between auxiliary enzymes and cellulases has also been described (Tenkanen *et al.*, 1999; Tabka *et al.*, 2006; Selig *et al.*, 2008). Backbone hydrolysing cellulases, xylanases and mannanases have been found to act synergistically in chemical pulp to solubilise cellulose, xylan and glucomannan (Tenkanen *et al.*, 1999). In addition, synergism has also been observed between the simultaneous hydrolysis of cellulose, xylan and ferulic acid esters, which form possible lignin-carbohydrate cross-linkages. Cellulases, xylanases and feruloyl esterase enhanced synergistically the hydrolytic action of the other enzyme preparations (Tabka *et al.*, 2006; Selig *et al.*, 2008).

1.4 Bottlenecks in enzymatic hydrolysis

Enzymatic hydrolysis of lignocellulose is still a serious bottleneck in the conversion of biomass to platform sugars (Wyman, 1999; Margeot *et al.*, 2009). This step is hindered by the complexity of lignocellulosic substrates as well as restrictions caused by the participating enzymes. Accordingly, efficient, optimised and economically competitive pretreatment and hydrolysis technologies are the most commonly studied approaches to overcome the challenges in biomass conversion (Himmel *et al.*, 2007). In addition, reducing the water consumption to generate high sugar and subsequently high ethanol concentrations also causes limitations in mixing and mass transfer due to the high substrate concentration (Jørgensen *et al.*, 2007). In this chapter, bottlenecks in enzymatic hydrolysis related to substrate

complexity, enzymes hydrolysing lignocellulose and high substrate consistency are discussed.

1.4.1 Substrate recalcitrance and complexity

Lignocellulosic biomass consists of a complex and inhomogeneous network of polymers – cellulose, hemicellulose and lignin. The highly ordered structure of cellulose and the complexity and heterogeneity of plant cell walls make biomass recalcitrant, *i.e.* resistant to hydrolysis by carbohydrases (Somerville *et al.*, 2004; Himmel *et al.*, 2007). Consequently, several pretreatment methods have been developed to loosen the structure of lignocellulose and to overcome its natural resistance to biological degradation (Mosier *et al.*, 2005). However, pretreatment methods at high temperatures are also known to lead to the formation of inhibitory compounds, which hinder both enzymatic hydrolysis and fermentation of sugars to ethanol (Palmqvist and Hahn-Hägerdal, 2000). In parallel, several attempts have been taken to identify the limitations of enzymatic hydrolysis which are related to the recalcitrance of lignocellulosic substrates (Gregg and Saddler, 1996; Zhu *et al.*, 2008). Some of these structurally limiting factors are addressed below.

1.4.1.1 Cellulose crystallinity and available surface area

It is widely accepted that highly organised crystalline cellulose is less accessible to cellulases than the amorphous regions are, and the crystallinity clearly hinders the enzymatic hydrolysis (Zhu *et al.*, 2008; Hall *et al.*, 2010). Hence, the major obstacles in the enzymatic hydrolysis of lignocellulose into sugars are partly attributed to the highly organised crystalline structure and partly the limited accessibility of cellulose (Nishiyama *et al.*, 2002; Hall *et al.*, 2010). Loosening the crystalline structure by fibrillating cellulose decreases the crystallinity and enhances the accessibility, and eventually leads to faster enzymatic hydrolysis (Ahola *et al.*, 2008). In particular, better hydrolysability might be a consequence of the increased surface area rather than a consequence of the reduced amount of crystalline units in the substrate. Although amorphous cellulose is generally considered less resistant to enzymatic hydrolysis, in most cases no increase in crystallinity has been observed during hydrolysis (Hall *et al.*, 2010). This suggests that crystalline and amorphous regions in cellulose are being attacked simultaneously and no accumulation of recalcitrant crystalline cellulose takes place. In addition, the length and width of cellulose crystals remain fairly constant during hydrolysis, suggesting that the crystals are hydrolysed sequentially (Ramos *et al.*, 1993).

The removal of amorphous components (lignin and hemicellulose) during pretreatment increases cellulose accessibility and concomitantly leads to a higher proportion of ordered cellulose within the biomass (Kim and Holtzapple, 2006). Because of this, contradictory effects of the crystallinity on enzymatic hydrolysability have been observed. In general, several parameters (such as particle size and lignin and hemicellulose content and distribution) influence the overall hydrolysa-

bility, and since the effects of these parameters are interrelated, it is difficult to study them separately (Zhu *et al.*, 2008).

1.4.1.2 Hemicellulose and lignin (matrices) as physical barriers

As discussed earlier, hemicellulose and lignin are in close physical and chemical association with cellulose via hydrogen bonds and covalent linkages. The highly ordered structure of cellulose as well as the complexity and heterogeneity of plant cell walls make biomass recalcitrant, *i.e.* resistant to enzymatic hydrolysis (Somerville *et al.*, 2004; Himmel *et al.*, 2007). Additionally, the densely packed three-dimensional structure with small average pore size hinders the penetration of enzymes into the lignocellulose matrix, restricting the surface area available for enzymatic action (Carpita, 1982). Hence, the hemicellulose and lignin matrix surrounding cellulose fibres needs to be at least partially broken down or loosened to allow the penetration of enzymes. Physicochemical treatment of biomass prior to enzymatic hydrolysis increases the exposure of polysaccharides and helps overcome its natural resistance to biological degradation (Mosier *et al.*, 2005).

In addition to lignin, hemicellulose may also prevent the hydrolysis of cellulose in the absence of auxiliary enzymes (Berlin *et al.*, 2005a). Hemicelluloses, *i.e.* xylans and glucomannans, are closely associated with cellulose bundles, and hence the simultaneous hydrolysis of cellulose and hemicelluloses seems essential for the efficient solubilisation of cellulose (Gübitz *et al.*, 1998; Somerville *et al.*, 2004).

1.4.1.3 Changes in substrate recalcitrance by various pretreatment methods

Efficient pretreatment is necessary to open up the structure of lignocellulose to allow enzymatic hydrolysis. The pretreatment of lignocellulose results in physical and chemical changes in the plant biomass: either hemicellulose or lignin is usually removed or modified (Mosier *et al.*, 2005; Kumar and Wyman, 2009). In particular, the role of pretreatment is typically to break down the macroscopic rigidity of biomass and to remove the physical barriers, *i.e.* lignin or hemicellulose (Mosier *et al.*, 2005; Himmel *et al.*, 2007). Therefore, various thermochemical pretreatments have been developed, as reviewed by, *e.g.*, Sun and Cheng (2002), aimed at lignin or hemicellulose removal (Sun and Cheng, 2002; Mosier *et al.*, 2005; Yang and Wyman, 2008).

The common pretreatment methods, such as steam explosion or especially hydrothermal treatments, produce substrates with improved enzymatic digestibility without extensive use of chemicals. These treatments result in deacetylation and partial removal of hemicelluloses. The extent of the hemicellulose removal largely depends on the severity of the pretreatment (Mosier *et al.*, 2005). During high-temperature pretreatments, the structure of lignin is altered and lignin is partially re-deposited on the surfaces of exploded cell wall fragments, hindering the enzymatic conversion of polysaccharides (Tanahashi *et al.*, 1983; Li *et al.*, 2007; Hansen *et al.*, 2011). In addition, pretreatment methods at high temperatures are

known to lead to liberation of soluble inhibitory compounds (Palmqvist and Hahn-Hägerdal, 2000). Weak acids such as acetic acid hydrolysed from hemicellulose, phenolic compounds generated by partial break-down of lignin, and furan derivatives from xylose oxidation, may hinder enzymatic hydrolysis and yeast fermentation. The inhibitory effects can, however, be removed or decreased by washing or by various detoxification methods.

To remove some of the lignin and to avoid undesired relocation, various chemicals such as alkalis, ammonia and ethanol have been exploited (Mosier *et al.*, 2005; Hendriks and Zeeman, 2009). The most wide-spread techniques are ammonia fibre expansion (AFEX) for herbaceous and agricultural residues, and alkaline extraction (kraft pulping) for woody materials (Kumar and Wyman, 2009). In both AFEX and alkaline pulping, the high pH causes fibre swelling and phase change in the crystalline structure of cellulose from I to III, making cellulose more accessible (Wyman *et al.*, 2005; Mosier *et al.*, 2005). Alkaline treatments may also result in solubilisation of hemicelluloses, which can lead to the relocation of hemicelluloses on cellulosic fibres, especially if the pH is decreased. However, while AFEX renders the substrate structure, it does not solubilise lignin and hemicellulose (Wyman *et al.*, 2005). In fact, a complete delignification is costly, and may not be necessary for an effective pretreatment. The structure of pretreated biomass is crucial for the subsequent hydrolysis, especially with regard to the accessibility and adsorption capacity of cellulases to the substrate (Wyman *et al.*, 2005). Optimal pretreatments need to be selected specifically for individual substrates due to large variations in structure and recalcitrance of various biomass types (Gregg and Saddler, 1996; Tengborg *et al.*, 2001).

1.4.2 Enzyme adsorption

Hydrolysis of cellulose is a heterogeneous reaction, in which the enzymes need to find and adsorb onto the insoluble substrates prior to successful catalysis. The adsorption of enzymes onto cellulose is a reversible process, and the equilibrium is usually described with Langmuir isotherms. The adsorption of enzymes is very quick, with the equilibrium of the adsorbed and desorbed enzymes being reached within the first 10–20 minutes of incubation irrespective of the temperature used (4 or 40–50°C), enzyme dosage or substrate composition (Nidetzky *et al.*, 1994; Medve *et al.*, 1998). On the other hand, the desorption of enzymes back into the hydrolysate liquid has been shown to be very slow due to strong adhesion forces between the enzymes and their substrates (Kurasin and Väljamäe, 2011).

Cellulases, in general, bind to their substrate mainly via the CBMs, but also bind with the catalytic site during the catalytic action (Jalak and Väljamäe, 2010). Since CBMs increase the probability of enzyme-substrate interaction, they have been shown to be essential appendices of cellulases for the efficient hydrolysis of insoluble substrates in aqueous systems (van Tilbeurgh *et al.*, 1986; Tomme *et al.*, 1988; Ståhlberg *et al.*, 1988; Reinikainen *et al.*, 1992). Although CBMs efficiently direct enzymes to organised carbohydrate structures such as crystalline cellulose,

they are also responsible for unproductive binding on lignin and other obstacles present in (pretreated) lignocelluloses, which cause a significant loss of enzymes available for hydrolysis (Palonen *et al.*, 2004; Kurasin and Väljamäe, 2011). Adsorption on lignin may be irreversible, and eventually leads to a loss of enzyme activity during hydrolysis (Palonen *et al.*, 2004; Rahikainen *et al.*, 2011).

Hydrolytic systems are dynamic systems, as the substrate is solubilised continuously during hydrolysis, leading to a gradually changing equilibrium of the adsorbed and free enzymes. In theory, two main phenomena take place during hydrolysis: a quick initial adsorption and a slow desorption-readsorption of the enzyme components (Nidetzky and Claeyssens, 1994; Boussaid and Saddler, 1999; Lu *et al.*, 2002; Tu *et al.*, 2007). On lignocellulosic substrates, however, the desorption of enzymes can seldom be observed due to irreversible adsorption and denaturing of the enzyme proteins on lignin (Rahikainen *et al.*, 2011). In addition, stacking of enzymes on obstacles in cellulose has been observed (Kurasin and Väljamäe, 2011; Igarashi *et al.*, 2011). On pretreated softwood with relatively high lignin content, the adsorption of cellulases has been found to increase continuously after a quick initial adsorption (Boussaid and Saddler, 1999; Lu *et al.*, 2002). Removal of lignin has been shown to result in decreased enzyme adsorption (Boussaid and Saddler, 1999; Lu *et al.*, 2002) and, in some cases, to lead to continuous desorption of enzyme proteins on delignified substrates (Boussaid and Saddler, 1999; Tu *et al.*, 2007).

The practical aim in the enzymatic conversion of lignocellulose to sugars has been to enhance enzyme desorption at the end of the hydrolysis for recovering and recycling the enzymes. Besides lignin removal with advanced pretreatment methods (Yang and Wyman, 2008), the application of surfactants is a commonly used technique to enhance enzyme efficiency and recovery by preventing unproductive binding of enzymes during hydrolysis (Ooshima *et al.,* 1986). In addition, the collection or engineering of high mobility enzymes with low affinity to lignin and high desorption constants has also been proposed (Berlin *et al.*, 2005b; Kurasin and Väljamäe, 2011). A low affinity to lignin could lead to a reduction in unproductive binding to lignin, which would lead to an enhancement in efficiency due to the increased ratio of enzymes available for hydrolysis; high desorption constants (*i.e.* faster desorption) increase the mobility of enzymes by accelerating the release of unproductively bound enzymes, also facilitating their recovery. Yet, obtaining a better understanding of enzyme adsorption-desorption during hydrolysis is necessary to potentially diminish the enzyme-related costs of biomass conversion to platform sugars by recovering and recycling enzymes (Margeot *et al.*, 2009).

1.4.3 Hydrolysis at high solid loading

On an industrial scale, high solid loadings are necessary in the conversion of biomass to ethanol or other products for economic and ecological reasons. The industrial-scale saccharification of biomass is currently uneconomic at substrate loadings below 15% (w/V); up to 30–40% concentrations are aimed at in order to

obtain a feasible end-product concentration (Wingren *et al.*, 2003; Jørgensen *et al.*, 2007). Reaching high sugar, and consequently high ethanol, concentrations of above 4% is a prerequisite for reducing energy consumption in the subsequent distillation. In addition, high solid loadings associated with lower water consumption result in reduced capital and operational costs due to the smaller equipment size needed (Wingren *et al.*, 2003). Additionally, reducing water consumption leads to improved sustainability by minimising the use of fresh water and the formation of waste water.

Pretreated raw materials such as wheat straw at solid loadings above 25% are highly viscous and have practically no water which can be separated from the solids in the initial phase of hydrolysis. For such materials, during the initial phase of hydrolysis the substrate is liquefied and the viscosity drops significantly (Szijártó *et al.*, 2011). Even so, the high thickness (*i.e.* viscosity) of this material causes challenges in mixing and consequently induces limitations on mass transfer. Horizontal gravity mixing has been developed to replace vertical mixing systems used in traditional low-consistency hydrolysis in order to increase mixing efficiencies and to partially overcome diffusion limitations occurring at high solid loadings (Jørgensen *et al.*, 2007).

1.4.4 End-product inhibition

As the hydrolysis progresses, the hydrolysis products accumulate, causing severe end-product inhibition of cellulases and resulting in a decreased rate of hydrolysis, especially at higher substrate consistencies (Holtzapple *et al.*, 1990; Xiao *et al.*, 2004; Jørgensen *et al.*, 2007; Kristensen *et al.*, 2009). Consequently, the hydrolysis slows down and the hydrolysis yields are decreased. Cellobiohydrolases are known to be inhibited by cellobiose, their hydrolysis product (Holtzapple *et al.*, 1990; Gruno *et al.*, 2004). This inibition is alleviated by supplementing cellulases with β-glucosidase cleaving cellobiose into glucose monomers. However, like cellobiose, glucose also exerts end-product inhibition on β-glucosidases (Xiao *et al.*, 2004). In addition, other sugars such as xylose, mannose and galactose have also been shown to have inhibitory effect on cellulase activity (Xiao *et al.*, 2004).

In order to overcome end-product inhibition caused by glucose and other hydrolysis products, the simultaneous fermentation of sugars to ethanol is applied (Gauss *et al.*, 1976). In contrast to separate hydrolysis and fermentation (commonly abbreviated as SHF), during simultaneous saccharification and fermentation (commonly abbreviated as SSF) the produced glucose (and xylose) can be subsequently taken up by the fermenting organism and transformed partially to alcohol, alleviating the inhibition retarding hydrolysis. On the other hand, the hydrolysis rate is decreased at the same time due to the lower temperature optimum of the fermenting organisms as compared with the hydrolytic enzymes. Separate and simultaneous fermentation methods have been compared, but the difference between their efficiency depends on the process parameters and enzyme preparations used (Wingren *et al.*, 2003).

2. Aims of the present study

The aim of this work is to investigate factors that limit the efficiency of the enzymatic saccharification of biomass, and consequently to search for solutions which could be relevant for solving these limitations and improving the biomass conversion process. This work investigates ideas with the potential to elevate hydrolysis yields and to enhance recovery of hydrolytic enzymes. In addition, enzyme inactivation and various rate limitations such as decreases in substrate reactivity and inaccessibility caused by lignin are also addressed with regard to the slow-down of saccharification.

Specifically, the aims of this thesis are:

- To observe changes in the composition and structure of the residual substrate during hydrolysis, especially focusing on particle size and cellulose crystallinity.
- To explore co-operativity of cellulases and auxiliary enzymes and to identify the enzyme components most useful for improving the total hydrolysis of lignocellulose.
- To study the desorption of individual enzyme components during hydrolysis of lignocellulosic materials with different lignin content.
- To investigate hydrolysis conditions and enzymes which facilitate efficient enzyme recovery, especially under industrial process conditions (such as at high substrate concentrations).

3. Materials and methods

This chapter describes the materials and methods used in this work. More detailed information can be found in the original Papers (I–V).

3.1 Enzymes

3.1.1 The preparation of enzymes

Commercial cellulase preparations, Celluclast 1.5 L and Novozym 188, were purchased from Novozymes, Denmark. In addition, individual types of purified cellulases and hemicellulases were used. Four types of *Trichoderma reesei* cellulases and their catalytic domains were purified according to Suurnäkki *et al.* (2000), namely cellobiohydrolases Cel7A (CBH I, EC 3.2.1.176) and Cel6A (CBH II, EC 3.2.1.91) and endoglucanases Cel7B (EG I, EC 3.2.1.4) and Cel5A (EG II, EC 3.2.1.4). *Aspergillus niger* β-glucosidase (Cel3A, EC 3.2.1.21) was purified from Novozym 188 according to Sipos *et al.* (Sipos *et al.*, 2009). *T. reesei* xylanase II (Xyn11A, EC 3.2.1.8) and mannanase (Man5A, EC.3.2.1.78) were purified according to Tenkanen *et al.* (Tenkanen *et al.*, 1992) and Stålbrand *et al.* (Stålbrand *et al.*, 1993) respectively.

3.1.2 Characterisation of the enzyme preparations

The protein content of the enzyme preparations was measured using the DC (detergent compatible) Protein Assay from Bio-Rad based on the method of Lowry (Lowry *et al.*, 1951). The molar mass of the purified enzyme proteins was identified by MALDI-ToF using a sinapic acid matrix with trifluoroacetic acid as the protonating agent. The purity of the purified enzyme preparations was confirmed by specific activity measurements and also by gel electrophoresis (SDS-PAGE), as described in Papers III and V.

The substrate specific activities of the commercial and purified enzyme preparations were measured. The substrates used were as follows: 4-methylumbelliferyl-β-D-lactoside (MUL) for the reducing end cellobiohydrolase activity and endoglucanase type I activity (van Tilbeurgh *et al.*, 1988), 4-methylumbelliferyl-β-D-

cellotrioside (MUG3) for the endoglucanase type II activity (Macarrón *et al.*, 1993), birch wood xylan for the xylanase activity (Bailey *et al.*, 1991), *p*-nitrophenyl-β-glucopyranoside (*p*NPG) for the β-glucosidase activity (Bailey and Nevalainen, 1981), and filter paper for the total cellulase activity, quantified using filter paper activity units (FPU) (IUPAC method, 1987). In order to measure the activity of individual enzymes in mixture, the interfering activity of other enzyme components present in the mixture was excluded. In the MUL and MUG3 assays, glucose was added at 100 mM concentration to suppress β-glucosidase activity; in the MUL assay, 5 mM cellobiose was used to inhibit cellobiohydrolase activity, which allowed the distinction to be made between reducing end cellobiohydrolase activity and endoglucanase type I activity (van Tilbeurgh *et al.*, 1988; Macarrón *et al.*, 1993). Cellobiohydrolase II has been shown to be active towards MUG3 and hence could interfere with the endoglucanase type II activity in the MUG3 assay (van Tilbeurgh et al., 1985), which should be considered when interpreting the results.

3.2 Lignocellulosic substrates

In the experiments, lignocellulosic substrates with high and low lignin content were used. The lignaceous substrates used were steam-pretreated washed spruce (SPS) from Lund University, Sweden (prepared as described in Paper II) and hydrothermally pretreated washed wheat straw from Inbicon, Denmark. The delignified substrates were as follows: catalytically oxidised spruce (COS, prepared as described in Paper III), elemental chlorine free bleached softwood chemical pulp (SCP) from UPM-Kymmene Oy, Pietarsaari, and nanofibrillated cellulose (NFC, produced from SCP as described in Paper IV). In addition, microcrystalline cellulose (Avicel from SERVA) was used as a model cellulosic substrates.

In Paper II, the substrates were obtained from SPS by first hydrolysing the SPS with a commercial enzyme mixture composed of Celluclast at 15 FPU/g dry weight and Novozym 188 at 1000 nkat/g dry weight loading for 2 days at 5% consistency at 45°C in 50 mM Na-acetate buffer (pH adjusted to 5.0) to obtain an extensively hydrolysed residue, SPS-RES. In the second step, both the SPS and SPS-RES were delignified using chlorite delignification based on the holocellulose preparation method by Yokoyama *et al.* (2002), as described in detail in Paper II.

3.2.1 Compositional analysis of lignocellulosic substrates

The substrate composition, in particular the carbohydrate composition, the lignin and extractive contents and the ash content, was determined according to standard procedures by NREL, USA (NREL Laboratory Analytical Procedures) with slight modifications as described in Paper II, and is summarised in Table 1.

3.2.2 Structural and morphological analysis of lignocellulosic substrates

The overall crystallinity and the crystal width were calculated from wide-angle X-ray scattering experiments (Paper I). The particle size distribution in the range of 0.04–2000 µm was measured using laser diffraction (Paper II). The pore size distribution was analysed with small-angle X-ray scattering (Paper I). The morphology was also evaluated with microscopic techniques, in particular with X-ray microtomography and transmission electron microscopy (TEM) (Paper I). A detailed description of the methods can be found in the original Papers I and II.

3.3 Enzymatic hydrolysis

3.3.1 Hydrolysis conditions

Enzymatic hydrolysis of each substrate was either carried out at 45°C (Papers I, II, IV and V) or 35°C (Paper III), at pH 5.0 in a 50 mM Na-citrate (Papers I, III–V) or Na-acetate (Paper II) buffer for various durations. Hydrolysis aliquots were mixed with magnetic stirrers at substrate loadings of 1%, 2% and 10% (w/w) when the total volume was below 5 ml, and were shaken above 5 ml (Papers I–V). The high-consistency (20% w/w) experiments were carried out using gravimetric mixing as described in Paper V. The hydrolysis experiments were performed using commercial or purified enzyme mixtures at a loading ranging from 5mg to 25mg protein/g d.w. (Papers I–V). The ratio of purified cellulases was set based on the ratio of enzyme components in the *T. reesei* culture broth (Gritzali and Brown, 1979).

After the hydrolysis, the samples were boiled for 10 min immediately prior to centrifugation when only the solubilised sugars were analysed. When the free enzymes were also analysed, the samples were chilled on ice, centrifuged, and then only a part of the recovered hydrolysate was boiled for 10 min in order to measure the solubilised sugars.

Table 1. The composition of the substrates used in this work. The composition is expressed as % of total dry weight (d.w.).

Substrates*	Lignin and extractives (% of d.w.)	Glucose (% of d.w.)	Xylose (% of d.w.)	Mannose (% of d.w.)	Arabinose (% of d.w.)	Galactose (% of d.w.)	Ash (% of d.w.)
SPS	43.4	51.0	0.21	0.23	<0.10	<0.10	0.11
SPS-DL	4.3	93.3	0.32	0.23	<0.18	<0.19	0.15
SPS-RES	69.8	28.4	0.32	0.64	<0.16	<0.16	0.23
SPS-RES-DL	15.2	79.4	0.41	<0.21	<0.20	<0.21	0.50
Wheat straw	26.4	58.6	3.48	0.22	0.06	0.08	n.d.
COS	4.5	85.3	3.42	6.94	0.31	0.11	n.d.
SCP	<1.0	69.1	6.87	6.27	0.64	0.27	n.d.
NFC	<1.0	72.5	6.83	6.24	0.63	0.27	n.d.
Avicel	<1.0	91.3	1.23	1.45	<0.10	<0.10	n.d.

n.d. not determined

* Abbreviations: SPS: steam-pretreated washed spruce; SPS-DL: delignified SPS; SPS-RES: enzymatic hydrolysis residue of SPS; SPS-RES-DL: delignified SPS-RES; Wheat straw: hydrothermally pretreated washed wheat straw; COS: catalytically oxidised spruce, SCP: elemental chlorine free bleached softwood chemical pulp; NFC: nanofibrillated cellulose.

3.3.2 Analysis of the hydrolysates and residues

Various characteristics of the hydrolysates and the hydrolysis residues were measured (Table 2). The degree of hydrolysis was calculated either from the amount of total reducing sugars or from the amount of solubilised sugars present in the hydrolysates. The reducing sugars were measured with a dinitrosalicylic acid (DNS) reagent; and the solubilised sugars were separated and quantified using either gas chromatography (Paper II) or high performance liquid chromatography (Papers III–V). Oligomeric sugars were hydrolysed into monomers with acid hydrolysis and the overall solubilised sugars quantified in Paper IV.

The hydrolysis residues were collected and analysed in Papers I and II in a similar manner to the non-hydrolysed substrates described above in Chapter 3.2.2 and Table 2.

Table 2. Methods used to characterise the accessibility of substrates and the performance of enzymes.

Substrate or enzyme characteristics	Method	Paper
ACCESSIBILITY OF SUBSTRATES		
Particle size	X-ray microtomography	I
	Laser diffraction	II
Pore size	Small-angle X-ray scattering	I
Cellulose crystallinity	Wide-angle X-ray scattering	I
Lignin	Chlorite delignification	II
Hemicellulose	Hydrolysis with combinations of purified cellulases and hemicellulases	II, IV
PERFORMANCE OF ENZYMES		
Adsorption-desorption	SDS-PAGE; enzyme activity on chromophoric substrates	III, V
Synergism	Hydrolysis with combinations of purified cellulases and hemicellulases	IV
The role of CBMs	Hydrolysis with intact or core cellulases	V

The adsorption of individual enzymes during hydrolysis was determined by measuring the residual enzyme activity in the hydrolysates using specific chromophoric substrates listed in Chapter 3.1.2, and by separation of the enzymes with SDS-PAGE followed by the quantification of the intensity of individual bands (Papers III and V).

4. Results

Enzymatic hydrolysis of various lignocellulosic substrates was studied with enzyme mixtures composed of either commonly used commercial enzyme preparations or purified enzymes from the lignocellulolytic system of *Trichoderma reesei*. The enzyme dosages and hydrolysis conditions are explained in detail in Papers I-V and summarised in the previous chapter. The main emphasis was on spruce-derived raw materials, as softwoods represent one of the most recalcitrant raw materials of all potential lignocellulosic raw materials.

4.1 Structural and enzyme related factors restricting enzymatic hydrolysis

Enzymatic hydrolysis is recognised to slow down as the hydrolysis proceeds. In order to understand the factors restricting and decreasing hydrolysis rate as a function of the degree of hydrolysis, the structure and composition of solid substrates and their enzymatic residues were characterised and compared (Table 2). In addition, the adsorption and desorption of enzymes was followed during hydrolysis on various softwood substrates in order to study the limitations of the hydrolysis relating to enzyme-substrate interactions.

4.1.1 Substrate structure and composition (Papers I, II)

Various methods were used to analyse the changes occurring in the substrates studied (Avicel and SPS) during enzymatic hydrolysis (Table 2). Avicel was hydrolysed with commercial enzyme mixtures to various extents, with the solid residues collected and compared to the original substrate to observe changes taking place in the physical structure of the material during hydrolysis (Paper I). On the other hand, SPS was hydrolysed with commercial ezyme mixtures to obtain extensively hydrolysed residue (SPS-RES); and then both the SPS and SPS-RES were delignified as described in the Materials and methods chapter, resulting in SPS-DL and SPS-RES-DL (Chapter 3.2); all four materials were then subjected to enzymatic hydrolysis with various enzyme mixtures composed of commercial mixtures or purified enzymes to compare the differences in their hydrolysability (Paper II).

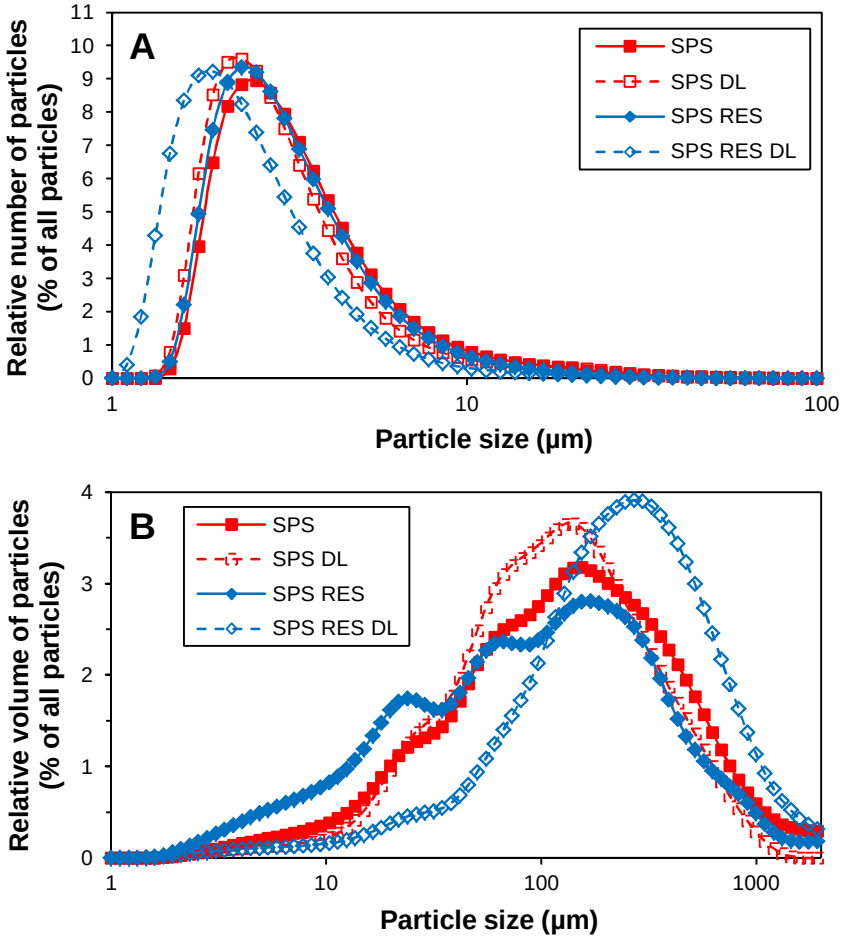

Figure 6. The (**A**) number- and (**B**) volume-based size distribution of particles in SPS, SPS-RES, SPS-DL and SPS-RES-DL (samples described in Paper II).

A gradual decrease in particle size during enzymatic hydrolysis of SPS and Avicel was detected (Paper II Table 2 and Paper I Figure 4; see also Figure 6 and Figure 7). The size distribution of particles in the SPS and SPS-RES was measured before and after delignification using laser diffraction. The results show that the hydrolysis (to form the residue SPS-RES) and the delignification of SPS (to form the SPS-DL) resulted in a general decrease in particle size (Figure 6 and Paper II Table 2). The number and volume based median diameter of the particles in the SPS decreased slightly from 3.0 to 2.9 µm and from 133 to 89 µm, respectively, when 64% of the total carbohydrate content (*i.e.* 38% of d.w.) in the SPS had been solubilised. In addition, delignification affected the particle size distribution of the SPS

and the residue SPS-RES differently, which indicates a clear difference in the structure. Delignification caused a more profound decrease in the median diameter of the particles in the residue (by 33% from 2.9 to 2.2 µm) than in the SPS (by 6.7% from 3.0 to 2.8 µm). At the same time, the volume-based median diameter decreased by 14% in the SPS (from 133 to 115 µm), whereas a 2.6-fold increase could be observed in the SPS-RES (from 89 to 235 µm). The decrease in the size of smaller particles in the SPS-RES presumably resulted in a relative increase in the mass of larger particles which were obviously more resistant towards enzymatic hydrolysis and which also retained their structure and size after lignin removal.

The changes in structure were evaluated microscopically during the hydrolysis of Avicel: X-ray microtomography revealed a general decrease in the particle size (Figure 7) while transition electron microscopy (TEM) demonstrated fine structural changes (Paper I Figure 5). Elongated and partially ramified structures (fibre bundles) with varying thickness can be observed in the TEM images, revealing that the enzymatic hydrolysis of Avicel caused both the enlarging of pores between individual microfibrils and the ramification of microfibril bundles. At the same time, almost intact particles were visible in the residual substrate even after reaching a hydrolysis yield of 67% (of the theoretical maximum yield, abbreviated as t.m.y.) (Paper I Figures 4 and 5), indicating the presence of larger particles and fibre bundles with smaller pores, obviously inaccessible to the hydrolytic enzymes.

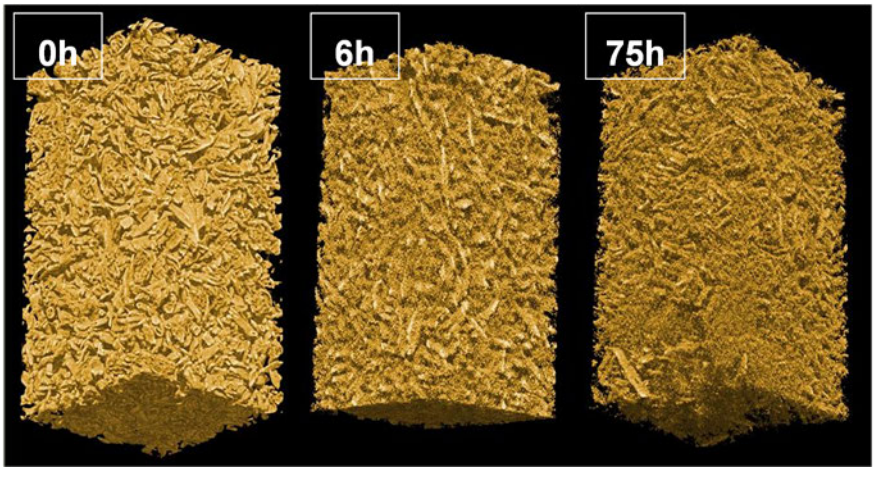

Figure 7. Changes in particle size during the hydrolysis of Avicel. Images by X-ray microtomography, the longer edge of the three-dimensional cuts is 1 µm.

At the nanoscale, however, no significant changes in the crystalline structure could be observed in Avicel. The crystallinity of cellulose remained unchanged even when reaching a 67% conversion (of t.m.y.) in the hydrolysis of Avicel with commercial enzyme mixtures (Paper I Table 1), implying amorphous regions of cellulose are not preferentially hydrolysed. In addition, the average crystal sizes were

found to be constant, indicating that cellulose crystals might have been digested sequentially due to the limited accessibility.

The analysis of the chemical composition of spruce samples, on the other hand, revealed structural changes during hydrolysis. The residual hemicelluloses remaining in spruce after steam pretreatment were inaccessible to the hydrolytic enzymes present in Celluclast: not only lignin but hemicelluloses accumulated in the SPS-RES (Paper II Tables 2 and 3). Whilst the amount of lignin and extractives increased from 43% to 70% of d.w., the mannose and xylose content increased from both being below 0.4% to respectively 2.2 and 1.1% of total polysaccharides. Most hemicelluloses, remaining in the SPS-RES after enzymatic hydrolysis, especially glucomannan, could, however, be removed along with lignin during delignification and hence seem to be associated with lignin as lignin-carbohydrate complexes.

4.1.2 Lignin as a physical barrier to the accessibility of polysaccharides (Paper II)

The hydrolysis of SPS and its hydrolysis residue (SPS-RES) were compared in order to reveal structural changes affecting hydrolysability in the residual substrate. Despite the accumulation of lignin, the SPS and SPS-RES could be hydrolysed to approximately the same degree with the commercial enzyme mixture (5 FPU) in 48 hours, yielding up to 34% and 30% of t.m.y. respectively (Figure 8.A and Paper II). On the other hand, the difference in the hydrolysability of SPS and SPS-RES was more noticeable with the purified enzyme mixtures, which lacked the auxiliary enzymes present in the commercial preparations and required for the total hydrolysis of lignocellulose. The most complete purified enzyme mixture resulted in hydrolysis degrees of 37% and 29% in the SPS and SPS-RES respectively (Figure 8.B and Paper II), indicating accumulation of polysaccharides more resistant to hydrolysis by the basic cellulases and xylanase TrXyn11A present in the purified enzyme mixtures.

Softwood lignin impeded the action of cellulases profoundly in the SPS and its enzymatic residue SPS-RES (Figure 8 and Paper II). After delignifying the substrates (producing SPS-DL and SPS-RES-DL respectively), the hydrolysis yield doubled irrespective of the enzyme mixtures used. After removing 93% of the lignin from SPS, the hydrolysis yield obtained with the commercial enzyme mixture (5 FPU) increased from only 32% (in the SPS) to 74% (in the SPS-DL) of total polysaccharides (Figure 8 and Paper II Table 2 and Figures 2 and 4). With the same enzyme mixture, the residue (SPS-RES) reached 29% conversion of t.m.y., while 73% of the remaining carbohydrates could be solubilised in the SPS-RES-DL after removing 92% of the lignin.

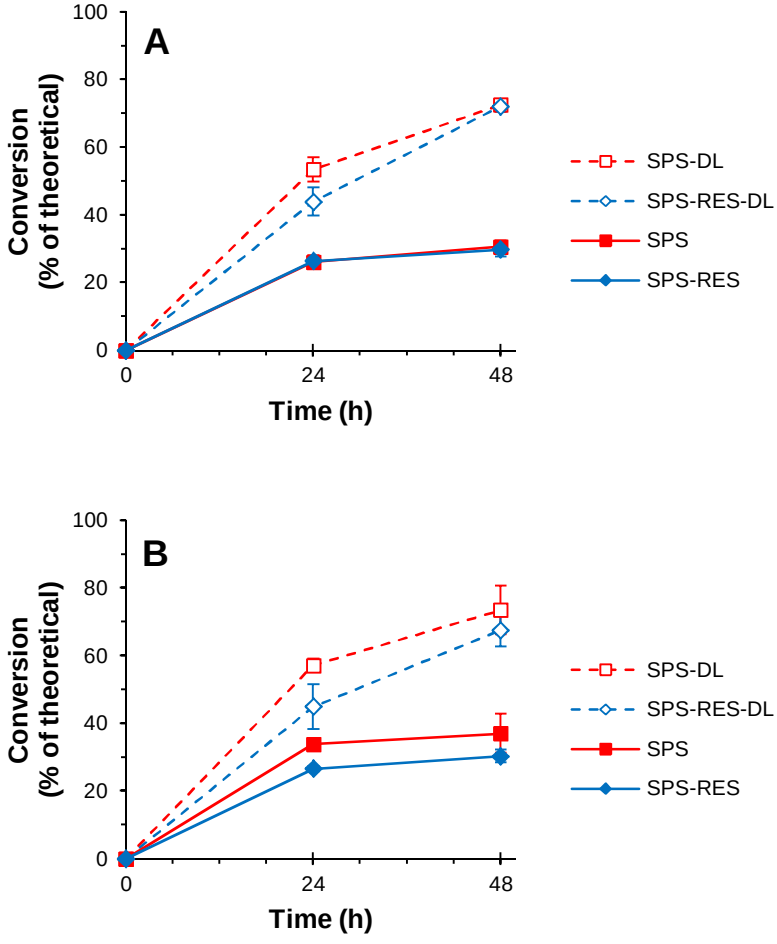

Figure 8. Hydrolysis of steam pretreated spruce (SPS), delignified SPS (SPS-DL), enzymatic residue of SPS (SPS-RES) and delignified SPS-RES (SPS-RES-DL) with (**A**) the commercial enzyme mixture (Celluclast at 5 FPU/g cellulose supplemented with Novozym 188 at 100 nkat/g cellulose); and (**B**) the most complete purified enzyme mixture (at a total protein loading of 18 mg/g cellulose composed of 58.4% TrCel7A, 19.5% TrCel6A, 19.5% TrCel5A and 2.7% TrXyn11A supplemented with β-glucosidase at 100 nkat/g cellulose). The hydrolysis was carried out at 45°C in 50 mM Na-acetate buffer (pH 5.0) at 1% cellulose concentration. The data are based on reducing sugars; error bars represent the standard deviation of the mean, with a sample size of 3.

Lignin played a major role in impeding the hydrolysis of polysaccharides in the SPS and SPS-RES. Hence, the hydrolysability of the delignified substrates (SPS-DL and SPS-RES-DL) with commercial and purified enzyme mixtures was com-

pared to reveal fine differences in the carbohydrate structure. In contrast to the hydrolysis of lignaceous substrates, the results reveal no major differences between the hydrolysability of polysaccharide content remaining after lignin removal in the SPS-DL and SPS-RES-DL. Although the hydrolysis yield was somewhat higher in the SPS-DL than in the SPS-RES-DL after 24 h (51 and 43% respectively with the commercial (5 FPU) enzyme mixture and 57 and 43% respectively with the most complete purified enzyme mixtures), the SPS-DL and SPS-RES-DL could be hydrolysed to the same extent at the end of the hydrolysis (74 and 73% respectively with the commercial (5 FPU) enzyme mixture and 70 and 68% respectively with the most complete purified enzyme mixtures, both after 48 h).

In accordance with the results obtained from the compositional analysis, the hydrolysis experiments using versatile enzyme mixtures revealed that the residual hemicellulose in the lignin-rich SPS and SPS-RES was less accessible to enzymes than after delignification in the SPS-DL and SPS-RES-DL. The ratio of the solubilised sugars by the commercial enzyme mixtures corresponded to the ratio in the average composition of the delignified substrates, whereas relatively less mannose and xylose was released from the lignaceous substrates (SPS and SPS-RES) as compared to the ratio of sugars in their substrate composition (Paper II Tables 3 and 7). Thus, the hemicelluloses resistant to enzymatic hydrolysis seem to be removed along with lignin during delignification.

4.1.3 Enzyme adsorption during hydrolysis (Papers III, V)

In addition to changes in the physical structure of the substrate during hydrolysis, the interaction between the enzymes and the substrate was investigated using substrates bearing different structural limitations. Steam pretreated spruce (SPS, lignaceous substrate), Avicel (microcrystalline cellulose) and catalytically oxidised spruce (COS, alkaline delignified softwood) were as substrates on which to compare the adsorption and desorption patterns of the hydrolytic enzymes. In addition, the adsorption of mixtures of purified enzymes containing or lacking CBMs was studied on hydrothermally pretreated wheat straw and Avicel at various substrate concentrations.

Similarly to previous observations, a quick initial enzyme adsorption took place during the initial phase of hydrolysis for each substrate. All enzymes were bound to a high extent, the major enzyme component, TrCel7A, being adsorbed to 95%, 99% and 95%, on the SPS, COS and Avicel respectively after 6h at 1% (w/w) substrate concentration (Paper III Figure 2). The adsorption profiles of individual enzymes varied with the type of substrate. For the delignified substrates COS and Avicel, the adsorption profiles of TrCel7A and TrCel5A were similar, while TrCel6A remained free in the hydrolysate to a higher extent than TrCel7A and TrCel5A (Paper III Tables 3 and 4). In the SPS containing 30% lignin, TrCel7A, TrCel6A and TrCel5A all adsorbed to a high degree (Paper III Table 2); in pretreated wheat straw TrCel7B was the least bound, followed by TrCel7A, TrCel5A and TrCel6A (Paper V Supplementary Table 2). The degree of adsorption, in general, increased

with the substrate concentration without influencing the ratio of individual enzymes (Paper V Supplementary Table 2).

After the initial adsorption, most enzymes remained bound in spite of the high degree of conversion (79% and 96% of cellulose hydrolysed, respectively) obtained on the SPS and Avicel (Paper III). Similarly, no desorption of cellulases was observed from hydrothermally pretreated wheat straw or from Avicel irrespective of the substrate concentration (Paper V). However, noticeable enzyme desorption was detected in the alkaline delignified spruce (COS) after solubilising 65% of the total carbohydrates (Paper III). At the end of the hydrolysis (88% of total carbohydrates hydrolysed), 16% of the loaded TrCel7A and 15% of TrCel5A could be recovered free in the supernatant.

On the other hand, a constant loss in activity of β-glucosidase occurred during the hydrolysis of all substrates (Paper III Figure 2). The measured β-glucosidase activity gradually decreased to 26%, 47% and 71% of the total by the end of the hydrolysis of Avicel, SPS and COS respectively. Enzyme inactivation was observed especially in the case of microcrystalline cellulose, Avicel (Papers III and V), where no stabilising substances were added or dissolved from the substrate to preserve the enzymes from denaturing. Incubation of purified enzyme mixtures at 45°C with magnetic mixing and without a polymeric substrate led to the complete loss of activity of TrCel7A (both intact and core) after two days, while wheat straw extract stabilised the enzymes (Paper V Supplementary Figure 1). Accordingly, the concentration of free TrCel7A core proteins decreased below 20% during the hydrolysis of Avicel after 48 h, presumably due to enzyme inactivation followed by precipitation (Paper V Figure 3.B). Enzyme inactivation could also be observed during the hydrolysis of Avicel at the lower temperature of 35°C: the activity of β-glucosidase decreased continuously throughout the hydrolysis to a residual activity of 26% (of the original activity) after 144 h (Paper III Figure 2.D).

4.2 Enzymes for efficient lignocellulose conversion

Substrate specificity and domain structure are important enzymatic properties affecting the efficiency of polysaccharide hydrolysing enzymes. To identify enzymes that are required for the efficient hydrolysis of softwood derived materials, cellulose and hemicelluloses were hydrolysed separately and together using combinations of purified enzymes considering the substrate specificity of cellulases. In addition, the ability of cellulases to hydrolyse lignocellulose was compared when using enzymes with or without CBMs.

4.2.1 Auxiliary enzymes (Papers II, IV)

Combinations of purified enzymes were exploited in order to analyse the structural interactions and limitations of various carbohydrate components in the hydrolysis in detail (Papers II and IV). In particular, the baseline cellulase mixture was composed of a minimal set of cellulases required for efficient hydrolysis: the cellobio-

hydrolases TrCel7A and TrCel6A, the endoglucanase TrCel5A and the β-glucosidase AnCel3A. The endoglucanase TrCel7B was omitted from the cellulase mixture because of its xylanolytic activity to avoid the concomitant solubilisation of xylan. Even so, the endoglucanase TrCel7B and the endo-β-xylanase TrXyn11A were used in this study to solubilise xylan while the endo-β-mannanase TrMan5A was included to solubilise glucomannan. The ratio of cellulases followed that of *T. reesei* culture broth (see Chapter 3.3). The xylanolytic endoglucanase TrCel7B was included in the cellulase mixture by replacing 50% of the TrCel5A to keep the endoglucanase level constant on a protein base. Endoxylanase and endomannanase were added to the cellulase mixture without altering its composition, leading to higher protein loading in the combined mixtures.

For the xylan containing substrates, cellulose was only partially degraded in the absence of hemicellulolytic enzymes, while supplementing xylanolytic or mannanolytic enzymes enhanced the solubilisation of cellulose (Papers II and IV). In the case of SPS containing very low amount of xylan (<0.5% of d.w.), the increase in the solubilisation of cellulose by the addition of xylanase TrXyn11A was almost unnoticeable (Paper II Figure 1.A and Paper IV Figure 3.D). However, in other substrates with a xylan content exceeding 1% of d.w., the positive effect of the xylanolytic enzymes on cellulose solubilisation was observed. Even in Avicel containing 1.2% of d.w. xylan, the hydrolysis of cellulose after 48 hours increased by nearly a third with the addition of TrXyn11A, from 44% to 58% of cellulose hydrolysed (Paper II Table 6).

Similarly, the endoglucanase TrCel7B, which is active towards xylan, improved the hydrolysis of cellulose when included in the cellulase mixture already containing the endoglucanase TrCel5A (Paper IV). The addition of TrCel7B increased the cellulose yield in Avicel after 96 hours by 39% (from 55% to 76% of t.m.y.) (Paper IV Tables 1 and 5). Analogously, after 48 hours of hydrolysis of COS, containing 3.4% of d.w. xylan, a 34% increase in the hydrolysis yield of cellulose (from 46% to 62% of t.m.y.) was obtained by including TrCel7B (Paper IV Tables 1 and 6). Further enhancement of cellulose hydrolysis by adding xylanase could be observed only in substrates with a xylan content above 5% of d.w., *i.e.* in nanofibrillated cellulose (NFC) and softwood chemical pulp (SCP) (Paper IV Figures 2 and 3). Addition of endomannanase resulted in a further increase of 16–35% in cellulose solubilisation (Paper IV Tables 2–6). In the case of SPS containing 0.23% of d.w. mannan, the cellulose yield increased by only 4%. In fact, the solubilisation of cellulose was found to have a linear correlation with the release of xylan and glucomannan (Paper IV Figure 4).

4.2.2 Synergism between cellulases and auxiliary enzymes (Paper IV)

Synergism between cellulases, endoxylanase and endomannanase was observed and, in general, resulted in increased conversion yields in SCP and NFC (Tables 3 and 4, and Paper IV). Endoxylanase and endomannanase (X and M) both contributed to a more extensive solubilisation of the other hemicellulose. The amount of

solubilised xylose in SCP increased from 25% to 33% of t.m.y. and that of solubilised mannose from 35% to 44% of t.m.y. as a result of the combined action of the enzymes (Table 3). Similarly, in NFC the solubilisation of xylose and mannose both increased synergistically from 39% to 44% of t.m.y. when the major hemicellulases, endoxylanase and endomannanase, acted together (Table 4).

Cellulases and xylanase (C and X) were also found to act synergistically. Endoxylanase, which alone did not release any glucose, increased the glucose yield from 40% to 76% of t.m.y. in NFC, and from 41% to 66% in SCP when combined with cellulases (Tables 3 and 4). At the same time, cellulases, although releasing alone only 10% and 13% of t.m.y. of xylan from NFC and SCP respectively, increased the solubilisation of xylans from 39% to 73% of t.m.y. of xylan in NFC and from 25% to 66% of t.m.y. of xylan in SCP when added together with endoxylanase (Tables 3 and 4).

Table 3. Solubilisation of polysaccharides from softwood chemical pulp (SCP) with combinations of cellulases, endoxylanase and endomannanase. The hydrolysis parameters are the same as in Paper IV. The values indicate total solubilised sugars, including mono- and oligomers.

Enzyme mix	Glucose (% of total)		Xylose (% of total)		Mannose (% of total)	
	0.5 h	12 h	0.5 h	12 h	0.5 h	12 h
X	n.d.	0.0	n.d.	25.2	n.d.	0.0
M	n.d.	0.8	n.d.	0.0	n.d.	34.5
C	11.7	41.0	1.0	12.9	5.6	31.6
XM	n.d.	1.1	n.d.	32.7	n.d.	43.6
CX	17.7	65.9	29.6	66.2	6.1	52.2
CM	10.6	43.6	1.2	13.9	20.4	62.4
CXM	22.4	104.1	32.3	88.5	32.7	97.8

n.d. not determined

C: Cellulase mixture, containing 0.150 µmol TrCel7A, 0.50 µmol TrCel6A, 0.50 µmol TrCel5A and 0.002 µmol AnCel3A per g dry weight

X: xylanase, 0.025 µmol/g d.w. TrXyn11A

M: mannanase, 0.010 µmol/g d.w. TrMan5A

Table 4. Solubilisation of polysaccharides from nanofibrillated cellulose (NFC) with combinations of cellulases, endoxylanase and endomannanase. The hydrolysis parameters are the same as in Paper IV. The values indicate total solubilised sugars, including mono- and oligomers.

Enzyme mix	Glucose (% of total)		Xylose (% of total)		Mannose (% of total)	
	0.5 h	12 h	0.5 h	12 h	0.5 h	12 h
X	n.d.	0.0	n.d.	39.3	n.d.	0.0
M	n.d.	0.9	n.d.	1.1	n.d.	38.8
C	15.3	39.6	1.2	10.0	6.8	36.2
XM	n.d.	1.1	n.d.	44.2	n.d.	43.7
CX	19.5	75.8	40.9	73.4	8.3	58.0
CM	15.4	45.3	1.3	17.2	31.1	58.5
CXM	23.4	97.3	43.2	84.3	39.6	94.2

n.d. not determined

C: Cellulase mixture, containing 0.150 µmol TrCel7A, 0.50 µmol TrCel6A, 0.50 µmol TrCel5A and 0.002 µmol AnCel3A per g dry weight

X: xylanase, 0.025 µmol/g d.w. TrXyn11A

M: mannanase, 0.010 µmol/g d.w. TrMan5A

Similarly to endoxylanase, supplementing endomannanase to the cellulase mixture (C, M and CM in Tables 3 and 4) synergistically improves the hydrolysis of cellulose in NFC and SCP. Endomannanase released 1% of t.m.y. of glucose in NFC and SCP, obviously resulting from the solubilisation of glucomannan. Although endomannanase releases glucose from glucomannan, the increase in glucose yield is clearly higher than the glucose release calculated from the yield of solubilised mannose. Due to supplementing endomannanase, the glucose yield increased from 40% to 45% in the case of NFC and from 41% to 44% of t.m.y. in the case of SCP. On the other hand, no synergism was detected in the mannose release between the endomannanase (M) and the cellulases (C) in the hydrolysis of NFC and SCP, as glucomannan was already extensively solubilised (36% and 32% of t.m.y. for NFC and SCP respectively) due to the versatile substrate specifity of the endoglucanase TrCel5A present in mix C (Tables 3 and 4). Adding endomannanase to the cellulase mixture (giving mix CM) improved the mannose release from 36% to 59% of t.m.y. in NFC and from 32% to 62% of t.m.y. in SCP. This increase, however, did not exceed the yield of mannose solubilised by endomannanase only, being 39% and 35% of t.m.y. respectively (Tables 3 and 4).

4.2.3 The role of carbohydrate binding modules in hydrolysis (Paper V)

The primary role of carbohydrate binding modules (CBMs) is to concentrate enzymes on the surface of cellulosic substrates. As observed earlier, without CBMs the performance of the studied cellulases, especially that of the CBHs, was impaired at 1% and 10% (w/w) substrate concentrations (Figure 9 and Paper V Figure 1). The excision of CBMs has a stronger impact on cellobiohydrolases than on endoglucanases. The overall hydrolysis yield at 1% concentration decreased from 93% to approximately 60% of d.w. when one of the cellobiohydrolases was substituted by only its core domain and to 27% of d.w. when both cellobiohydrolases lacked CBMs. At 10% (w/w) substrate concentration, the effect was reduced: the overall yield decreased from 69% to 59% of d.w. and to 41% of d.w. respectively when one or both cellobiohydrolases lacked CBMs. On the other hand, practically no decrease in the yield could be detected when endoglucanases were replaced by the corresponding core enzymes.

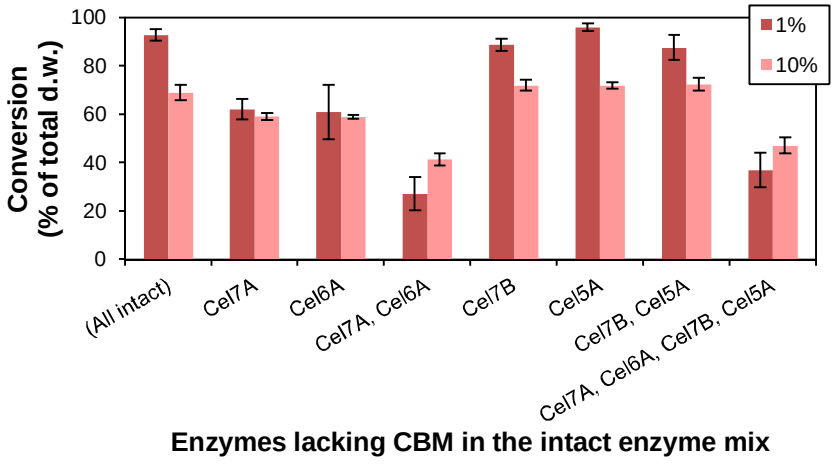

Figure 9. Hydrolysis of Avicel with purified enzyme mixtures. The major cellulase components (TrCel7A, TrCel6A, TrCel7B and TrCel5A) were replaced one-by-one, in pairs or altogether by their catalytic core domains. Avicel was hydrolysed for 48 hours at 1% and 10% (w/w) substrate concentrations with enzyme loadings 0.150 µmol TrCel7A, 0.50 µmol TrCel6A, 0.25 µmol TrCel7B, 0.25 µmol TrCel5A, 0.025 µmol TrXyn11A 0.010 µmol TrMan5A and 0.002 µmol AnCel3A per g dry weight. Error bars indicate the standard deviation of the mean, with a sample size of 3.

When increasing the substrate concentration from 1% to 20% (w/w), the difference between the performance of intact and core enzyme mixtures disappeared on Avicel and hydrothermally pretreated wheat straw (Paper V Figure 2). When the substrate concentration was increased from 1% to 10% (w/w), the conversion yield

achieved with the intact mixture gradually decreased from 85% to 63% of t.m.y. in wheat straw and from 90% to 67% of t.m.y. in Avicel. However, the core mixture was more efficient at higher substrate loadings: although the yield was as low as 52 and 29% of t.m.y. at 1% (w/w) concentration in wheat straw and Avicel respectively, the core mixture resulted in 54% and 50% of t.m.y. at 10% (w/w) substrate loading. Further increasing the substrate loading lowered the yield by both enzyme mixtures. The impact was, however, less profound when using the core mixture. At 20% (w/w) substrate concentration, conversion yields of 38% and 41% of t.m.y. were obtained in wheat straw and of 30% and 23% of t.m.y. in Avicel with the intact and core enzyme mixtures respectively.

4.3 Enhanced enzyme recovery (Papers III, V)

The quantity of enzymes recovered could be increased in two different ways: as a result of enzyme desorption at the end of hydrolysis (from the alkaline delignified substrate COS – Paper III) or by applying cellulases without their binding modules (Paper V). With desorption, less than 20% of the originally loaded enzyme activities could be recovered free in the supernatant when reaching a conversion yield of above 90% of COS. In addition, based on the quantity of free proteins in the supernatant, 34% of TrCel6A could be recovered. For the other enzymes, the enzyme activities and the quantity of proteins measured in the hydrolysates were in good agreement, implying that all free enzymes remained active in the hydrolysate (Paper III Figure 2 and Table 4).

The excision of the CBMs of cellulases resulted in a weaker adsorption affinity of the core (catalytic) domains on cellulose, and presumably also on lignin. Hence, a higher concentration of free enzymes was recoverable in the supernatant, although conversion yields remained low at low substrate loadings, 52% and 29% of t.m.y. in wheat straw and Avicel respectively, at 1% subtrate loading (Paper V Figures 1–3). In the case of wheat straw, less than 30% of the intact TrCel7A could be recovered, whereas over 60% of the core TrCel7A was detected in the supernatant after a hydrolysis of 48 hours at substrate loadings ranging from 1% to 20% w/w (Paper V Figure 3). In the case of Avicel, the intact and core enzymes lost activity and precipitated out of the dilute hydrolysis solution (Paper V Figures 2, 3 and S1). At higher substrate loadings (10–20% w/w), less than 40% of the intact TrCel7A and more than 90% of the core TrCel7A was recovered from the supernatant after hydrolysing Avicel for 48 hours.

5. Discussion

5.1 The accessibility of enzymes to substrates hinders the hydrolysis of cellulose

5.1.1 Particle size

The morphology of cellulosic substrates, in particular the particle size and available surface area, plays an important role in the accessibility of lignocellulose to enzymes (Zhang and Lynd, 2004; Zhu *et al.*, 2008). Several pretreatment methods have been developed to loosen and break down the compact structure of plant cell walls, leading to increased pore size and reduced particle size, both of which increase the available surface area (Mosier *et al.*, 2005). Distinct fibre bundles could be observed microscopically in Avicel, which is holocellulose produced from coniferous pulp (Paper I). During hydrolysis, loosening of the fibre structure continues enzymatically, as was visualised microscopically. The particle size in Avicel decreased during hydrolysis (Paper I Figure 4) while bigger pores available for enzyme penetration were found to be enlarged due to enzymatic attack (Paper I Figure 5). Enzymes seem to access cellulose both from the outer surface of the particles, and from the inner surface through the pores. Considering that cellulose crystallinity was found to remain unchanged during hydrolysis, it could be hypothesized that during the enzymatic hydrolysis of fibre bundles the outer cellulose chains are peeled off layer-by-layer, exposing inner and hence previously hidden structures to enzymatic hydrolysis, as also proposed earlier by Ramos *et al.* (1993).

The changes in particle size distributions of Avicel and SPS during hydrolysis were followed using X-ray microtomography (Figure 7) and laser diffraction (Figure 6). Both methods show a general decrease in particle size during hydrolysis. The laser diffraction reveals that the relative number of finer particles in SPS increased during hydrolysis, although the decrease in the mass (or volume) of particles was not uniform (Figure 6). Similarly, almost intact fibre bundles could still be observed in the residue of Avicel even after a conversion of 67% was reached (Figure 7). Lignin removal from SPS and its hydrolysis residue (SPS-RES) decreased the particle size further (Figure 6) and led to increased hydrolysability of the substrates (Figure 8). Reduced particle size and increased enzymatic digestibility

have also been reported previously as a result of lignin removal (Mooney *et al.*, 1998). Lignin removal also results in an increase in pore volume (Mooney *et al.*, 1998). Although reducing the particle size is important in order to increase the available surface area, the substrate structure, including microfibril aggregation, cellulose crystallinity and the presence of lignin and hemicellulose, also needs to be considered in order to improve accessibility of cellulases to cellulose (Zhu *et al.*, 2008).

5.1.2 Cellulose crystallinity

Cellulose crystallinity has long been studied to understand the structure and hydrolysis of cellulose at a molecular level. However, the impact of crystallinity on the hydrolysability is uncertain. On the one hand, the highly ordered structure of crystalline cellulose is considered to hinder the hydrolysis of cellulose due to the reduced accessibility of cellulases to the cellulose chains (Lynd *et al.*, 2002). On the other hand, the cellulose binding site of CBMs requires 2–3 organised cellulose chains aligned parallel (*i.e.* crystalline regions) for efficient substrate recognition (Boraston *et al.*, 2004).

The exact mechanism of cellulose hydrolysis by cellulases is still uncertain. At a molecular level, two mechanisms have been proposed for the degradation of cellulose crystals (Ramos *et al.*, 1993). According to the first mechanism, individual cellulose crystals are digested sequentially; and according to the second, all cellulose crystals are digested and narrowed at the same time. The first mechanism would result in no change in crystal size, while the second would lead to a measurable decrease in crystal width. In this study, the hydrolysis of Avicel was followed up to 67% conversion, and X-ray microtomography showed a general decrease in particle size throughout the hydrolysis (Paper I). At the same time, however, no changes in the average crystal size or crystallinity in the residual substrate were observed due to enzymatic hydrolysis despite the morphological changes. This contradiction could be explained by the fact that the available techniques analyse whole bulky materials and only data on the average crystal size or the overall crystallinity can be obtained (Park *et al.*, 2010). In fibre aggregates, the microfibrils are hydrolysed sequentially, by a “peeling-off” type of mechanism. Consequently, local changes in the substate often cannot be visualised, such as peeling off cellulose chains layer-by-layer from the outer crystals of microfibril aggregates. This could explain why no change in crystal size was observed when cellulose crystals were digested at the same time (Paper I). In a related study, no increase in the crystallinity has been observed during the hydrolysis of Avicel, suggesting that crystalline and amorphous regions in cellulose are being attacked simultaneously and that no accumulation of crystalline cellulose takes place during hydrolysis (Hall *et al.*, 2010). Accordingly, the hydrolysability, *i.e.* the initial hydrolysis rate, of Avicel remains unchanged independent of the conversion (Yang *et al.*, 2006). In any case, it could be assumed that the slow-down of hydrolysis is not caused by a relative increase in cellulose crystallinity. Nonetheless, the degree of crystallinity clearly determines the initial hydrolysis rate, and reduced crystallinity

leads to faster hydrolysis, presumably due to an increase in the reactivity and accessibility of cellulose by disrupting the tight structure of cellulose chains (Hall *et al.*, 2010).

5.1.3 Lignin

During most pretreatments at high temperature, lignin is retained or partially redeposited on the surfaces of the substrate fragments, hindering the accessibility of enzymes to polysaccharides (Tanahashi *et al.*, 1983; Li *et al.*, 2007; Kristensen *et al.*, 2008). Accordingly, lignin was found to impede the hydrolysis of cellulose profoundly in steam pretreated spruce (Figure 8 and Paper II). Removal of lignin from the substrate surface has been found earlier to uncover the cellulose fibrillar structure using atomic force microscopy (Kristensen *et al.*, 2008), which implies increased accessibility of cellulases to the polysaccharide network. Accordingly, in this study, the removal of lignin increased the accessibility of enzymes to the substrate, leading to a doubling of the hydrolysis yield irrespective of the enzyme mixture used (Paper II).

Accumulation of lignin on the substrate surface has been proposed to reduce the hydrolysis rate (Hansen *et al.*, 2011). Atomic force microscopy images reveal that lignin droplets precipitated on the cell walls of wheat straw during hydrothermal pretreatment gradually form a covering layer on the substrate surface as the hydrolysis of carbohydrates proceeds and lignin accumulates in the residue (Hansen *et al.*, 2011). In this study, however, hydrolysing 38% of d.w. (equivalent to a 64% polysaccharide yield) of SPS (to form SPS-RES) was not found to decrease hydrolysability: SPS and SPS-RES could be hydrolysed to a similar extent with commercial enzyme mixtures, suggesting that the hydrolysis of polysaccharides remains at the same rate despite the accumulation of lignin (Figure 8 and Paper II). Previously it has been shown on Avicel that the reaction rate of cellulose does not decrease as a function of time or conversion (up to 78% of cellulose hydrolysed) when restarting the hydrolysis with fresh enzymes (Yang *et al.*, 2006). Therefore it could be anticipated that neither accumulation of lignin nor conversion of cellulose are responsible for the slow-down of hydrolysis.

Although the cellulose hydrolysability did not seem to be affected after 64% conversion of polysaccharides in SPS, the carbohydrate composition of the solids were changed by the enzymatic action (Paper II Table 3). Not only lignin, but also hemicelluloses, accumulated during the hydrolysis of SPS. A detailed structural analysis using purified enzymes revealed that the residual hemicellulose was poorly solubilised by the enzymes studied; the residual hemicellulose in the lignin-rich SPS (and SPS-RES) was eventually resistant to hydrolysis by the hydrolytic enzymes (Paper II). These hemicelluloses remaining in SPS after pretreatment and in SPS-RES after pretreatment and enzymatic hydrolysis, especially glucomannan, could, however, be removed along with lignin during delignification (Paper II Table 3) and hence seem to be associated with lignin as lignin-carbohydrate complexes. These results are in accordance with previous observation by Ahlgren

and Goring (1971), that some glucomannan can be dissolved from black spruce along with lignin by extensive delignification. Later, galactoglucomannan has been found to be involved in lignin-carbohydrate linkages, especially in spruce (Eriksson *et al.*, 1980; Choi *et al.*, 2007).

5.1.4 Hemicellulose and auxiliary enzymes

Cellulose, together with mainly hemicelluloses, form a complex three-dimensional polysaccharide network in plant cell walls (Sjöström, 1993). Accordingly, hemicelluloses present in the lignocellulosic substrates were found to hinder the accessibility of cellulose. In particular, cellulose, xylan and glucomannan could only be hydrolysed to less than 50% without the simultaneous hydrolysis of the other polysaccharides (Tables 3 and 4 and Paper IV). Various hemicellulases and other auxiliary enzymes have been recognised as being necessary for the total hydrolysis of lignocellulose, with the requirements depending on the composition of the substrates (Berlin *et al.*, 2007; Banerjee *et al.*, 2010). The effect of endoxylanase TrXyn11A and endomannanase TrMan5A on hydrolysis yield also depends on the composition of the softwood substrates (Papers II and IV). Hemicellulases were found not to enhance the hydrolysis of SPS containing less than 0.5% of d.w. hemicellulose, whereas a clear increase could be detected in the cellulose yield for other substrates containing over 1% xylan or glucomannan.

Co-operativity was also observed between cellulases and hemicellulases in Paper IV, as previously observed by Tenkanen *et al.* (1999). The simultaneous hydrolysis of cellulose, xylan and glucomannan leads to almost complete solubilisation of the polysaccharides: the hydrolysis yields were seen to reach close to 100% (Tables 3 and 4 and Paper IV). In particular, the solubilisation of cellulose, xylan and glucomannan were found to be in linear correlation independent of the composition of the enzyme preparations. Analogously, Selig *et al.* (2008) have found a linear correlation between the release of cellobiose and xylobiose. These observations support the idea that cellulose, xylan and glucomannan are interlinked in softwood, and hence their simultaneous hydrolysis is necessary to increase the overall hydrolysis yield. Recently, Hu *et al.* (2011) have reported that xylanase treatment increases the fibre surface area and hence the accessibility of cellulose, presumably due to xylan removal. In addition, the fibre swelling increases as a result of the synergistic co-operation of cellulases and xylanases (Hu *et al.*, 2011). Simultaneous hydrolysis of cellulose and hemicelluloses loosens the structure of the polysaccharide network of lignocellulose, uncovering new action sites for the enzymes.

Co-operation between the glycoside hydrolases in solubilising various polysaccharides from an interlinked network could be described as synergism. This synergism, however, differs from the classical definition of synergism described earlier between cellulases or between hemicellulases and their accessory (sidegroup cleaving) enzymes. As cellulases and hemicellulases act on different substrates, hemicellulases alleviate the steric hindrance of cellulases caused by hemicellu-

loses limiting the accessibility of cellulose. This type of synergism may be distinguished from the ones described earlier (intramolecular synergism) by referring to the hydrolysis of different polysaccharides by the enzymes contributing to the other's action as intermolecular synergism.

5.2 Enzyme adsorption and desorption

The adsorption and desorption of cellulases is a reversible process, with an equilibrium between the amount of enzymes free in the liquid phase and those bound to the lignocellulosic substrate or residue. During the hydrolysis of lignocellulose, in general, cellulases have been found to adsorb promptly onto the solid substrate, followed by slow desorption and readsorption of the enzymes (Nidetzky and Claeyssens, 1994; Boussaid and Saddler, 1999; Lu *et al.*, 2002; Tu *et al.*, 2007). As previously observed, a quick initial adsorption of cellulases (95–99% of the major enzyme component TrCel7A) could be detected on the tested lignocellulosic substrates independent of their composition (Paper III and Paper V). On the other hand, enzyme adsorption and desorption during the later phase of hydrolysis was strongly influenced by the nature of the substrate. Continuous desorption could be observed from alkaline delignified spruce (COS) after 60% conversion of the theoretical maximum yield, whereas the quantity of free enzymes remained below 1% throughout the hydrolysis of steam pretreated spruce (SPS) (Paper III). Likewise, removal of lignin has been shown to result in decreased enzyme adsorption (Boussaid and Saddler, 1999; Lu *et al.*, 2002) and, in some cases, to lead to gradual desorption of enzyme proteins on delignified substrates (Boussaid and Saddler, 1999; Tu *et al.*, 2007).

Despite the reversibility of adsorption and desorption on cellulose, most enzymes remain bound throughout the hydrolysis, even at high degrees of conversion on high lignin containing substrates (Papers III and V). Lignin has been recognised to adsorb cellulases unproductively (Palonen *et al.*, 2004), resulting in lower free enzyme concentrations and consequently lower quantities of efficient enzymes available for hydrolysis. The adsorption of cellulases increases continuously throughout the hydrolysis of pretreated softwood with relatively high lignin content (Boussaid and Saddler, 1999; Lu *et al.*, 2002), which could be explained by the irreversibility of unproductive adsorption. Unproductive adsorption can be diminished by adding surfactants which reduce hydrophobic interactions between cellulases and lignin surfaces, leading to higher cellulase recovery from the liquid phase (Tu *et al.*, 2007).

The adsorption pattern of cellulases changes not only with the type of substrate but with the individual enzymes (Nidetzky and Claeyssens, 1994). Accordingly, analysis of hydrolysates with SDS-PAGE revealed that pure *T. reesei* cellulases in the enzyme mixture adsorbed to substrates with different lignin contents to a varying degree (Paper III and Paper V). The relative amounts of free TrCel7A, TrCel6A and TrCel5A in the hydrolysate corresponded well to each other on the two delignified substrates, Avicel and COS (Paper III). The adsorption profile of the individ-

ual enzymes, however, varies with the type of substrate although independent of the substrate concentration (Paper V). Similarly, Priwobo *et al.* (2012) has observed that the adsorption and activity profiles of specific enzymes differ among the constituents of the commercial cellulase mixture Accelerase.

In addition to the adsorption-desorption phenomena, enzyme inactivation by denaturing may occur during hydrolysis and lead to reduced hydrolytic efficiency and enzyme recovery. Enzyme inactivation has been observed in dilute solution, which could be overcome by the addition of surfactants (Ouyang *et al.*, 2010). The addition of surfactants to the hydrolysis system facilitates enzyme desorption and seems to prevent inactivation of desorbed enzymes in the liquid phase, thus increasing enzyme recovery. When no stabilising substances (neither supplemented nor dissolved from the substrate) were present in the hydrolysate to prevent enzymes from denaturing, a substantial loss in the protein amount and activity of free (and hence recoverable) enzymes was detected in the hydrolysate during the hydrolysis of Avicel at low concentration (Paper V). On the other hand, the same enzymes remained free and active on hydrothermally pretreated wheat straw when small soluble compounds dissolved from the substrate protected them from inactivation. In a related study, Priwobo *et al.* (2012) has revealed that enzyme components which remain primarily free in the liquid phase during the hydrolysis of steam pretreated corn stover at 2% solid loading are more vulnerable to inactivation than enzymes which are adsorbed to a higher extent. Both studies confirmed that the activity of recovered free enzymes correspond well with the protein data, implying that only active enzymes remain free in solution (Paper V; Priwobo *et al.*, 2012).

Recently, adsorption on lignin has been proposed to lead to slow inactivation of the bound enzymes (Rahikainen *et al.*, 2011). Unproductive and irreversible binding of cellulases on lignin, and consequent inactivation of the bound cellulases, were proposed to hinder the desorption of enzymes from lignin-rich residues, which could partially contribute to the gradual decrease in enzyme recovery during hydrolysis reported previously (Boussaid and Saddler; 1999; Tu *et al.*, 2007). Estimating the extent of enzyme inactivation during hydrolysis and its impact on the enzyme recovery is difficult, as enzyme inactivation takes place in parallel to enzyme adsorption. The degree of enzyme inactivation in hydrolytic systems should be evaluated by also taking into account the enzyme fraction bound to the residual substrate (Paper V; Priwobo *et al.*, 2012), which makes the quantification challenging.

5.3 Improving enzymatic conversion

5.3.1 Improving the conversion yield

The major target in the total exploitation of biomass is to increase the efficiency of the conversion of biomass to its basic components. In particular, the aim is to increase the conversion yield and shorten the residence time while maintaining a

relatively low enzyme load. Enhancement of the rate and the final conversion yield of enzymatic hydrolysis can be achieved by increasing the accessibility of cellulose. The accessibility of cellulose can be increased primarily by efficient pretreatments of the biomass which lead to partial removal of the cell wall components, for example, lignin or xylan removal from the lignocellulose matrix.

To increase the overall sugar yield from biomass, pentoses, mostly xylose, need to be recovered along with glucose. Depending on the pretreatment conditions, xylose can be recovered partly from the pretreatment liquor and partly after enzymatic hydrolysis of the pretreated biomass (Mosier *et al.*, 2005). In order to increase the recovery of hemicellulose derived sugars in lignocellulose hydrolysis, the inclusion of xylanolytic and mannanolytic enzymes is necessary in case of softwood (Paper IV). The hemicellulose fraction remaining in the pretreated substrate was found to be only partially solubilised (below 40%) by the action of xylanolytic and mannanolytic enzymes in the absence of cellulases (Tables 3 and 4). Similarly, the simultaneous hydrolysis of hemicellulose was required to obtain high cellulose conversion, implying that cellulose, xylan and glucomannan are interlinked in softwood. Thus, hydrolysing hemicellulose seems to loosen the structure of lignocellulose and to uncover new action sites for cellulases during hydrolysis, further increasing the accessibility of cellulose.

In addition to hemicellulose, lignin, and especially softwood lignin, inhibits the hydrolysis of polysaccharides profoundly (Paper II). The removal of lignin increases the substrate accessibility and was found to double the hydrolysis yield by commercial and purified enzyme mixtures. Apart from hindering the accessibility to carbohydrates, lignin has also been shown to cause unproductive binding of cellulases (Palonen *et al.,* 2004) and to cause slow inactivation of the bound enzymes (Rahikainen *et al.,* 2011) leading to a reduction in the quantity of enzymes available for hydrolysis and consequently decreasing the hydrolysis yield. Interestingly, although the presence of lignin hinders enzymatic hydrolysis, this hindrance does not strengthen as a function of hydrolysis time and degree. SPS and its hydrolysis residue (SPS-RES) could be hydrolysed to the same extent with commercial enzyme preparations. As expected, the purified enzyme mixtures were not as efficient as the commercial enzyme preparations due to the absence of restricting auxiliary enzymes which are present in commercial preparations (Paper II). Identification and supplementation of basic enzyme preparations with these restricting components is needed when designing novel enzyme mixtures.

5.3.2 Enzyme recovery at high solid loadings

Despite the reported reduction in the production costs of enzymes over the last decade, enzymes can still comprise 15% or more of the overall cost of biomass conversion to ethanol (Aden and Foust, 2009; Humbird *et al.*, 2011). Further reduction of the cost of biomass conversion could potentially be achieved by recycling enzymes. After hydrolysis, however, most of the loaded enzymes remain bound to the residual substrate. The addition of a fresh substrate on the partially

hydrolysed residue has been developed as a method to reuse the adsorbed enzymes, and is applicable for relatively pure cellulose materials (Lee *et al.*, 1995). However, this method might not be applicable to lignin-containing plant raw materials due to the consequent accumulation of lignin in each successive hydrolysis round. In addition, readsorption onto a fresh substrate is feasible only if the substrate has a high enough adsorption capacity. Therefore, desorption of adsorbed cellulases from the solid residue into the liquid phase is an appealing option for reasonable enzyme recycling after the hydrolysis of substrates containing lignin.

The adsorption of two-domain cellulases to lignocellulose is mostly directed by their CBMs (Palonen *et al.*, 1999). At low concentrations, CBMs are essential for the high performance of cellulases as they concentrate the enzymes onto the surface of their substrate (Boraston *et al.*, 2004). On the other hand, CBMs are also thought to be responsible for unproductive adsorption (Palonen *et al.*, 2004), which leads to a decrease in the quantity of enzymes available to hydrolysis and also leads to extensive enzyme adsorption throughout the hydrolysis. The beneficial and disadvantageous effects of the CBMs on binding seems to level off at higher substrate concentrations (Paper V). Eventually, the performance of the core enzymes lacking CBMs was shown to reach that of the intact cellulases with their CBMs. The conversion yields, although fairly low at high substrate concentrations, were similar with the intact and core enzymes. Hence, core enzymes provide a competitive alternative to the two-domain enzymes in cellulose conversion on industrial scale where high solid loadings are used while also enabling enzyme recycling. Core enzymes could be recovered and recycled from the liquid phase by simple ultrafiltration. However, the hydrolysis process using core enzymes still needs to be optimised, including optimising the hydrolysis parameters and recycling techniques, in order to reach higher conversion yields and to be able to evaluate the feasibility of these enzymes.

5.4 The role of water in microbial cellulolytic systems

Cellulose degrading organisms expressing different cellulose degrading mechanisms have been isolated from various (aqueous and non-aqueous) environments worldwide (Lynd *et al.*, 2002). Based on the results obtained in *'in vitro'* hydrolysis experiments at varying substrate concentrations in Paper V, it can be anticipated that microorganisms living in environments with varying water content might have developed different cellulose degrading mechanisms, and that the environmental conditions related to water may play a central role in the evolutionary development of cellulose degrading microbial systems. Aquatic anaerobic bacteria living in *e.g.* rumens or hot springs have evolved cell-bound cellulosomes which anchor the substrate to the cell. Hence, the enzymes bound to the cellulosomes are not lost in the surrounding liquid and the sugars formed can be taken up by the organism without being washed away by aqueous streams. On the other hand, in less dilute (semi-aqueous or terrestrial) environments, litter degraders can secrete cellulases

without risk of them being washed away from the vicinity of the organisms by aqueous streams.

Surprisingly, about 60% of cellulases were found to lack cellulose binding modules (CBMs) or dockerins, which connect cellulosomal enzymes to scaffoldins, and indirectly, to cellulose binding modules (Paper V). Previous analyses have also reported similar ratios of CBM-less enzymes (Martinez *et al.*, 2009; Medie *et al.*, 2012). This indicates that CBMs may not be needed as essential domains of secreted cellulases, depending on the specific natural habitat of the organism. Cellulases with CBMs are superior compared to cellulases without CBMs in dilute systems (Tomme *et al.*, 1988). On the other hand, the difference between cellulases containing and lacking CBMs was shown to be negligible at higher substrate concentrations, *i.e.* at reduced water content (Paper V). Accordingly, it could be hypothesized that CBMs might not provide an evolutionary advantage in terrestrial environments with low water content.

CBMs anchor enzymes to a substrate and consequently are responsible for enzyme adsorption on cellulose and lignin throughout the hydrolysis (Palonen *et al.*, 1999 and 2004). When omitting CBMs, free hydrolytic enzymes could be recovered from the hydrolysate at the end of the hydrolysis (Paper V) due to the reduced affinity of the core enzyme to the insoluble substrate (Palonen *et al.*, 1999). Thus, hydrolytic enzymes without CBMs represent good candidates for recyclable enzymes on an industrial scale (*i.e.* at a high substrate concentration) without compromising hydrolysis performance. Therefore, hydrolytic enzymes could be screened for their potential for biomass conversion based on the natural environment of the producing organisms. In addition, enzyme screening should be carried out at elevated substrate concentrations corresponding to industrial conditions.

6. Conclusions and future perspectives

Lignocellulosic biomass, available worldwide in plant cell walls, is a promising alternative feedstock for production of liquid fuels. The function of plant cell walls is to support the plant structure and to protect the plants from environmental impacts. Consequently, plant cell walls contain extremely resistant structures, which complicates the conversion to sugar monomers. The aim of this thesis is to investigate the limitations hindering enzymatic saccharification of lignocellulosic materials, and to propose solutions to overcome these limitations.

In lignocellulose, cellulose, hemicelluloses and lignin were found to be physically interlinked with each other. Therefore, pretreatment of lignocellulose is essential in order to increase the enzymatic accessibility of cellulose and hemicellulose. Accordingly, extensive removal of lignin and hemicellulose enhances the hydrolysis of cellulose. Removal of lignin from steam pretreated spruce was found to accelerate enzymatic hydrolysis of cellulose. In addition, the simultaneous hydrolysis of cellulose and hemicelluloses was shown to be necessary to increase the accessibility of cellulases and hemicellulolytic enzymes to the polysaccharides, presumably by reciprocally uncovering new action sites. In fact, the solubilisation of cellulose, xylan and glucomannan takes place synergistically and the released glucose, xylose and mannose are linearly correlated.

Changes in the residual substrates were observed throughout hydrolysis to reveal structures hindering the action of the cellulases. Although lignin was found to impede cellulose hydrolysis, the accumulation of lignin during the hydrolysis of steam pretreated spruce does not seem to slow down the hydrolysis. In microcrystalline cellulose, the particle size decreases gradually during hydrolysis. Despite extensive hydrolysis and morphological changes in the residual substrate, however, no alteration in cellulose structure was observed at nanoscale. The average crystallinity and crystal size of cellulose were found to remain constant during hydrolysis. Hence, cellulose microfibrils comprising fibre bundles are predicted to be hydrolysed sequentially by a “peeling-off” type of mechanism.

In addition to substrate accessibility, enzyme adsorption and desorption during hydrolysis have to be considered. While the adsorption of cellulases is required for an efficient catalysis, more efficient desorption of cellulases could contribute to better enzyme recovery and hence to a reduction in the cost of the total hydrolysis of biomass. Accordingly, cellulases were found to adsorb to the substrate to a

varying extent, depending on the substrate composition. Cellulases remained adsorbed throughout the hydrolysis of the lignin-rich steam pretreated spruce; slow desorption of cellulases was detected only in delignified spruce. This implies that the composition of the substrate has a profound role in enabling enzyme recovery. The adsorption profile of individual cellulases varied with the type of substrate and with the individual enzymes. Thus, the recovered enzymes would need to be completed with the limiting components prior to recycling. In addition, unproductive binding of enzymes and enzyme inactivation could be diminished during hydrolysis by supplementing the enzymes with surfactants to increase the hydrolytic efficiency and enzyme recovery.

An efficient way to change the equilibrium between adsorbed and desorbed enzymes without compromising the yield would provide a great potential for recycling cellulases. For the first time, it was demonstrated that cellulases lacking CBMs reach a similar hydrolysis yield to cellulases containing CBMs at a high substrate loading (20% concentration). In addition, using cellulases lacking CBMs instead of the two-domain cellulases enabled enzyme recovery for recycling. Therefore, hydrolytic enzymes without CBMs represent promising candidates for recyclable enzymes in commercial-scale biomass utilisation without compromising the hydrolytic performance. The absence of CBMs, however, makes these enzymes less efficient at low substrate concentrations. Consequently, enzyme screening and enzymatic hydrolysis should be designed and performed at high concentrations in future in order to test enzymes under industrially-relevant conditions. To confirm the final relevance of this novel approach, the hydrolysis conditions still remain to be optimised in order to reach satisfactorily high conversion yields, and the hydrolytic efficiency of cellulases lacking CBMs need to be confirmed at higher overall yields.

Our current knowledge about the exact mechanism of cellulose degradation by various microorganisms is still incomplete. This work focuses on the well-characterised purified enzymes of *T. reesei*, which still serve as fundamental, versatile and industrially applicable tools for the study of hydrolytic mechanisms. Further studies into hydrolytic enzymes from other organisms, however, need to be conducted to prove the role of CBMs. Approximately 60% of the identified and putative glycoside hydrolases were found to lack carbohydrate binding modules. Genetic libraries are expanded continuously with newly available data, and unknown enzyme components such as the GH 61 oxidoreductases are continually being discovered which could potentially enhance lignocellulose hydrolysis. The available enzyme databases and gene libraries, complemented by genetic engineering, provide an excellent resource for the discovery of enzymes with improved hydrolytic properties.

References

Aden, A. and Foust, T. 2009. Technoeconomic analysis of the dilute sulfuric acid and enzymatic hydrolysis process for the conversion of corn stover to ethanol. *Cellulose* **16**, pp. 535–545.

Ahlgren, P.A. and Goring, D.A.I. 1971. Removal of wood components during chlorite delignification of black spruce. *Can. J. Chem.* **49**, pp. 1272–1275.

Ahola, S., Turon, X., Österberg, M., Laine, J. and Rojas, O.J. 2008. Enzymatic hydrolysis of native cellulose nanofibrils and other cellulose model films: effect of surface structure. *Langmuir* **24**, pp. 11592–11599.

Andersson, S., Wikberg, H., Pesonen, E., Maunu, S.L. and Serimaa, R. 2004. Studies of crystallinity of Scots pine and Norway spruce cellulose. *Trees* **18**, pp. 346–353.

Arantes, V. and Saddler, J.N. 2010. Access to cellulose limits the efficiency of enzymatic hydrolysis: the role of amorphogenesis. *Biotech. Biofuels* **3**, art. no. 4.

Bailey, M.J., Biely, P. and Poutanen, K. 1991. Interlaboratory testing of methods for assay of xylanase activity. *J. Biotechnol.* **23**, pp. 257–270

Bailey, M.J. and Nevalainen, K.M.H. 1981. Induction, isolation and testing of stable *Trichoderma reesei* mutants with improved production of solubilising cellulose. *Enzyme Microb. Technol.* **3**, pp. 153–157.

Banerjee, G., Car, S., Scott-Craig, J.S., Borrusch, M.S. and Walton, J.D. 2010. Rapid optimization of enzyme mixtures for deconstruction of diverse pretreatment/biomass feedstock combinations. *Biotechnol. Biofuels* **3**, art. no. 22.

Bansal, P., Hall, M., Realff, M.J., Lee, J.H. and Bommarius, A.S. 2010. Multivariate statistical analysis of X-ray data from cellulose: A new method to determine degree of crystallinity and predict hydrolysis rates. *Bioresour. Technol.* **101**, pp. 4461–4471.

Berlin, A., Gilkes, N., Kilburn, D., Markov, A., Skomarovsky, A., Okunev, O., Gusakov, A., Maximenko, V., Gregg, D., Sinitsyn, A. and Saddler, J. 2005a. Evaluation of novel fungal cellulase preparations for ability to hydrolyze softwood substrates – evidence for the role of accessory enzymes. *Enzyme Microb. Technol.* **37**, pp. 175–184.

Berlin, A., Gilkes, N., Kurabi, A., Bura, R., Tu, M., Kilburn, D. and Saddler, J. 2005b. Weak lignin-binding enzymes: a novel approach to improve activity of cellulases for hydrolysis of lignocellulosics. *Appl. Biochem. Biotechnol.* **121**, pp. 163–170.

Berlin, A., Maximenko, V., Gilkes, N. and Saddler, J. 2007. Optimization of enzyme complexes for lignocellulose hydrolysis. *Biotechnol. Bioeng.* **97**, pp. 287–296.

Bertaud, B. and Holmbom, B. 2004. Chemical composition of earlywood and latewood in Norway spruce heartwood, sapwood and transition zone wood. *Wood Sci. Technol.* **38**, pp. 245–256.

Biely, P. 1985. Microbial xylanolytic systems. *Trends Biotechnol.* **3**, pp. 286–289.

Biely, P. 2003. Xylanolytic enzymes. In: Whitaker, J.R., Voragen, A.G.J. and Wong, D.W.S. (Eds.). *Handbook of Food Enzymology*. Marcel Dekker, Inc., New York. pp. 879–916.

Biely, P., Vršanská, M. and Claeyssens, M. 1991. The endo-1,4-β-glucanase I from *Trichoderma reesei*. Action on β-1,4-oligomers and polymers derived from D-glucose and D-xylose. *Eur. J. Biochem.* **200**, pp. 157–163.

Bondeson, D., Mathew, A. and Oksman, K. 2006. Optimization of the isolation of nanocrystals from microcrystalline cellulose by acid hydrolysis. *Cellulose* **13**, pp. 171–180.

Bonin, C. and Lal, R. 2012. Bioethanol potentials and life-cycle assessments of biofuel feedstocks. *Crit. Rev. Plant Sci.* **31**, pp. 271–289.

Boraston, A.B., Bolam, D.N., Gilbert, H.J. and Davies, G.J. 2004. Carbohydrate-binding modules: Fine-tuning polysaccharide recognition. *Biochem. J.* **382**, pp. 769–781.

Boussaid, A. and Saddler, J.N. 1999. Adsorption and activity profiles of cellulases during hydrolysis of two Douglas fir pulps. *Enzyme Microb. Technol.* **24**, pp. 138–143.

Bu, L., Beckham, G.T., Crowley, M.F., Chang, C.H., Matthews, J.F., Bomble, Y.J., Adney, W.S., Himmel, M.E. and Nimlos, M.R. 2009. The energy landscape for interaction of the family 1 carbohydrate-binding module and the cellulose surface is altered by hydrolyzed glycosidic bonds. *J. Phys. Chem. B* **113**, pp. 10994–11002.

Buranov, A.U. and Mazza, G. 2008. Lignin in straw of herbaceous crops. *Ind. Crops Prod.* **28**, pp. 237–259.

Campbell, M.M. and Sederoff, R.R. 1996. Variation in lignin content and composition. Mechanisms of control and implications for the genetic improvement of plants. *Plant Physiol.* **110**, pp. 3–13.

Cantarel, B.L., Coutinho, P.M., Rancurel, C., Bernard, T., Lombard, V. and Henrissat, B. 2009. The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics. *Nucleic Acids Res.* **37**, pp. D233–D238.

Carpita, N.C. 1982. Limiting diameters of pores and the surface structure of plant cell walls. *Science* **218**, pp. 813–814.

Choi, J.W., Choi, D.-H. and Faix, O. 2007. Characterization of lignin–carbohydrate linkages in the residual lignins isolated from chemical pulps of spruce (*Picea abies*) and beech wood (*Fagus sylvatica*). *J. Wood Sci.* **53**, pp. 309–313.

Collins, T., Gerday, C. and Feller, G. 2005. Xylanases, xylanase families and extremophilic xylanases. *FEMS Microbiol. Rev.* **29**, pp. 3–23.

Davies, G. and Henrissat, B. 1995. Structures and mechanisms of glycosyl hydrolases. *Structure* **3**, pp. 853–859.

Ding, S.-Y. and Himmel, M.E. 2006. The maize primary cell wall microfibril: A new model derived from direct visualization. *J. Agric. Food Chem.* **54**, pp. 597–606.

Ebringerová, A. and Heinze, T. 2000. Xylan and xylan derivatives–biopolymers with valuable properties, 1. Naturally occurring xylans structures, isolation procedures and properties. *Macromol. Rapid Comm.* **21**, pp. 542–556.

EPA (United States Environmental Protection Agency) 2009. EPA Lifecycle analysis of greenhouse gas emissions from renewable fuels. Available at: http://www.epa.gov/oms/renewablefuels/420f09024.pdf.

Eriksson, Ö., Goring, D.A.I. and Lindgren, B.O. 1980. Structural studies on the chemical bonds between lignins and carbohydrates in spruce wood. *Wood Sci. Technol.* **14**, pp. 267–279.

Eriksson, T., Karlsson, J. and Tjerneld, F. 2002. A model explaining declining rate in hydrolysis of lignocellulose substrates with cellobiohydrolase I (Cel7A) and endoglucanase I (Cel7B) of *Trichoderma reesei*. *Appl. Biochem. Biotechnol.* **101**, pp. 41–60.

Faulds, C.B., Mandalari, G., Lo Curto, R.B., Bisignano, G., Christakopoulos, P. and Waldron, K.W. 2006. Synergy between xylanases from glycoside hydrolase family 10 and family 11 and a feruloyl esterase in the release of phenolic acids from cereal arabinoxylan. *Appl. Microbiol. Biotechnol.* **71**, pp. 622–629.

Fergus, B.J. and Goring, D.A.I. 1970. The distribution of lignin in birch wood as determined by ultraviolet microscopy. *Holzforschung* **24**, pp. 118–124.

Fox, J.M., Levine, S.E., Clark, D.S. and Blanch, H.W. 2012. Initial- and processive-cut products reveal cellobiohydrolase rate limitations and the role of companion enzymes. *Biochem.* **51**, pp. 442−452.

Gauss, W.F., Suzuki, S. and Takagi, M. 1976. Manufacture of alcohol from cellulosic materials using plural ferments. *U.S. Pat. No. 3,990,944.*

Gilbert, H.J. 2007. Cellulosomes: microbial nanomachines that display plasticity in quaternary structure. *Mol. Microbiol.* **63**, pp. 1568–1576.

Gilkes, N.R., Henrissat, B., Kilburn, D.G., Miller, R.C. and Warren, R.A.J. 1991. Domains in microbial β-1,4-glycanases: sequence conservation, function, and enzyme families. *Microbiol. Rev.* **55**, pp. 303–315.

Gregg, D.J. and Saddler, J.N. 1996. Factors affecting cellulose hydrolysis and the potential of enzyme recycle to enhance the efficiency of an integrated wood to ethanol process. *Biotechnol. Bioeng.* **51**, pp. 375–383.

Gritzali, M. and Brown, R.D. Jr. 1979. The cellulase system of *Trichoderma*. Relationships between purified extracellular enzymes from induced or cellulose-grown cells. *Adv. Chem. Ser.* **81**, pp. 237–260.

Gruno, M., Väljamäe, P., Pettersson, G. and Johansson, G. 2004. Inhibition of the *Trichoderma reesei* cellulases by cellobiose is strongly dependent on the nature of the substrate. *Biotechnol. Bioeng.* **86**, pp. 503–511.

Gübitz, G.M., Stebbing, D.W., Johansson, C.I. and Saddler, J N. 1998. Lignin-hemicellulose complexes restrict enzymic solubilization of mannan and xylan from dissolving pulp. *Appl. Microbiol. Biotechnol.* **50**, pp. 390–395.

Hall, M., Bansal, P., Lee, J.H., Realff, M.J. and Bommarius, A.S. 2010. Cellulose crystallinity – a key predictor of the enzymatic hydrolysis rate. *FEBS J.* **277**, pp. 1571–1582.

Hansen, M.A.T., Kristensen, J.B., Felby, C. and Jørgensen, H. 2011. Pretreatment and enzymatic hydrolysis of wheat straw (*Triticum aestivum L.*) – The impact of lignin relocation and plant tissues on enzymatic accessibility. *Bioresour. Technol.* **102**, pp. 2804–2811.

Hatfield, R.D., Ralph, J. and Grabber, J.H. 1999. Cell wall cross-linking by ferulates and diferulates in grasses. *J. Sci. Food Agric.* **79**, pp. 403–407.

Hendriks, A.T.W.M. and Zeeman, G. 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresour. Technol.* **100**, pp. 10–18.

Henrissat, B. 1991. A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J.* **280**, pp. 309–316.

Henrissat, B. and Davies, G. 1997. Structural and sequence-based classification of glycoside hydrolases. *Curr. Op. Struct. Biol.* **7**, pp.637–644.

Henrissat, B., Driguez, H., Viet, C. and Schülein, M. 1985. Synergism of cellulases from *Trichoderma reesei* in the degradation of cellulose. *Bio/Technol.* **3**, pp. 722–726.

Himmel, M.E., Ding, S.Y., Johnson, D.K., Adney, W.S., Nimlos, M.R., Brady, J.W. and Foust, T.D. 2007. Biomass recalcitrance: engineering plants and enzymes for biofuels production. *Science* **315**, pp. 804–807.

Holtzapple, M., Cognata, M., Shu, Y. and Hendrickson, C. 1990. Inhibition of *Trichoderma reesei* cellulase by sugars and solvents. *Biotechnol. Bioeng.* **36**, pp. 275–287.

Hu, J., Arantes, V. and Saddler, J.N. 2011. The enhancement of enzymatic hydrolysis of lignocellulosic substrates by the addition of accessory enzymes such as xylanase: is it an additive or synergistic effect? *Biotechnol. Biofuels* **4**, art. no. 36.

Hult, E.-L., Larsson, P.T. and Iversen, T. 2001. Cellulose fibril aggregation – an inherent property of kraft pulps. *Polymer* **42**, pp. 3309–3314.

Humbird, D., Davis, R., Tao, L., Kinchin, C., Hsu, D., Aden, A., Schoen, P., Lukas, J., Olthof, B., Worley, M., Sexton, D. and Dudgeon, D. 2011. Process design and economics for biochemical conversion of lignocellulosic biomass to ethanol. NREL Technical report: NREL/TP-5100-47764. Available at: http://www.nrel.gov/biomass/pdfs/47764.pdf.

Igarashi, K., Koivula, A., Wada, M., Kimura, S., Penttilä, M. and Samejima, M. 2009. High speed atomic force microscopy visualizes processive movement of *Trichoderma reesei* cellobiohydrolase I on crystalline cellulose. *J. Biol. Chem.* **284**, pp. 36186–36190.

Igarashi, K., Uchihashi, T., Koivula, A., Wada, M., Kimura, S., Okamoto, T., Penttilä, M., Ando, T., and Samejima, M. 2011. Traffic jams reduce hydrolytic efficiency of cellulase on cellulose surface. *Science* **333**, pp. 1279–1282.

Iiyama, K., Lam, T.B.-T. and Stone, B.A. 1990. Phenolic acid bridges between polysaccharides and lignin in wheat internodes. *Phytochem.* **29**, pp. 733–737.

Iiyama, K., Lam, T.B.-T. and Stone, B.A. 1994. Covalent cross-links in the cell wall. *Plant Physiol.* **104**, pp. 315–320.

IUPAC (International Union of Pure and Applied Chemistry). 1987. Measurement of cellulase activities. *Pure & Appl. Chem.* **59**, pp. 257–268.

Jalak, J. and Väljamäe, P. 2010. Mechanism of initial rapid rate retardation in cellobiohydrolase catalyzed cellulose hydrolysis. *Biotechnol. Bioeng.* **106**, pp. 871–883.

Jeffries, T.W. 1990. Biodegradation of lignin-carbohydrate complexes. *Biodegrad.* **1**, pp. 163–176.

Jørgensen, H., Vibe-Pedersen, J., Larsen, J. and Felby, C. 2007. Liquefaction of lignocellulose at high-solids concentrations. *Biotechnol. Bioeng.* **96**, pp. 862–870.

Kabel, M.A., Carvalheiro, F., Garrote, G., Avgerinos, E., Koukios, E., Parajó, J.C., Gírio, F.M., Schols, H.A. and Voragen, A.G.J. 2002. Hydrothermally treated xylan rich by-products yield different classes of xylo-oligosaccharides. *Carbohydr. Polym.* **50**, pp. 47–56.

Karlsson, J., Medve, J. and Tjerneld, F. 1999. Hydrolysis of steam-pretreated lignocellulose. *Appl. Biochem. Biotechnol. - Part A Enzyme Eng. Biotechnol.* **82**, pp. 243–258.

Karlsson, J., Siika-aho, M., Tenkanen, M. and Tjerneld, F. 2002. Enzymatic properties of the low molecular mass endoglucanases Cel12A (EG III) and Cel45A (EG V) of *Trichoderma reesei*. *J. Biotechnol.* **99**, pp. 63–78.

Kim, S. and Holtzapple, M.T. 2006. Effect of structural features on enzyme digestibility of corn stover. *Bioresour. Technol.* **97**, pp. 583–591.

Kipper, K., Väljamäe, P. and Johansson, G. 2005. Processive action of cellobiohydrolase Cel7A from *Trichoderma reesei* is revealed as 'burst' kinetics on fluorescent polymeric model substrates. *Biochem. J.* **385**, pp. 527–535.

Klemm, D., Kramer, F., Moritz, S., Lindström, T., Ankerfors, M., Gray, D. and Dorris, A. 2011. Nanocelluloses: a new family of nature-based materials. *Angew. Chem. Int. Ed.* **50**, pp. 5438–5466.

Kolenová, K., Vršanská, M. and Biely, P. 2006. Mode of action of endo-β-1,4-xylanases of families 10 and 11 on acidic xylooligosaccharides. *J. Biotechnol.* **121**, pp. 338–345.

Kotiranta, P., Karlsson, J., Siika-aho, M., Medve, J., Viikari, L., Tjerneld, F. and Tenkanen, M. 1999. Adsorption and activity of *Trichoderma reesei* cellobiohydrolase I, endoglucanase II and the corresponding core proteins on steam pretreated willow. *Appl. Biochem. Biotechnol.* **81**, pp. 81–90.

Kristensen, J.B., Felby, C. and Jörgensen, H. 2009. Yield-determining factors in high-solids enzymatic hydrolysis of lignocellulose. *Biotechnol. Biofuels* **2**, art. no. 11.

Kristensen, J.B., Thygesen, L.G., Felby, C., Jørgensen, H. and Elder, T. 2008. Cell-wall structural changes in wheat straw pretreated for bioethanol production. *Biotechnol. Biofuels* **1**, art. no. 5.

Kumar, R. and Wyman, C.E. 2009. Cellulase adsorption and relationship to features of corn stover solids produced by leading pretreatments. *Biotechnol. Bioeng.* **103**, pp. 252–267.

Kurasin, M. and Väljamäe, P. 2011. Processivity of cellobiohydrolases is limited by the substrate. *J. Biol. Chem.* **286**, pp. 169–177.

Kyoto protocol to the United Nations Framework Convention on Climate Change. 1998. Available at: http://unfccc.int/resource/docs/convkp/kpeng.pdf.

Lam, T.B., Kadoya, K. and Iiyama, K. 2001. Bonding of hydroxycinnamic acids to lignin: ferulic and *p*-coumaric acids are predominantly linked at the benzyl position of lignin, not the β-position, in grass cell walls. *Phytochem.* **57**, pp. 987–992.

Lawoko, M., Henriksson, G. and Gellerstedt, G. 2003. New method for quantitative preparation of lignin-carbohydrate complex from unbleached softwood kraft pulp: lignin-polysaccharide networks I. *Holzforschung* **57**, pp. 69–74.

Lawoko, M., Henriksson, G. and Gellerstedt, G. 2006. Characterisation of lignin-carbohydrate complexes (LCCs) of spruce wood (*Picea abies L.*) isolated with two methods. *Holzforschung* **60**, pp. 156–161.

Lawoko, M., Nutt, A., Henriksson, H., Gellerstedt, G. and Henriksson, G. 2000. Hemicellulase activity of aerobic fungal cellulases. *Holzforschung* **54**, pp. 497–500.

Lee, D., Yu, A.H.C. and Saddler, J.N. 1995. Evaluation of cellulase recycling strategies for the hydrolysis of lignocellulosic substrates. *Biotechnol. Bioeng.* **45**, pp. 328–336.

Li, J., Henriksson, G. and Gellerstedt, G. 2007. Lignin depolymerization/ repolymerization and its critical role for delignification of aspen wood by steam explosion. *Bioresour. Technol.* **98**, pp. 3061–3068.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, pp. 265–275.

Lu, Y., Yang, B., Gregg, D., Saddler, J.N. and Mansfield, S.D. 2002. Cellulase adsorption and an evaluation of enzyme recycle during hydrolysis of steam-exploded softwood residues. *Appl. Biochem. Biotechnol.* **98–100**, pp. 641–654.

Lundqvist, J., Teleman, A., Junel, L., Zacchi, G., Dahlman, O., Tjerneld, F. and Stålbrand, H. 2002. Isolation and characterization of galactoglucomannan from spruce (*Picea abies*). *Carbohydr. Polym.* **48**, pp. 29–39.

Lynd, L.R., Weimer, P.J., van Zyl, W.H. and Pretorius, I.S. 2002. Microbial cellulose utilization: fundamentals and biotechnology. *Microbiol. Mol. Biol. Rev.* **66**, pp. 506–577.

Macarrón, R., Acebal, C., Castillón, M.P., Domínguez, J.M., de la Mata, I., Pettersson, G., Tomme, P. and Claeyssens, M. 1993. Mode of action of endoglucanase III from *Trichoderma reesei*. *Biochem. J.* **289**, pp. 867–873.

Margeot, A., Hahn-Hagerdal, B., Edlund, M., Slade, R. and Monot, F. 2009. New improvements for lignocellulosic ethanol. *Curr. Opin. Biotechnol.* **20**, pp. 372–380.

Martinez, D., Berka, R.M., Henrissat, B., Saloheimo, M., Arvas, M., Baker, S.E., Chapman, J., Chertkov, O., Coutinho, P.M., Cullen, D., Danchin, E.G., Grigoriev, I.V., Harris, P., Jackson, M., Kubicek, C.P., Han, C.S., Ho, I.,

Larrondo, L.F., de Leon, A.L., Magnuson, J.K., Merino, S., Misra, M., Nelson, B., Putnam, N., Robbertse, B., Salamov, A.A., Schmoll, M., Terry, A., Thayer, N., Westerholm-Parvinen, A., Schoch, C.L., Yao, J., Barbote, R., Nelson, M.A., Detter, C., Bruce, D., Kuske, C.R., Xie, G., Richardson, P., Rokhsar, D.S., Lucas, S.M., Rubin, E.M., Dunn-Coleman, N., Ward, M. and Brettin, T.S. 2008. Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. *Hypocrea jecorina*). *Nature Biotechnol.* **26**, pp. 553–560.

Martinez, D., Challacombe, J., Morgenstern, I., Hibbett, D., Schmoll, M., Kubicek, C.P., Ferreira, P., Ruiz-Duenas, F.J., Martinez, A.T., Kersten, P., Hammel, K.E., Vanden Wymelenberg, A., Gaskell, J., Lindquist, E., Sabat, G., BonDurant, S.S., Larrondo, L.F., Canessa, P., Vicuna, R., Yadav, J., Doddapaneni, H., Subramanian, V., Pisabarro, A.G., Lavín, J.L., Oguiza, J.A., Master, E., Henrissat, B., Coutinho, P.M., Harris, P., Magnuson, J.K., Baker, S.E., Bruno, K., Kenealy, W., Hoegger, P.J., Kües, U., Ramaiya, P., Lucas, S., Salamov, A., Shapiro, H., Tu, H., Chee, C.L., Misra, M., Xie, G., Teter, S., Yaver, D., James, T., Mokrejs, M., Pospisek, M., Grigoriev, I.V., Brettin, T., Rokhsar, D., Berka, R. and Cullen, D. 2009. Genome, transcriptome, and secretome analysis of wood decay fungus *Postia placenta* supports unique mechanisms of lignocellulose conversion. *Proc. Natl. Acad. Sci. U.S.A.* **106**, pp. 1954–1959.

McCleary, B.V. and Matheson, N.K. 1983. Action patterns and substrate-binding requirements of β-D-mannanase with mannosaccharides and mannan-type polysaccharides. *Carbohydr. Res.* **119**, pp. 191–219.

Medie, F.,M., Davies, G.J., Drancourt, M. and Henrissat, B. 2012. Genome analyses highlight the different biological roles of cellulases. *Nat. Rev. Microbiol.* **10**, pp. 227–234.

Medve, J., Karlsson, J., Lee, D. and Tjerneld, F. 1998. Hydrolysis of microcrystalline cellulose by cellobiohydrolase I and endoglucanase II from *Trichoderma reesei*: adsorption, sugar production pattern, and synergism of the enzymes. *Biotechnol. Bioeng.* **59**, pp. 621–634.

Mooney, C.A., Mansfield, S.D., Touhy, M.G. and Saddler, J.N. 1998. The effect of initial pore volume and lignin content on the enzymatic hydrolysis of softwoods. *Bioresour. Technol.* **64**, pp. 113–119.

Moreira, L.R.S. and Filho, E.X.F. 2008. An overview of mannan structure and mannan-degrading enzyme systems. *Appl. Microbiol. Biotechnol.* **79**, pp. 165–178.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M. and Ladisch, M. 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresour. Technol.* **96**, pp. 673–686.

Mueller, S.C. and Brown, R.M. Jr. 1980. Evidence for an intramembranous component associated with a cellulose microfibril synthesizing complex in higher plants. *J. Cell Biol.* **84**, pp. 315–326.

Nidetzky, B. and Claeyssens, M. 1994. Specific quantification of *Trichoderma reesei* cellulases in reconstituted mixtures and its application to cellulase-cellulose binding studies. *Biotechnol. Bioeng.* **44**, pp. 961–966.

Nidetzky, B., Hayn, M., Macarron, R. and Steiner, W. 1993. Synergism of *Trichoderma reesei* cellulases while degrading different celluloses. *Biotechnol. Lett.* **15**, pp. 71–76.

Nidetzky, B., Steiner, W. and Claeyssens, M. 1994. Cellulose hydrolysis by the cellulases from *Trichoderma reesei*: adsorptions of two cellobiohydrolases, two endocellulases and their core proteins on filter paper and their relation to hydrolysis. *Biochem J.* **303**, pp. 817–823.

Nishiyama, Y., Langan, P. and Chanzy, H. 2002. Crystal structure and hydrogen-bonding system in cellulose Iβ from synchrotron X-ray and neutron fiber diffraction. *J. Am. Chem. Soc.* **124**, pp. 9074–9082.

NREL Chemical Analysis and Testing Task, Laboratory Analytical Procedures 001-005. Downloaded from: http://engineering.purdue.edu/~lorre/16/research.

Obst, J.R. and Landucci, L.L. 1986. The syringyl content of softwood lignin. *J. Wood Chem. Technol.* **6**, pp. 311–327.

Ooshima, H., Sakata, M. and Harano, Y. 1986. Enhancement of enzymatic hydrolysis of cellulose by surfactant. *Biotechnol. Bioeng.* **28**, pp. 1727–1734.

von Ossowski, I., Ståhlberg, J., Koivula, A., Piens, K., Becker, D., Boer, H., Harle, R., Harris, M., Divne, C., Mahdi, S., Zhao, Y., Driguez, H., Claeyssens, M., Sinnott, M.L. and Teeri, T.T. 2003. Engineering the exo-loop of *Trichoderma reesei* cellobiohydrolase, Cel7A. A comparison with *Phanerochaete chrysosporium* Cel7D. *J. Mol. Biol.* **333**, pp. 817–829.

Ouyang, J., Dong, Z., Song, X., Lee, X., Chen, M. and Yong, Q. 2010. Improved enzymatic hydrolysis of microcrystalline cellulose (Avicel PH101) by polyethylene glycol addition. *Bioresour. Technol.* **101**, pp. 6685–6691.

Palmqvist, E. and Hahn-Hägerdal, B. 2000. Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. *Bioresour. Technol.* **74**, pp. 25–33.

Palonen, H., Tenkanen, M. and Linder, M. 1999. Dynamic interaction of *Trichoderma reesei* cellobiohydrolases Cel6A and Cel7A and cellulose at equilibrium and during hydrolysis. *Appl. Environ. Microbiol.* **65**, pp. 5229–5233.

Palonen, H., Tjerneld, F., Zacchi, G. and Tenkanen, M. 2004. Adsorption of *Trichoderma reesei* CBH I and EG II and their catalytic domains on steam pretreated softwood and isolated lignin. *J. Biotechnol.* **107**, pp. 65–72.

Park, S., Baker, J.O., Himmel, M.E., Parilla, P.A. and Johnson, D.K. 2010. Cellulose crystallinity index: measurement techniques and their impact on interpreting cellulase performance. *Biotechnol. Biofuels* **3**, art. no. 10.

Pollet, A., Delcour, J.A. and Courtin, C.M. 2010. Structural determinants of the substrate specificities of xylanases from different glycoside hydrolase families. *Crit. Rev. Biotechnol.* **30**, pp. 176–191.

Priwobo, A., Arantes, V. and Saddler, J.N. 2012. The adsorption and enzyme activity profiles of specific *Trichoderma reesei* cellulase/xylanase components when hydrolyzing steam pretreated corn stover. *Enzyme Microb. Technol.* **50**, pp. 195–203.

Quinlan, R.J., Sweeney, M.D., Lo Leggio, L., Otten, H., Poulsen, J.-C.N., Johansen, K.S., Krogh, K.B.R.M., Jørgensen, C.I., Tovborg, M., Anthonsen, A., Tryfona, T., Walter, C.P., Dupree, P., Xu, F., Davies, G.J. and Walton, P.H. 2011. Insights into the oxidative degradation of cellulose by a copper metalloenzyme that exploits biomass components. *Proc. Natl. Acad. Sci. U.S.A.* **108**, pp. 15079–15084.

Rahikainen, J., Mikander, S., Marjamaa, K., Tamminen, T., Lappas, A., Viikari, L. and Kruus, K. 2011. Inhibition of enzymatic hydrolysis by residual lignins from softwood—study of enzyme binding and inactivation on lignin-rich surface. *Biotechnol. Bioeng.* **108**, pp. 2823–2834.

Ralph, J., Grabber, J.H. and Hatfield, R.D. 1995. Lignin–ferulate crosslinks in grasses: active incorporation of ferulate polysaccharide esters into ryegrass lignins. *Carbohydr. Res.* **275**, pp. 167–178.

Ramos, L.P. 2003. The chemistry involved in the steam treatment of lignocellulosic materials. *Quim. Nova* **26**, pp. 863–871.

Ramos, L.P., Nazhad, M.M. and Saddler, J.N. 1993. Effect of enzymatic hydrolysis on the morphology and fine structure of pretreated cellulosic residues. *Enzyme Microb. Technol.* **15**, pp. 821–831.

Reinikainen, T., Ruohonen, L., Nevanen, T., Laaksonen, L., Kraulis, P., Jones, T.A., Knowles, J.K.C. and Teeri, T.T. 1992. Investigation of the function of mutated cellulose-binding domains of *Trichoderma reesei* cellobiohydrolase I. *PROTEINS Struct. Funct. Genet.* **14**, pp. 475–482.

Saka, S. 1991. Chemical composition and distribution. In: Hon, D.N.-S. and Shiraishi, N. (Eds.). *Wood and Cellulosic Chemistry*, Marcel Dekker, Inc., New York. Pp. 59–88.

Scalbert, A., Monties, B., Lallemand, J.-Y., Guittet, E. and Rolando, C. 1985. Ether linkage between phenolic acids and lignin fractions from wheat straw. *Phytochem.* **24**, pp. 1359–1362.

Selig, M.J., Knoshaug, E.P., Adney, W.S., Himmel, M.E. and Decker, S.R. 2008. Synergistic enhancement of cellobiohydrolase performance on pretreated corn stover by addition of xylanase and esterase activities. *Bioresour. Technol.* **99**, pp. 4997–5005.

Shallom, D. and Shoham, Y. 2003. Microbial hemicellulases. *Curr. Op. Microbiol.* **6**, pp. 219–228.

Sipos, B., Benkő, Z., Dienes, D., Réczey, K., Viikari, L. and Siika-aho, M. 2009. Characterisation of specific activities and hydrolytic properties of cell-wall degrading enzymes produced by *Trichoderma reesei* Rut C30 on different carbon sources. *Appl. Biochem. Biotechnol.* **161**, pp. 347–364.

Sjöström, E. 1993. Wood Polysaccharides: Wood Chemistry, Fundamentals and Applications. Academic Press Inc., San Diego. Pp. 1–20, 51–70.

Solomon, B.D. 2010. Biofuels and sustainability. *Ann. N.Y. Acad. Sci.* **1185**, pp. 119–134.

Somerville, C., Bauer, S., Brininstool, G., Facette, M., Hamann, T., Milne, J,. Osborne, E., Paredez, A,. Persson, S., Raab, T., Vorwerk, S. and Youngs, H. 2004. Towards a systems approach to understanding plant cell walls. *Science* **306**, pp. 2206–2211.

Ståhlberg, J., Johansson, G. and Pettersson, G. 1988. A binding-site-deficient, catalytically active, core protein of endoglucanase III from the culture filtrate of *Trichoderma reesei*. *Eur. J. Biochem.* **173**, pp. 179–183.

Ståhlberg, J., Johansson, G. and Pettersson, G. 1991. A new model for enzymatic hydrolysis of cellulose based on the two-domain structure of cellobiohydrolase I. *Bio/Technol.* **9**, pp. 286–290.

Stålbrand, H. 2003. Enzymology of endo-1,4-β-mannanases. In: *Handbook of Food Enzymology*. Whitaker, J.R., Voragen, A.J.G. and Wong, D.W.S. (Eds.). Marcel Dekker, Inc., New York. Pp. 961–969.

Stålbrand, H., Siika-aho, M., Tenkanen, M. and Viikari, L. 1993. Purification and characterization of two β-mannanases from *Trichoderma reesei*. *J. Biotechnol.* **29**, pp. 229–242.

Suh, J.-H. and Choi, Y.-J. 1996a. Synergism among endo-xylanase, β-xylosidase and acetyl xylan esterase from *Bacillus stearothermophilus*. *J. Microbiol. Biotechnol.* **6**, pp. 173–178.

Suh, J.-H. and Choi, Y.-J. 1996b. Synergic effects among endo-xylanase, β-xylosidase, and α-L-arabinofuranosidase from *Bacillus stearothermophilus*. *J. Microbiol. Biotechnol.* **6**, pp. 179–183.

Sun, Y. and Cheng, J. 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresour. Technol.* **83**, pp. 1–11.

Suurnäkki, A., Tenkanen, M., Siika-Aho, M., Niku-Paavola, M.-L., Viikari, L. and Buchert, J. 2000. *Trichoderma reesei* cellulases and their core domains in the hydrolysis and modification of chemical pulp. *Cellulose* **7**, pp. 189–209.

Szijártó, N., Siika-aho, M., Sontag-Strohm, T. and Viikari, L. 2011. Liquefaction of hydrothermally pretreated wheat straw at high-solids content by purified Trichoderma enzymes. *Bioresour. Technol.* **102**, pp. 1968–1974.

Tabka, M.G., Herpoël-Gimbert, I., Monod, F., Asther, M. and Sigoillot, J.C. 2006. Enzymatic saccharification of wheat straw for bioethanol production by a combined cellulase xylanase and feruloyl esterase treatment. *Enzyme Microb. Technol.* **39**, pp. 897–902.

Tanahashi, M., Takada, S., Aoki, T., Goto, T., Higuchi, T. and Hanai, S. 1983. Characterization of explosion wood. 1. Structure and physical properties. *Wood Res.* **69**, pp. 36–51.

Teeri, T.T. 1997. Crystalline cellulose degradation: new insight into the function of cellobiohydrolases. *Trends Biotechnol.* **15**, pp. 160–167.

Teeri, T.T., Koivula, A., Linder, M., Wohlfahrt, G., Divne, C. and Jones, T.A. 1998. *Trichoderma reesei* cellobiohydrolases: why so efficient on crystalline cellulose? *Biochem. Soc. Trans.* **26**, pp. 173–178.

Tengborg, C., Galbe, M. and Zacchi, G. 2001. Influence of enzyme loading and physical parameters on the enzymatic hydrolysis of steam-pretreated softwood. *Biotechnol. Prog.* **17**, pp. 110–117.

Tenkanen, M., Puls, J. and Poutanen, K. 1992. Two major xylanases of *Trichoderma reesei*. *Enzyme Microb. Technol.* **14**, pp. 566–574.

Tenkanen, M., Thornton, J. and Viikari, L. 1995. An acetylglucomannan esterase of *Aspergillus oryzae*; purification, characterization and role in the hydrolysis of *O*-acetyl-galactoglucomannan. *J. Biotechnol.* **42**, pp. 197–206.

Tenkanen, M., Makkonen, M., Perttula, M., Viikari, L. and Teleman, A. 1997. Action of *Trichoderma reesei* mannanase on galactoglucomannan in pine kraft pulp. *J. Biotechnol.* **57**, pp. 191–204.

Tenkanen, M., Tamminen, T. and Hortling, B. 1999. Investigation of lignin-carbohydrate complexes in kraft pulps by selective enzymatic treatments. *Appl. Microbiol. Biotechnol.* **51**, pp. 241–248.

van Tilbeurgh, H., Pettersson, G., Bhikabhai, R., De Boeck, H. and Claeyssens, M. 1985. Studies of the cellulolytic system of *Trichoderma reesei* QM 9414. Reaction specificity and thermodynamics of interactions of small substrates and ligands with the 1,4-β-glucan cellobiohydrolase II. *Eur. J. Biochem.* **148**, pp. 329–334.

van Tilbeurgh, H., Tomme, P., Claeyssens, M., Bhikhabhai, R. and Pettersson, G. 1986. Limited proteolysis of the cellobiohydrolase I from *Trichoderma reesei*. Separation of functional domains. *FEBS Lett.* **204**, pp. 223–227.

van Tilbeurgh, H., Loontiens, F.G., de Bruyne, C.K. and Claeyssens, M. 1988. Fluorogenic and chromogenic glycosides as substrates and ligands of carbohydrases. *Methods Enzymol.* **160**, pp. 45–59.

Timell, T.E. 1967. Recent progress in the chemistry of wood hemicelluloses. *Wood Sci. Technol.* **1**, pp. 45–70.

Tomme, P., van Tilbeurgh, H., Pettersson, G., van Damme, J., Vandekerckhove, J., Knowles, J., Teeri, T. and Claeyssens, M. 1988. Studies of the cellulolytic system of *Trichoderma reesei* QM 9414. Analysis of domain function in two cellobiohydrolases by limited proteolysis. *Eur. J. Biochem.* **170**, pp. 575–581.

Tu, M., Chandra, R.P. and Saddler, J.N. 2007. Recycling cellulases during the hydrolysis of steam exploded and ethanol pretreated Lodgepole pine. *Biotechnol. Progr.* **23**, pp. 1130–1137.

Väljamäe, P., Sild, V., Nutt, A., Pettersson, G. and Johansson, G. 1999. Acid hydrolysis of bacterial cellulose reveals different modes of synergistic action between cellobiohydrolase I and endoglucanase I. *Eur. J. Biochem.* **266**, pp. 327–334.

Verbruggen, M.A., Beldman, G. and Voragen, A.G.J. 1998. Enzymic degradation of sorghum glucuronoarabinoxylans leading to tentative structures. *Carbohydr. Res.* **306**, pp. 275–282.

Vlasenko, E., Schülein, M., Cherry, J. and Xu, F. 2010. Substrate specificity of family 5, 6, 7, 9, 12, and 45 endoglucanases. *Bioresour. Technol.* **101**, pp. 2405–2411.

Vogel, J. 2008. Unique aspects of the grass cell wall. *Curr. Op. Plant Biol.* **11**, pp. 301–307.

de Vries, R.P. and Visser, J. 2001. Aspergillus enzymes involved in degradation of plant cell wall polysaccharides. *Microbiol. Mol. Biol. Rev.* **65**, pp. 497–522.

de Vries, R.P., Kester, H.C.M., Poulsen, C.H., Benen, J.A.E. and Visser, J. 2000. Synergy between enzymes from Aspergillus involved in the degradation of plant cell wall polysaccharides. *Carbohydr. Res.* **327**, pp. 401–410.

de Vries, B.J.M., van Vuuren, D.P. and Hoogwijk, M.M. 2007. Renewable energy sources: Their global potential for the first-half of the 21st century at a global level: An integrated approach. *Energy Policy* **35**, pp. 2590–2610.

Wang, L., Zhang, Y. and Gao, P. 2008. A novel function for the cellulose binding module of cellobiohydrolase I. *Sci. China Ser. C-Life Sci.* **51**, pp. 620–629.

Willför, S., Sundberg, A. and Hemming, J. 2005a. Polysaccharides in some industrially important softwood species. *Wood Sci. Technol.* **39**, pp. 245–258.

Willför, S., Sundberg, A., Pranovich, A. and Holmbom, B. 2005b. Polysaccharides in some industrially important hardwood species. *Wood Sci. Technol.* **39**, pp. 601–617.

Wingren, A., Galbe, M. and Zacchi, G. 2003. Techno-economic evaluation of producing ethanol from softwood: Comparison of SSF and SHF and identification of bottlenecks. *Biotechnol. Progr.* **19**, pp. 1109–1117.

Wyman, C.E. 1999. Biomass ethanol: technical progress, opportunities, and commercial challenges. *Annu. Rev. Energy Environ.* **24**, pp. 189–226.

Wyman, C.E., Dale, B.E., Elander, R.T., Holtzapple, M., Ladisch, M.R. and Lee, Y.Y. 2005. Comparative sugar recovery data from laboratory scale application of leading pretreatment technologies to corn stover. *Bioresour. Technol.* **96**, pp. 2026–2032.

Xiao, Z., Zhang, X., Gregg, D.J. and Saddler, J.N. 2004. Effects of sugar inhibition on cellulases and β-glucosidase during enzymatic hydrolysis of softwood substrates. *Appl. Biochem. Biotechnol.* **113–116**, pp. 1115–1126.

Yang, B. and Wyman, C.E. 2008. Pretreatment: the key to unlocking low-cost cellulosic ethanol. *Biofuels, Bioprod. Bioref.* **2**, pp. 26–40.

Yang, B., Willies, D.M. and Wyman, C.E. 2006. Changes in the enzymatic hydrolysis rate of Avicel cellulose with conversion. *Biotechnol. Bioeng.* **94**, pp. 1122–1128.

Yokoyama, T., Kadla, J.F. and Chang, H.-M. 2002. Microanalytical method for the characterization of fiber components and morphology of woody plants. *J. Agric. Food Chem.* **50**, pp. 1040–1044.

Zhang, Y.-H.P. and Lynd, L.R. 2004. Toward an aggregated understanding of enzymatic hydrolysis of cellulose: Noncomplexed cellulase systems. *Biotechnol. Bioeng.* **88**, pp. 797–824.

Zhu, L., O'Dwyer, J.P., Chang, V.S., Granda, C.B. and Holtzapple, M.T. 2008. Structural features affecting biomass enzymatic digestibility. *Bioresour. Technol.* **99**, pp. 3817–3828.

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PAPER I

Changes in submicrometer structure of enzymatically hydrolyzed microcrystalline cellulose

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Changes in Submicrometer Structure of Enzymatically Hydrolyzed Microcrystalline Cellulose

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To understand the limitations occurring during enzymatic hydrolysis of cellulosic materials in renewable energy production, we used wide-angle X-ray scattering (WAXS), small-angle X-ray scattering (SAXS), X-ray microtomography, and transmission electron microscopy (TEM) to characterize submicrometer changes in the structure of microcrystalline cellulose (Avicel) digested with the *Trichoderma reesei* enzyme system. The microtomography measurements showed a clear decrease in particle size in scale of tens of micrometers. In all the TEM pictures, similar elongated and partly ramified structures were observed, independent of the hydrolysis time. The SAXS results of rewetted samples suggested a slight change in the structure in scale of 10−20 nm, whereas the WAXS results confirmed that the degree of crystallinity and the crystal sizes remained unchanged. This indicates that the enzymes act on the surface of cellulose bundles and are unable to penetrate into the nanopores of wet cellulose.

Introduction

Cellulose, the most abundant biopolymer on earth, can be used as a renewable energy source. Cellulosic materials can be hydrolyzed into fermentable sugars, which can be further utilized as raw material for biofuel production. In second gereneration biorefineries one of the aims is to use agricultural or forest industry waste materials not competing with food production. Until today, most of the raw materials for production of biofuels originate from food plants, which cannot be considered a sustainable solution.[1,2]

The hydrolysis of cellulose can be carried out with the aid of acids or cellulase enzymes. Though the processes have been investigated already for several decades, unanswered questions still remain, concerning especially the reaction rates and the physicochemical interactions between the enzymes and the substrate material.[3] It has been discovered that the reactions are strongly affected by the physicochemical properties and the morphology of the substrate.[4] These parameters influence the accessibility of the enzymes to the substrate and they may also change during the reaction. The changes in cellulase activity and availability might be responsible for the gradual slowing down of the reaction, which is presently one of the main factors retarding the full use of the method. The accessibility of cellulose is also an important factor related to this subject, because the hydrolysis is a two-phase reaction.[5,6]

Several properties such as crystallinity, degree of polymerization, surface area, porosity, and particle size and their effects on the rates of hydrolysis have been investigated.[2,4,5,7] For instance, in the case of a partly crystalline substrate, such as microcrystalline cellulose (MCC), a higher degree of crystallinity seems to reduce the surface area suitable for binding of the enzymes and in this way hinders the reaction.[5] However, this is not necessarily true for all cellulosic substrates.[6] Concerning the possible change in the degree of crystallinity or the dimensions of the crystallites during hydrolysis, diverse results for different substrates have been obtained. Some studies have shown that the crystallinity has slightly increased,[7,8] while others have reported that it has remained unchanged during the hydrolysis.[4] Likewise, the average crystal size determined from the 200 reflection, that is, the width of the crystallites, has decreased,[4,7] remained unchanged,[4] or even increased with the hydrolysis.[8] A logical explanation for most of the changes would be the more readily hydrolyzable amorphous parts in cellulose microfibrils, which would be hydrolyzed faster, leaving proportionally more crystalline cellulose behind.[8] However, this hypothesis has been questioned, because some findings indicate that the crystallinity of cellulose does not change appreciably during enzymatic hydrolysis.[9]

In a larger scale, the morphology of the cellulose particles becomes an important factor. Because the average diameter of cellulolytic enzymes is as high as 5.9 nm,[10] the accessibility of the enzymes to the substrate is highly dependent on the roughness of the surface and on the pore structure of the substrate particles. According to previous studies, in which the crystallinities remained the same and the surface roughness increased, the hydrolysis of microcrystalline cellulose is supposed to take place in the outer layer of the particle surface

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and to proceed layer by layer.[7] Furthermore, the smaller and more readily accessible particles would be hydrolyzed faster, which causes that the average parameters remain as there is always a larger proportion of completely intact particles in the sample.[4]

In this study, the effects of enzymatic hydrolysis on the structure of microcrystalline cellulose were investigated with a purpose to find new insights into the limiting factors, especially related to the substrate structure, responsible for the gradual slowing down of the process. The used enzymes originated from the *Trichoderma reesei* species and the hydrolysis times varied between 6 and 75 h, eventually leading to a 68% degree of hydrolysis. The main characterization was done with X-ray scattering methods, wide- and small-angle X-ray scattering (WAXS and SAXS). They have been widely used for similar purposes and they have proved to be suitable for studies of partly crystalline and highly hierarchical biological soft matter.[4,7,11,12] In addition to the scattering methods, the morphology of the samples was studied with X-ray microtomography and transmission electron microscopy (TEM).

Experimental Section

Enzymatic Hydrolysis. The starting material of the hydrolysis was commercially available microcrystalline cellulose, Avicel (14204, SERVA Electrophoresis), with a particle size of 0.05 mm. Avicel was hydrolyzed with a mixture of commercial enzyme preparations (Celluclast 1.5 L and Novozym 188 by Novozymes, Denmark) at a loading of Celluclast (10 FPU/g dry weight substrate) supplemented with Novozym 188 β-glucosidase (100 nkat/g dry weight), corresponding to a total load of 20 mg protein/g of glucan. The cellulases originated from the *Trichoderma reesei* enzyme system and the β-glucosidase from *Aspergillus niger*. The hydrolyses were carried out in 50 mM sodium-citrate buffer, pH adjusted to 5.0, shaken at 130 rpm at 45 °C, with 1% substrate consistency. After hydrolysis, the samples were boiled for 10 min to inactivate the enzymes, then filtered through a 60 μm filter. The hydrolysis residue was freeze-dried for the X-ray and TEM measurements.

The enzymatic hydrolyses for samples after 6, 24, and 75 h of hydrolysis were evaluated on the basis of reducing sugars liberated, using the DNS method.[13] The degree of hydrolysis was calculated as the percentage solubilized of the total dry weight. The hydrolysis was originally done in duplicates, but the parallel hydrolysis residues were mixed before freeze-drying.

WAXS Measurements. For the WAXS measurements, the samples were prepared by pressing the sample powders by hand into metal rings of a thickness of 1.5 mm and covering them on both sides with Mylar foil. The radiation was produced by an X-ray generator (UltraX18S, Rigaku) with a rotating copper anode. The beam was monochromated with a Si(111) crystal and a totally reflecting mirror, thus, singling out the Cu Kα radiation (λ = 1.541 Å). After the sample, the scattered radiation was detected with an image plate detector (MAR345, Marresearch) in perpendicular transmission geometry. Each sample was measured for 30 min in room temperature. In addition, the air scattering with Mylar foils and the "dark current" produced mainly by the image reading process of the detector were measured and taken into account in the analysis of the results. The instrumental broadening of the measurement device, which was determined to be approximately 0.3° at the scattering angle $2\theta = 28°$ by measuring a silicon sample, was also considered during the analysis.

The 004 reflection ($2\theta = 35°$) was measured separately with a four-circle diffractometer, which included a four-circle goniometer (420/511, Huber), an X-ray generator (Siemens), a sealed X-ray tube with a copper anode, a ground, and bent germanium monochromator, and a NaI(Tl) scintillation counter. For these measurements, the sample powders were pressed as thin plates in metal holders and they were measured in symmetrical transmission geometry. The instrumental broadening was determined by measuring a sample of hexamethyltetramine ($C_6H_{12}N_4$).

SAXS Measurements. The SAXS measurements were carried out on the same samples as the WAXS measurements. In addition, the measurements were repeated with rewetted samples. These were prepared by immersing the once freeze-dried powders for a week in water with a concentration of approximately 3 wt %. The excess water was removed right before the measurements and the swollen powders were placed in metal rings and sealed with Mylar foil, similarly to the dry samples.

The SAXS measurement apparatus consisted of an X-ray generator (Siemens) with an X-ray tube with a copper anode (Panalytical) and a two-dimensional wire detector (HI-STAR, Bruker AXS). The operational voltage and current of the generator were 36 kV and 25 mA, respectively. The monochromated beam was point-collimated and the measurement geometry was a perpendicular transmission. Besides the three dry and three wet samples, the scattering of an empty metal ring covered with Mylar foil was measured for the background correction. The measurement time was 60 min for each. The measured q-values were in the range of 0.014−0.22 1/Å, with the definition $q = (4\pi \sin \theta)/(\lambda)$ for the length of the scattering vector. A piece of Lupolen was used as a standard in scaling the intensities to absolute scale.[12] The measurement program made spatial and flood-field corrections for the data.

Microtomography Measurements. The measurement system was a custom-made X-ray microtomography device (nanotom180 NF, Phoenix|X-ray Systems + Services), including a flat panel CMOS detector (Hamamatsu) with 2304 × 2304 pixels (pixel size 50 μm). The X-ray tube had a tungsten anode and its operational voltage and current were 60 kV and 400 μA, respectively. The X-ray beam was approximately conical in shape, making it possible to image samples in μm-resolution by utilizing geometric magnification (see, e.g., Stock[14]). Here this magnification was 50.

The sample powders were encapsulated into Eppendorf tubes which were attached to the sample holder of the tomography device using beeswax. In the measurements, 1440 absorption images (projections) were acquired in 0.25 degree steps over a full circle of rotation. Each projection was an average of five individual images of 750 ms exposure time. Three-dimensional reconstructions were made with the program datos|x (version 1.3.2.11) supplied by Phoenix|X-ray Systems + Services. The voxel size of the reconstructions was the same as the pixel size of the projections, 1.00 μm. The reconstructed volumes were visualized using the program VGStudio MAX 1.2.1 (Volume Graphics GmbH, Germany).

Cryo-TEM Measurements. The sample powders were immersed in water for about one week before the measurements. Thereafter, 3 mL of sample solution was applied on the holey carbon EM grids and vitrified using a FEI Vitrobot: excess water was removed by blotting the grids several times for a duration of 1 s, after which the grids were plunged in a mixture of liquid ethane and propane at temperature of −180 °C. The grids with vitrified sample solution were maintained at liquid nitrogen temperature and then cryo transferred into the microscope using Gatan 910 cryo-transfer holder at temperature of about −185 °C. The samples were imaged with a FEI Tecnai 12 bright-field transmission electron microscope with a LaB_6 filament. The electrons were accelerated with a voltage of 120 kV and they were detected after the sample with a CCD camera (Ultrascan 1000, Gatan) with a pixel size of 14 μm and a 16 bit dynamic range. The image magnifications varied from less than 1000× to 62000×.

Data Analysis. *WAXS Analysis.* The integrated intensities were corrected for absorption, measuring geometry, and scattering by air and Mylar foils. When determining the crystal sizes, an amorphous background intensity was subtracted from the data. The background was the measured scattering of a sulfate lignin sample, which has proved to be a suitable approximation for the amorphous background of MCC samples.[15]

Table 1. Crystal Size and Degree of Crystallinity of Enzymatically Hydrolyzed MCC Determined by the WAXS Analysis

		crystal size (nm) corresponding to reflections				
hydrolysis time (h)	degree of hydrolysis	$1\bar{1}0$	110	200	004	degree of crystallinity (%)
0[a]		5.0 ± 0.3	5.0 ± 0.4	5.34 ± 0.10		48 ± 3
6	30.8 ± 0.3	4.7 ± 0.3	5.5 ± 0.4	5.36 ± 0.10	21 ± 2	49 ± 3
24	57.9 ± 0.8	4.8 ± 0.3	5.6 ± 0.4	5.24 ± 0.10	22 ± 2	50 ± 3
75	67.6 ± 0.5	4.8 ± 0.3	5.7 ± 0.4	5.27 ± 0.10	24 ± 2	49 ± 3

[a] The corresponding values for nonhydrolyzed MCC, measured earlier.

The crystal sizes were calculated by fitting Gaussian functions to peaks corresponding to the reflections $1\bar{1}0$, 110, and 200 of cellulose Iβ. From the full width at half-maximum (fwhm) and the position of the maximum of the fit, the crystal size corresponding to each reflection could be calculated using the Scherrer equation (eq 1, see Supporting Information for equations and more details on the analysis of scattering data).

The degree of crystallinity was determined by a curve fitting procedure, in which altogether 24 Gaussian functions were fitted to the data corresponding to the theoretical reflections of cellulose.[16] The degree of crystallinity was then calculated as the ratio of the intensity scattered by the crystalline part and the measured intensity, using sulfate lignin again as an approximation for the amorphous part.[15]

The crystal sizes corresponding to the 004 reflection, that is, the average lengths of the crystallites, were determined from the four-circle diffractometer measurements. The amorphous background was assumed to be linear near this reflection and the crystal lengths were calculated using the Scherrer equation (eq 1).

SAXS Analysis. The SAXS intensity of a system of two phases with smooth interfaces obeys the Porod law ($I(q) \propto q^{-4}$). When knowing the invariant $Q = \int_0^\infty I(q)q^2 \mathrm{d}q$ and the volume fractions of the phases, the specific surface of the system can be calculated from eq 2 in the Supporting Information. One way to gain the invariant Q is presented in eq 3. This approach requires the intensities to be on absolute scale, which can be reached by measuring a reference sample (see eq 4). The specific surfaces for each sample, that is, the surface area in a mass unit of the solid material, presented later in this article, were calculated with eq 5.[12,17,18]

With the Porod law it is also possible to determine the Porod lengths, that is, average chord lengths of the system. These measures tell about the average distances averaged in all directions between interfaces in the system. The average chord lengths for the whole system and separately for both phases were calculated with eqs 6 and 7.[17,19,20]

If the data obeys a power law $I(q) \propto q^{-\alpha}$ with some other power than $\alpha = 4$, the system may contain fractal structures. Such power laws can be visible in any q-region, for which holds $qR_g \gg 1$. Here R_g can be the radius of gyration of the particles in the sample or some other length typical for the dimensions of the phases. The fractal structures can be either mass or surface fractals, which appear as powers $\alpha \leq 3$ and $3 < \alpha < 4$, respectively. The fractal dimensions D_m and D_s for these structures were calculated with eq 8. Large D_s values correspond to smooth surfaced and internally dense particles, smaller D_s values to internally dense particles with rough surfaces. A smaller D_m represents a more open or ramified structure. The special case of integer power $\alpha = 1$ corresponds to thin cylinders or strings and the power $\alpha = 2$ to thin planes or disks.[17,21]

Results

Degrees of Hydrolysis. The samples studied were hydrolyzed for 6, 24, and 75 h, which corresponded to 30.8, 57.9, and 67.6% of hydrolysis degrees, respectively (Table 1). The behavior of the degree of hydrolysis as a function of hydrolysis time is typical: the rate of hydrolysis is relatively fast in the beginning and gradually decreases with time.[7]

WAXS Results. The WAXS curves are presented as the integrated and corrected intensity of the scattered beam I as a function of the scattering angle 2θ (Figure 1). The shapes of the curves are almost identical and their vertical displacements are most likely due to different sample masses. The calculated average crystal sizes in four directions and the degree of crystallinity are shown in Table 1. The error estimates were mainly determined by slightly varying the fitting parameters and observing their influence on the eventual values.

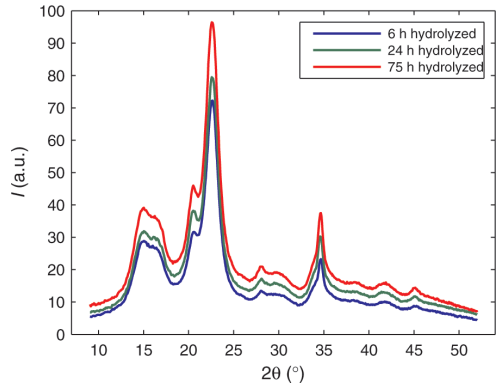

Figure 1. WAXS curves of MCC hydrolyzed enzymatically for various times.

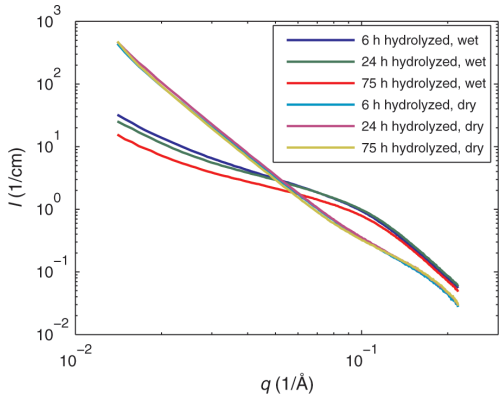

Figure 2. Logarithmic SAXS curves of MCC hydrolyzed enzymatically for various times. A small constant based on Porod's law has been subtracted from the intensities.

SAXS Results. The SAXS curves for both dry and wet samples are presented on a logarithmic scale (Figure 2). The intensities were calculated from the experimental data with eq 4 and they are thus in absolute units (cm^{-1}). The optical thickness of each sample was calculated using values of 3.33 l/cm,[12] 12 l/cm, and 10.4 l/cm[22] for the linear absorption coefficients of Lupolen, cellulose, and water, respectively. The volume fractions were determined with the masses and volumes of the samples, using the density of crystalline cellulose (1.6 g/cm^3) as the density of the solid phase.

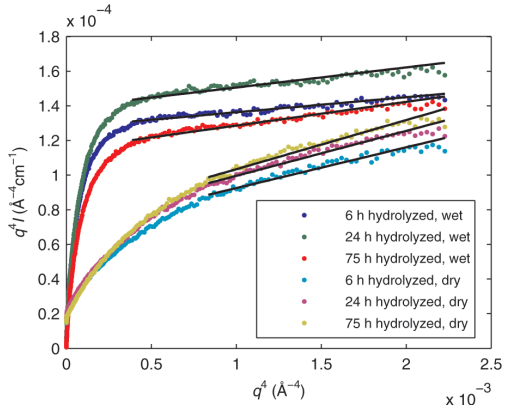

Figure 3. Porod fits for all samples.

Table 2. Powers α Gained from the Power Law Fits on Regions [q_{min}, q_{max}] and the Calculated Fractal Dimensions *D*

	6 h wet	24 h wet	75 h wet	6 h dry	24 h dry	75 h dry
q_{min} (1/Å)	0.027	0.027	0.027	0.019	0.019	0.019
q_{max} (1/Å)	0.077	0.077	0.077	0.074	0.071	0.071
α	1.5	1.3	1.2	3.5	3.7	3.6
D[a]	1.5 (m)	1.3 (m)	1.2 (m)	2.5 (s)	2.3 (s)	2.4 (s)

[a] The letter "m" in brackets stands for mass fractals and the letter "s" stands for surface fractals.

Table 3. Specific Surfaces ($S_{s,m}$) and the Average Chord Lengths for Whole Samples (L_c) and Separately for the Matrix (L_m) and Solid (L_s) Phases

	6 h wet	24 h wet	75 h wet	6 h dry	24 h dry	75 h dry
$S_{s,m}$ (m^2/g)	330	410	220	3.3	3.5	3.6
L_c (nm)	6.4	5.2	9.0	140	140	160
L_m (nm)				570	510	430
L_s (nm)				180	200	270

The fitting of Porod's q^{-4} law was carried out for all samples (Figure 3). A constant of order 10^{-2} l/cm was subtracted from the intensity in order to improve the fit.[23] The fitting ranges were $q = 0.140-0.218$ 1/Å for the wet and $q = 0.170-0.218$ 1/Å for the dry samples. These ranges were chosen by following the criteria presented by Vickers et al.[20]

Power laws with other powers than $\alpha = 4$ were fitted to the data on smaller *q*-values. They were found on slightly different regions for wet and dry samples. These regions as well as the α values and fractal dimensions are presented in Table 2. The fractal dimensions show that the nanoscale structure of the particles in the dry samples is much denser than in the wet samples.

The specific surfaces and average chord lengths calculated from the Porod law fit with eqs 5, 6, and 7 are shown in Table 3. The average chord lengths were calculated from the specific surfaces (eqs 6 and 7) with the aid of the volume fractions of the two phases. Because some inaccuracy was involved in the determination of the volume fractions for the wet samples, the average chord lengths of the phases (L_m and L_s) are not presented in the table. This is also why the results for wet samples in Table 3 should not be compared with each other. On the contrary, the results for dry samples are well comparable between different hydrolysis times, but they may include systematic error of maximum 50% due to the density value of the solid phase used in the calculations. The magnitudes and possible sources of error in the results of Table 3 are further discussed in the Discussion.

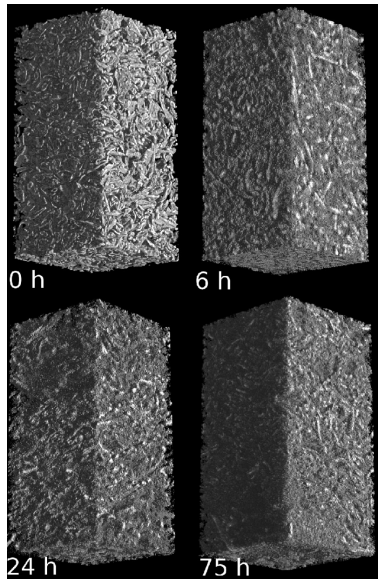

Figure 4. Three-dimensional X-ray microtomography reconstructions from enzymatically hydrolyzed MCC with various hydrolysis times. The native MCC sample was imaged earlier. The dimensions of the reconstructions are 0.5 × 0.5 × 1.0 mm^3.

Microtomography and Cryo-TEM Images. The micrometer-size structure of the samples was evaluated using three-dimensional microtomographic reconstructions (Figure 4) as well as cross sections of these reconstructions. The microtomography images are suitable for qualitative comparison between the samples with various hydrolysis degrees. From the images a clear decrease of particle size could be seen in the micrometer scale. Also, the boundaries of the particles were more irregular and fuzzy in the hydrolyzed samples. However, because the voxel size of the reconstruction was 1.00 μm, structures smaller than that could not be distinguished in the images.

The TEM images showed elongated sheet-like structures in various length scales (Figure 5). In pictures with lower magnification, these particles had widths in the order of 1 μm, whereas their lengths were approximately 10× higher (Figure 5b). These particles were interpreted as bundles or aggregates of several parallel microfibrils. Some of these bundles were ramified or frayed on their ends or sometimes also on their sides (Figure 5a). The individual strings were as thick as a microfibril on their thinnest and the cavities between them could extend a length of several micrometers in the longitudinal direction of the fibres (Figure 5d). The thicknesses of the particles were interpreted to vary, since individual microfibrils inside a bundle were to be seen in some pictures, whereas in some pictures the particles were completely opaque (Figure 5c). In addition to the larger particles, few individual microfibrils were seen in the samples (Figure 5e).

Discussion

According to the WAXS results, the average crystal sizes of cellulose and the crystallinity of the sample did not change during hydrolysis. The obtained values are in agreement with earlier results for similar types of celluloses; according to Andersson et al., the degree of crystallinity in Avicel is below

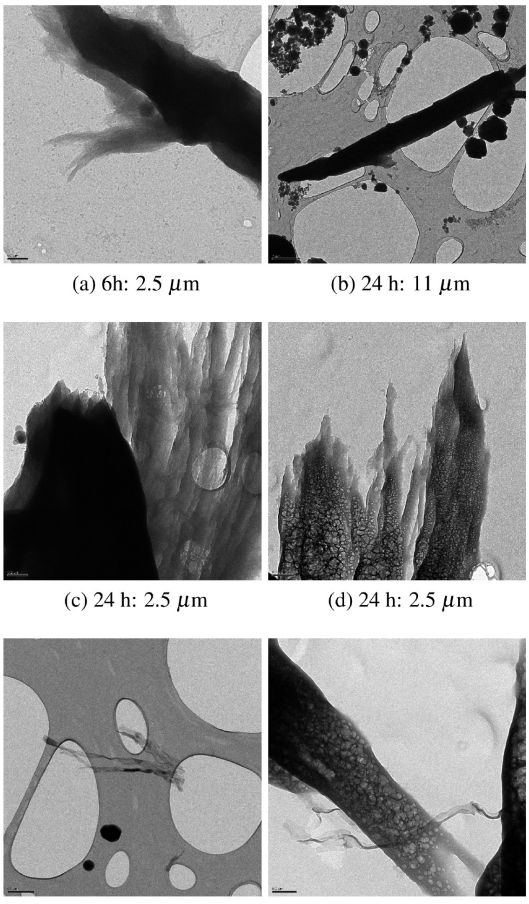

Figure 5. Collection of TEM pictures of enzymatically hydrolyzed MCC after various hydrolysis times. The length below each image indicates the length of one side of the image. The dark round-shaped objects are ice contamination and the big light circles holes in the carbon film, with which the grid was covered. The microfibril bundles are seen as partly ramified or smooth-surfaced and elongated particles. The bubbles inside of some of them are due to beam damage. Possible individual microfibrils can be seen in (e) and (f).

60%.[24] Previous studies have also shown that the crystallinity of MCC remains unchanged during hydrolysis,[4,9] which can be interpreted so that, independent of the hydrolysis time, most of each sample consists of intact cellulose fibrils. Based on this, many studies have proposed that the hydrolysis solubilizes the outermost fibrils of the aggregates one by one,[4,6] which seems a plausible interpretation also in this work. In addition, a small change in the crystallinity, as reported for instance by Wang et al. for cotton fibers during enzymatic hydrolysis,[7] could have stayed inside the error margins of this study. Concerning the crystal sizes, their behavior during hydrolysis seems to be strongly affected by the origin of the cellulosic substrate.[4,7,8] According to Gama and Mota, the crystal width of Avicel remained unchanged, whereas it decreased for the less crystalline Sigmacell during enzymatic hydrolysis.[4]

The power law behavior of the SAXS intensities was investigated on smaller q-values (Table 2). Based on the power laws, structures between 10−20 nm in wet samples and between 10−30 nm in dry samples could be described by fractal dimensions. The surface fractal dimensions of the dry samples corresponded to fairly smooth surfaces,[25] meaning that the particles were dense inside. The mass fractal dimensions of the wet samples corresponded to a more open structure formed by loose aggregates.[26] This mass fractal dimension diminished during the hydrolysis, which could mean an opening of the structures caused for instance by the enlargening of pores between the individual microfibrils or a ramification of the microfibril bundles, leading to structures like the ones observed in the TEM pictures (e.g., Figure 5d).

At this point, it must be noted that the theory concerning the interaction between water and MCC in submicrometer scale is not known exactly. A question arises, whether water can penetrate into the amorphous parts of the cellulose fibrils or not. This is an important question for the SAXS analysis, because only differences in electron density are seen by this method. If water can penetrate into the amorphous parts, the two phases visible for SAXS would be pieces of crystalline cellulose and a water matrix surrounding them. This was the assumption used in this study and its usage has been justified elsewhere.[20] The dry samples, on the other hand, would be seen as solid cellulose in air, having both the amorphous and the crystalline parts included in the same phase.

As already stated in Results, the SAXS results presented for the wet samples in Table 3 may contain some experimental error. The major source of this error was the imperfect determination of the volume fractions of water and cellulose. In the preparation of these samples, it was not guaranteed that the water would be equally dispersed in the whole sample and the total volume of the sample was not precisely the same for all samples. Thus, the actual proportion of water in the beam was hard to determine afterward and the values shown in Table 3 for the wet samples are not comparable between different hydrolysis times. For the dry samples, the determination of volume fractions was easier and the results for them are more reliable.

A minor source of error in the results of Table 3 might be the use of the density of crystalline cellulose as the density of the solid phase in all samples. A smaller density value of 1.4 g/cm^3 (the "true density" of MCC according to Sun[27]) in the analysis was observed to increase the specific surfaces of the dry samples by 50% and the same of the wet samples by 190%. Also, the average chord lengths of whole samples would have been 20 and 60% smaller for dry and wet samples, respectively. Especially the possible errors for the wet samples seem quite large, but, on the other hand, if the two phases in the wet samples are supposed to be water and crystalline cellulose, the use of the density of crystalline cellulose is justified. Also, the real density of the solid phase in the dry samples most likely settles somewhere between these extremes. In any case, the choice of the density value should have the same effect on the values from all samples, regardless of hydrolysis time.

Despite the possible inaccuracy in the actual values, the orders of magnitude of the specific surfaces and average chord lengths shown in Table 3 are representative. Similar results for dry and wet cellulose materials have been published earlier[20,28−30] and it is known that the pore structure of wet cellulose changes during drying and rewetting.[20] Wet cellulose contains a large proportion of narrow pores between individual microfibrils or small bundles of them. These pores occupy a large proportion of the total volume and yield a high specific surface. In dry cellulose, the pores are fewer and larger in their cross sections.[28−31] This explains the fact that the average chord lengths, that is, the average distances between interfaces of the phases, were 1−2 orders of magnitude larger in the dry samples

than in the wet samples. The specific surfaces were also 2 orders of magnitude smaller in the dry samples, because larger pores have substantially less surface area per volume than smaller pores.

No definite conclusions on the effects of hydrolysis on the structure of MCC can be drawn based on the results presented in Table 3. Especially the values for the wet samples behaved inconsistently as a function of hydrolysis time, which is most probably caused by the inaccuracy of the volume fractions. In spite of all, it might be possible, that the change in the values of the dry samples would correspond to a small change in the morphology. The specific surface seemed to increase slightly during hydrolysis, as the average chord length for the solid phase increased and the average chord length for the air phase decreased. Gupta and Lee as well as Wang et al. have both reported previously an increase in the specific surface of cellulose as a function of hydrolysis time,[5,7] but taking into account the inaccuracy of the results of this study, it is impossible to make further conclusions about any eventual changes.

Thus, in the SAXS measurements the only quantity which was seen to change undisputedly during hydrolysis was the fractal dimension of the wet samples. The reason why this change was seen only in the wet samples could be due to the different pore structures of wet and dry cellulose. So it might be possible that the changes during hydrolysis could be hidden inside the solid phase when the cellulose is dried and come visible again when the substance is rewetted. This phenomenon could be studied in the future by carrying out the measurements in real time or without drying. In addition, the Porod fit on larger q-values might have revealed more information on the structural changes of MCC during the hydrolysis, though the samples were not measured on that interval in this work.

In a recent study, Kent et al. studied the structure of Avicel FD100 hydrolyzed with cellulases from the *Trichoderma viride* enzyme system, accompanied by β-glucosidase from almond in some of the experiments.[9] Their main method was small-angle neutron scattering (SANS), but the samples were also characterized with scanning electron microscopy (SEM) and X-ray diffraction. The fundamental difference compared to this work was that the enzymes reacted either in a static state, that is, without any stirring or shaking, or in a dynamic state under constant agitation and motion through the sample chamber, whereas in this work the bottles were only shaken with a low rate. Despite a different enzyme origin and hydrolysis methods, their results were at least partly congruent with our results. In the SANS curves, they saw power laws on slightly different q-ranges, even though their fractal dimensions were higher and increased during the dynamic hydrolysis. They interpreted the fractal dimensions to describe the water distribution rather than the solid material, which then indicated a more compact and dense distribution of water inside the fibers. Their curves also showed a "roll-off" at $q = 0.08$ 1/Å, which disappeared in the beginning of the hydrolysis. In our results, a "roll-off" was seen at $q = 0.10$ 1/Å and it did not change during the hydrolysis. Similar to our results, their SEM images showed no change in the scale of hundreds of nanometers and according to their X-ray diffraction experiments the crystallinity index was preserved.

The results of this study support a common view of enzymatic hydrolysis of cellulosic materials.[6,7,10] According to our results, it seems that cellulase enzymes cannot penetrate into the nanopores of all MCC particles or at least cannot act synergistically in them. In contrast, the enzymes may proceed layer by layer on the outer surface of the cellulose bundles or possibly on the surfaces of larger pores inside them. Despite this, some of the bundles are already open enough toward the enzymes to enter or they may transform into such forms due to hydrolysis on their outer surface or on the surface of larger pores. This would result in an increased accessible surface for the enzymes and, hence, lead to a fast degradation of the whole bundle. This proportion of the substrate could even yield most of the total amount of glucose produced by the hydrolysis. In a similar way, Kent et al.[9] concluded that under agitation the cellulose, amorphous and crystalline on equal extents was digested around the larger pores throughout the particles. The smaller pores would not be large enough for the enzymes to penetrate. These ideas are also supported by the TEM pictures of this study, which besides giving a picture of the morphology of the micrometer-sized MCC particles, proved that there were still almost intact particles even in the last sample, where 68% of the original cellulose was degraded into glucose. The actual change between the samples was to be seen on a larger scale, for which the microtomography images provided an undisputable evidence.

Conclusions

Overcoming the bottlenecks in efficient biorefining is of a great importance to utilize the cellulosic biomass completely as a renewable energy source. Possibly the most critical step in the process is the degradation of cellulose into fermentable sugars, which requires efficient enzymatic hydrolysis. It has been shown previously that the structure and morphology of the substrate play a crucial role in the process. Therefore, the knowledge of this structure and the effect of hydrolysis on it are valuable information when searching for ways to improve the efficiency of the entire process.

In this work, the modification of the submicrometer structure of microcrystalline cellulose during the enzymatic hydrolysis was studied using X-ray scattering methods, X-ray microtomography, and transmission electron microscopy. Most of the results support the perception that the cellulase enzymes are not generally able to penetrate into the nanosized pores of cellulose. Instead, they degrade the bundles of microfibrils from the outer surface or from the surface of larger pores, proceeding layer by layer. The more easily accessible bundles are degraded faster, whereas the less easily accessible bundles can remain almost intact.

This interpretation was supported by the unchanged degree of crystallinity and crystal sizes in the WAXS results as well as the unaltered average dimensions in scale of 1−100 nm in the SAXS results. Besides the decrease of the microscale particle size seen in the tomography images, the only measure that was seen to change during the hydrolysis was the fractal dimension of the SAXS curves for wet samples in scale of 10−20 nm. This was interpreted to rise from a change in the pore structure corresponding to this length scale or a ramification of the bundles of microfibrils, which was also observed for some particles in the TEM pictures.

The results of this study may offer explanations for the gradual slowing down of enzymatic hydrolysis. Some of the substrate particles are impossible for the enzymes to penetrate into, at least without agitation. The questions apparently need more investigation, but each step is important on the way to the full usage of renewable energy sources.

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Supporting Information Available. All equations referred to in the Experimental Section and a more detailed description on the analysis of scattering data. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Himmel, M. E.; Ding, S.-Y.; Johnson, D. K.; Adney, W. S.; Nimlos, M. R.; Brady, J. W.; Foust, T. D. *Science* **2007**, *315*, 804–807.
(2) Jørgensen, H.; Kristensen, J. B.; Felby, C. *Biofuels, Bioprod. Biorefin.* **2007**, *1*, 119–134.
(3) Teeri, T. T. *Tibtech* **1997**, *15*, 160–167.
(4) Gama, F. M.; Mota, M. *Biocatal. Biotransform.* **1997**, *15*, 221–236.
(5) Gupta, R.; Lee, Y. Y. *Biotechnol. Bioeng.* **2009**, *102*, 1570–1581.
(6) Zhou, W.; Schüttler, H.-B.; Hao, Z.; Xu, Y. *Biotechnol. Bioeng.* **2009**, *104*, 261–274.
(7) Wang, L.; Zhang, Y.; Gao, P.; Shi, D.; Liu, H.; Gao, H. *Biotechnol. Bioeng.* **2006**, *93*, 443–456.
(8) Cao, Y.; Tan, H. *Enzyme Microb. Technol.* **2005**, *36*, 314–317.
(9) Kent, M. S.; Cheng, G.; Murton, J. K.; Carles, E. L.; Dibble, D. C.; Zendejas, F.; Rodriquez, M. A.; Tran, H.; Holmes, B.; Simmons, B. A.; Knierim, B.; Auer, M.; Banuelos, J. L.; Urquidi, J.; Hjelm, R. P. *Biomacromolecules* **2010**, *11*, 357–368.
(10) Gama, F. M.; Teixeira, J. A.; Mota, M. *Biotechnol. Bioeng.* **1994**, *43*, 381–387.
(11) Jungnikl, K.; Paris, O.; Fratzl, P.; Burgert, I. *Cellulose* **2008**, *15*, 407–418.
(12) Vainio, U.; Maximova, N.; Hortling, B.; Laine, J.; Stenius, P.; Simola, L. K.; Gravitis, J.; Serimaa, R. *Langmuir* **2004**, *20*, 9736–9744.
(13) Miller, G. L. *Anal. Chem.* **1959**, *31*, 426–428.
(14) Stock, S. R. *Int. Mater. Rev.* **1999**, *44*, 141–164.
(15) Andersson, S.; Serimaa, R.; Paakkari, T.; Saranpää, P.; Pesonen, E. *J. Wood Sci.* **2003**, *49*, 531–537.
(16) Nishiyama, Y.; Langan, P.; Chanzy, H. *J. Am. Chem. Soc.* **2002**, *124*, 9074–9082.
(17) Porod, G. General theory. In *Small Angle X-ray Scattering*; Glatter, O., Kratky, O., Eds.; Academic Press: London, 1982.
(18) Spalla, O.; Lyonnard, S.; Testard, F. *J. Appl. Crystallogr.* **2003**, *36*, 338–347.
(19) Ishii, D.; Kanazawa, Y.; Tatsumi, D.; Matsumoto, T. *J. Appl. Polym. Sci.* **2007**, *103*, 3976–3984.
(20) Vickers, M. E.; Briggs, N. P.; Ibbett, R. N.; Payne, J. J.; Smith, S. B. *Polymer* **2001**, *42*, 8241–8248.
(21) Schmidt, P. W. *J. Appl. Crystallogr.* **1991**, *24*, 414–435.
(22) NIST, 4.8; http://physics.nist.gov/, accessed 2009.
(23) Svergun, D. I.; Koch, M. H. J. *Rep. Prog. Phys.* **2003**, *66*, 1735–1782.
(24) Andersson, S.; Wikberg, H.; Pesonen, E.; Maunu, S. L.; Serimaa, R. *Trees* **2004**, *18*, 346–353.
(25) Ishii, D.; Tatsumi, D.; Matsumoto, T. *Biomacromolecules* **2003**, *4*, 1238–1243.
(26) Lenz, J.; Schurz, J. *Holzforschung* **1990**, *44*, 227–228.
(27) Sun, C. C. *Int. J. Pharm.* **2008**, *346*, 93–101.
(28) Crawshaw, J.; Bras, W.; Mant, G. R.; Cameron, R. E. *J. Appl. Polym. Sci.* **2002**, *83*, 1209–1218.
(29) Papirer, E.; Brendle, E.; Balard, H.; Vergelati, C. *J. Adhes. Sci. Technol.* **2000**, *14*, 321–337.
(30) Yu, X.; Atalla, R. H. *Powder Technol.* **1998**, *98*, 135–138.
(31) Luukkonen, P.; Maloney, T.; Rantanen, J.; Paulapuro, H.; Yliruusi, J. *Pharm. Res.* **2001**, *318*, 1562–1569.

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PAPER II

Restriction of enzymatic hydrolysis of pretreated spruce by lignin

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Restriction of the enzymatic hydrolysis of steam-pretreated spruce by lignin and hemicellulose

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ABSTRACT

The presence of lignin is known to reduce the efficiency of the enzymatic hydrolysis of lignocellulosic raw materials. On the other hand, solubilization of hemicellulose, especially of xylan, is known to enhance the hydrolysis of cellulose. The enzymatic hydrolysis of spruce, recognized among the most challenging lignocellulosic substrates, was studied by commercial and purified enzymes from *Trichoderma reesei*. Previously, the enzymatic hydrolysis of steam pretreated spruce has been studied mainly by using commercial enzymes and no efforts have been taken to clarify the bottlenecks by using purified enzyme components.

Steam-pretreated spruce was hydrolyzed with a mixture of Celluclast and Novozym 188 to obtain a hydrolysis residue, expectedly containing the most resistant components. The pretreated raw material and the hydrolysis residue were analyzed for the enrichment of structural bottlenecks during the hydrolysis. Lignin was removed from these two materials with chlorite delignification method in order to eliminate the limitations caused by lignin. Avicel was used for comparison as a known model substrate. Mixtures of purified enzymes were used to investigate the hydrolysis of the individual carbohydrates: cellulose, glucomannan and xylan in the substrates. The results reveal that factors limiting the hydrolysis are mainly due to the lignin, and to a minor extent by the lack of accessory enzymes. Removal of lignin doubled the hydrolysis degree of the raw material and the residue, and reached close to 100% of the theoretical within 2 days. The presence of xylan seems to limit the hydrolysability, especially of the delignified substrates. The hydrolysis results also revealed significant hemicellulose impurities in the commonly used cellulose model substrate, making it questionable to use Avicel as a model cellulose substrate for hydrolysis experiments.

1. Introduction

Fuels from lignocellulose biomass have a high potential to reduce green house gas emissions, and hence are important means to fulfil the road transport CO_2 emissions targets [1,2]. An increasingly important aspect is to utilize wastes and raw materials which do not compete with food production, such as agricultural residues or woody biomass. A further option is to produce new high yield crops on set-aside agricultural land or grassland areas [2].

The hydrolysis of the biomass to give fermentable sugars is still, however, a considerable bottleneck in the production of fuel-ethanol from lignocellulosic raw materials, especially from softwood [3]. The problem relates both to the enzymatic regulations and to the compact structure of the substrate itself [4].

Biomass is a complex material consisting of three major organic fractions, with representative compositions in softwood on a dry weight basis being as follows: 45–50% cellulose, 25–35% hemicellulose, and 25–35% lignin [5]. Biomass also contains smaller amounts of minerals (ash) and various extractives. In the Scandinavian countries spruce (*Picea* sp.), pine (*Pinus* sp.) and birch (*Betula* sp.) are the most widespread woody feedstocks. Spruce consists of 45–47% cellulose, 24–26% hemicellulose, 27–28% lignin and 0.4–0.9% extractives [6].

Woody cell walls are built up by several layers; the middle lamella, primary and secondary cell walls, and the warty layer. The middle lamella, located between the cells, is highly lignified, and is suggested to bind the cells together. The primary cell wall is formed by an irregular network of cellulose microfibrils combined with hemicellulose, pectin and proteins, embedded in lignin matrix. The primary cell wall is supported by a thicker secondary cell wall, composed of three distinct layers, from which the middle layer (S_2) forms the main portion. These layers consist of highly organised cellulosic microfibrils with an orientation nearly perpendicular to

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each other in the adjacent layers. The concentration of lignin is the highest in the middle lamella and the primary wall but, the overall amount of lignin is the highest in the S_2 because of its thickness [7].

The major hemicellulose constituents in softwood are glucomannans and xylans; galactoglucomannans making up 15–20% and xylans 7–10% of the biomass [8]. Recently, Willför et al. reported that, based on the overall hemicellulose sugar composition, galactoglucomannans and arabinoglucuronoxylans in Norway spruce sapwood and heartwood make up 14 and 8% of the total dry weight, respectively [9]. The backbone of softwood glucomannan is composed of β-(1→4)-linked D-glucopyranose and D-mannopyranose units, and it is partially substituted by α-galactose and acetyl units. About one-third of the mannose units of spruce glucomannan is acetylated [10]. In softwood, two types of glucomannans have been identified which differ in their solubility and molar ratio of galactose:glucose:mannose. The main glucomannan in softwoods has low galactose content with a ratio of 0.1:1:4 [7]. In the high-galactose containing galactoglucomannan the corresponding ratio is 1:1:3 [7]. In addition to galactoglucomannans, also linear and highly branched galactans have been found in softwood [11,12]. Softwood arabino-4-O-methylglucuronoxylan is composed of D-xylopyranose units connected via β-(1→4)-glycosidic linkages. The average molar ratio of arabinose:methylglucuronic acid:xylose sugar units in softwood xylan is 1.3:2:10 [7]. In spruce sapwood and heartwood, the galactose:glucose:mannose ratio was (0.5–0.9):1.1:3 and the arabinose:methylglucuronic acid:xylose ratio was (2.4–2.5):1.7:10, respectively [9]. Softwood xylans are not acetylated [13].

Linkages between hemicellulose and lignin have been reported in wood and other plant materials: in spruce, lignin is reported to be linked through covalent bonds to the major hemicellulose components; *i.e.* arabinoglucuronoxylan, galactoglucomannan and glucomannan [14]. Previously, Eriksson et al. found in black spruce that glucuronoxylans function primarily to stabilize the cell wall through hydrogen-bonding interactions with cellulose and covalent interactions with lignin [15]. On the other hand, Choi et al. found recently that galactose and mannose are predominantly linked to lignin fragments in residual lignins of spruce wood [16]. The proposed lignin–carbohydrate bonds include ether or ester linkages between the lignin unit and the hydroxyl or carboxylic acid group of the carbohydrate [17].

In order to improve the accessibility of the raw material to the enzymes, a thermal or chemical pretreatment is necessary. For the last 30 years, a wide range of pretreatment technologies has been developed combining thermal treatments with chemicals, such as SO_2, acid or ammonia (as reviewed *e.g.* by Sun and Cheng [4]; Mosier et al. [18]). Steam explosion with or without acid catalyst has been the most extensively studied method [19,20]. According to Morjanoff and Gray, addition of H_2SO_4 (or SO_2) or CO_2 in steam explosion can effectively improve enzymatic hydrolysis , decrease the production of inhibitory compounds, and lead to more complete removal of hemicellulose [21]. The chemical composition and structure of the feedstock are strongly affected by the pretreatment method. The high-pressure steam radically modifies the plant cell wall structure, yielding a dark brown material from which partially hydrolyzed hemicelluloses are easily recovered by water-washing, leaving a water-insoluble fraction composed of cellulose, residual hemicelluloses and chemically modified lignin [22,23].

The common pretreatment methods do not remove significant amounts of lignin. However, many studies have shown that relocation of lignin takes place during the high-temperature treatment [22,24,25]. Already in 1983, the formation of small particles, brown oily substances both inside and outside the exploded cell walls was detected by photomicrographs and it was suggested that lignin is first depolymerised and then repolymerised [24]. These results are consistent with the softening and agglomeration of lignaceous materials and extractives. Later, it was found that when these particles are smeared out by mild alkali, the hydrolysis is considerably reduced [26]. It thus appears that due to the high temperature prevailing in many pretreatments, lignin is separated from the original location although not removed from the substrate, causing severe problems in the hydrolyzability. More recent observations also support the hypothesis of lignin solubilization and relocation [27]. Thus, a complete delignification seems not to be necessary for an effective pretreatment whereas the location of lignin may be crucial for the hydrolysis.

A complete delignification, although efficient for improving the hydrolyzability, is usually considered too expensive as an industrial scale pretreatment method. Various chemicals such as mild alkali, dioxane, ethanol, alkaline hydrogen peroxide and sodium chlorite [23] are commonly used for analytical purposes to extract lignin. Among these, chlorite delignification is considered as the most selective one [28]. Chlorite delignification is also used for holocellulose isolation [29].

Several attempts have been taken to obtain a better understanding on the bottlenecking factors in the enzymatic hydrolysis of pretreated cellulosic substrates [30]. The major obstacles in the enzymatic hydrolysis of lignocellulose into sugars are related to the crystalline structure of cellulose and to the complexicity of the raw materials; *i.e.* the presence of hemicellulose and/or lignin in the pretreated raw material. Grohmann et al. related the improvement in enzyme digestibility of pretreated wood to the removal of hemicellulose which, according to Grethlein, results in an increase in both the accessible pore volume and the specific surface area [31,32]. Increasing the severity of the pretreatment usually leads to increased solublization of hemicelluloses in water and improved glucose yields in enzymatic hydrolysis [33–36].

The aim of the present study was to investigate the bottlenecks of the enzymatic hydrolysis of softwood, *i.e.* Norway spruce (*Picea abies*). Softwood is generally considered among the most difficult and recalcitrant raw materials for the enzymatic hydrolysis and therefore represents a challenge for improvement. The goal was to analyze the compositional changes and structural limitations of pretreated spruce, using purified enzyme components and commercial enzyme preparations in detailed enzymatic hydrolysis experiments, complemented by chemical delignification of the raw material.

2. Materials and methods

2.1. Raw materials and preparation of substrates

Lignocellulosic softwood raw material was used in the experiments. Unpretreated spruce sawdust (abbreviated as S) was kindly provided by KCL, Finland and the steam-pretreated and washed spruce by Lund University, Sweden. The spruce chips were impregnated with 3% SO_2, steam-pretreated at 215 °C for 5 min and the solid fraction was washed with hot water. The pretreated feedstock (SPS) was enzymatically hydrolyzed and washed; the residue after the hydrolysis (SPS-Res) was analyzed and used for further hydrolysis tests. The eventually adsorbed enzymes were inactivated by boiling after the primary hydrolysis. The two latter substrates were also delignified (SPS-DL and SPS-Res-DL, respectively). As reference to the delignified spruce samples, microcrystalline cellulose (Avicel) was used.

2.2. Delignification

The steam pretreated, washed raw material and the residue of the enzymatic hydrolysis were delignified with chlorite delignification according to the holocellulose preparation method of Yokoyama et al. but increasing the scale 10-fold [29]. The extraction of the samples (3–4 g dry weight) was carried out in a Soxhlet apparatus with 200 ml acetone for 5 h, followed by complete evaporation of acetone under hood. The extractive-free samples were delignified without previous milling. Sodium chlorite/acetic acid solution was added six times to the acetone-extracted spruce residue to achieve a complete delignification because of the high concentration of lignin in the sample.

2.3. Carbohydrate analysis of solid samples

Carbohydrates were determined according to the NREL-method with slight modifications [37]. Approximately 100 mg (d.w.) samples were measured into tubes and 1.0 ml 72% sulphuric acid was added. After mixing, the tubes were submerged in a water bath maintained at 30 °C, mixed every 15 min and finally diluted with 14 ml ultra-pure water and autoclaved for 50 min at 121 °C. The samples were filled to 25 ml and filtered and analyzed for monosaccharides by DNS and GC-FID system (Agilent 6890N) using Agilent HP-5 column [38] or a Dionex ICS-3000 gradient HPLC system (Dionex ICS-3000, Sunnyvale, CA) using CarboPac PA-1 column and 1 ml/min eluent flow and 30 °C column temperature [39]. The carbohydrate composition is expressed as percentage of total identified sugars in the acid hydrolysates.

2.4. Carbohydrate analysis of liquid samples

Carbohydrates liberated in enzymatic hydrolysis experiments were analyzed by DNS and GC or HPLC as described above.

2.5. Lignin content

The samples (3–5 g) were first extracted with acetone as above and the extractive-free samples were further hydrolyzed with sulphuric acid to remove all carbohydrates, according to the NREL-methods for acid-insoluble (Klason-) and acid-soluble lignin in the biomass with slight modification [37]. The 72% sulphuric acid hydrolysis was carried out in vacuum for 2 h at room temperature, followed by a second hydrolysis step for 4 h at room temperature by diluting the samples 3.5-fold with distilled water. After this, the samples were refluxed with the addition of 300 ml distilled water for 5 h. The acid-soluble lignin was measured at 203 nm against 4% H_2SO_4 blank. The lignin was calculated as the sum of the acid-insoluble lignin and acid-soluble lignin.

2.6. Ash content

The dried samples (0.5–1.0 g) were combusted in a furnace at 575 °C according to the NREL-method [37], and the remaining ash was measured.

2.7. Enzymatic hydrolysis

Enzymatic hydrolysis experiments were carried out in two stages. In the first stage, the hydrolysis of the pretreated (non-delignified and delignified) substrate was studied by using commercial preparations (Celluclast and Novozym 188 by Novozymes, Denmark) at dosages of 5 and 10 FPU/g of cellulose (d.w.), supplemented with β-glucosidase, 100 nkat/g cellulose. The hydrolysis was carried out in 50 mM sodium-acetate buffer, pH adjusted to 5.0, at 45 °C, with 1% cellulose content. In addition to the commercial enzymes, three mixtures of purified enzymes were used based on cellobiohydrolases CBH I (Cel 7A, EC 3.2.1.91.) and CBH II (Cel 6A, EC 3.2.1.91.), endoglucanase EG II (Cel 5A, EC 3.2.1.4.) from *T. reesei*, purified according to Suurnäkki et al., *Aspergillus niger* β-glucosidase (Cel 3A, EC 3.2.1.21.) purified from Novozym 188 according to Sipos et al. and *T. reesei* xylanase II (Xyn 11, EC 3.2.1.8.), purified according to Tenkanen et al. [40–42]. The enzymes used were pure as judged by SDS-PAGE.

Endoglucanase II was chosen instead of endoglucanase I, which is known to hydrolyze efficiently also xylan [43]. On the other hand, endoglucanase II is known to have catalytic activity towards glucomannans as well as to glucans [44]. Thus, based on the composition of softwood (both glucomannan and xylan), the set of enzymes was chosen to be able to differentiate xylan and glucomannan hydrolysis.

All mixtures of purified enzymes were applied using the same protein amount content per substrate; 18 mg/g cellulose, corresponding to about 10 FPU/g cellulose in the commercial Celluclast preparation. The most complete preparation of purified enzymes (abbreviated as CBH-EG-XYL) was designed to resemble the enzyme composition commonly present in culture broths of *T. reesei*, containing 58.4% CBH I, 19.5% CBH II, 19.5% EG II and 2.7% XYL II. In the second preparation (abbreviated as CBH-EG), the xylanase activity was omitted and it contained 60% CBH I, 20% CBH II and 20% EG II. In the third preparation (abbreviated as CBH) only the two cellobiohydrolases were present; 75% CBH I and 25% CBH II (CBH I to CBH II ratio 3:1). To all enzyme preparations, β-glucosidase, 100 nkat/g cellulose, was added. The enzyme preparations used in the experiments are described in Table 1.

To obtain a hydrolysis residue enriched in lignin and non-hydrolyzed carbohydrates, as well as potential lignin–carbohydrate complexes, the hydrolysis was repeated in a larger scale with the commercial enzyme preparations Celluclast and Novozym 188 (referred to as primary hydrolysis) at a dosage of about 30 FPU/g cellulose and 1000 nkat/g dry weight, respectively, at 5% consistency at 45 °C, pH 5 for 2 days. During the primary hydrolysis, a relatively higher dosage of β-glucosidase was used to completely exclude the product inhibition of CBH's by cellobiose. After this primary hydrolysis, the composition was analyzed and the residue was also delignified. The yield, composition and particle size of the spruce fractions are summarized in Tables 2 and 3. The hydrolysis was further studied in a secondary hydrolysis stage, using the same commercial and purified enzymes as for the non-hydrolyzed pretreated spruce substrate. The enzymatic hydrolyses were evaluated on the basis of sugars liberated.

Table 1
Enzyme preparations used in the hydrolysis experiments.

Enzyme mixtures	Enzyme	Ratio in mix	Protein to cellulose (mg/g cellulose)
Cel-Nov 10 FPU	Celluclast	100%	–
	Novozym 188		–
Total			18.1
Cel-Nov 5 FPU	Celluclast	50%	–
	Novozym 188		–
Total			9.1
CBH + EG + XYL	CBH I	58.4%	10.9
	CBH II	19.5%	3.6
	EG II	19.5%	3.6
	XYL II	2.7%	0.5
	β-glucosidase		0.053
Total			18.6
CBH + EG	CBH I	60%	10.9
	CBH II	20%	3.6
	EG II	20%	3.6
	β-glucosidase		0.053
Total			18.2
CBH	CBH I	75%	13.6
	CBH II	25%	4.5
	β-glucosidase		0.053
Total			18.2

The protein ratio in the enzyme mixes is given as the percentage of the total protein content in the Celluclast enzyme preparation with 10 FPU/g cellulose. The enzyme mixes were supplemented with Novozym 188 and β-glucosidase, dosed on their catalytic activity as 100 nkat/g cellulose.

2.8. Enzyme activity measurements

The enzyme activities were measured as follows: the cellobiohydrolase activity using methyl umbelliferyl lactoside (MULac) as substrate according to van Tilbeurgh et al. [45,46]; the endoglucanase activity using HEC according to Bailey and Nevalainen [47]; the xylanase activity using birch xylan according to Bailey et al. [48]; the β-glucosidase activity using *p*-nitrophenyl-β-glucopyranoside according to Bailey and Nevalainen [47]; as well as the filter paper activity according to the IUPAC method [49].

2.9. Particle size analysis

The particle size distribution of the various non-hydrolyzed samples and partially hydrolyzed residues was analyzed by laser diffraction using a Coulter LS 230 (Beckman Coulter, Fullerton, CA) instrument equipped with polarization intensity differential scattering (PIDS) assembly for particles smaller than 0.4 μm. The laser diffraction (laser 750 nm) involved the particle sizes 0.4–2000 μm. The samples were suspended in ethanol and then fed to the equipment. The measurement screened the sample for 90 s in the range of 0.040–2000 μm; the background was ethanol. Duplicate measurements of each sample were performed to check the stability of the sample in the alcoholic suspension. The results were calculated using an optical model, where the particles were assumed to be spherical in shape with a refractive index of 1.5.

3. Results and discussion

3.1. Composition and particle size of non-delignified and delignified steam-pretreated spruce

The samples including the starting material, native spruce, were first analyzed for their main constituents. The steam-pretreated spruce sample was delignified in order to study the role of lignin in the enzymatic hydrolysis. The composition and the particle size of S, SPS and SPS-DL were determined (Tables 2 and 3). Comparing the composition of the SPS and SPS-DL samples, it can be seen that the delignification was effective, as 93% of the lignin was removed, decreasing the lignin level to 4.3% (Table 2).

The composition of S and SPS differs mostly in their hemicellulose content (Table 3). The galactoglucomannans in the original spruce contained both low and high-galactose glucomannans, based on the approximate galactose:mannose ratio of about 1:6.

Table 2
The yields of dry weight in the primary enzymatic hydrolysis, the composition and median particle size of unpretreated, native spruce (S), steam-pretreated spruce (SPS), delignified steam-pretreated spruce (SPS-DL) spruce, enzymatically hydrolyzed steam-pretreated spruce residue (SPS-Res) and enzymatically hydrolyzed and delignified steam-pretreated spruce residue (SPS-Res-DL) samples.

Sample/Composition	Yield (% of SPS)	Total polysaccharide (% of d.w.)	Lignin (% of d.w.)	Lignin and extractives (% of d.w.)	Ash (% of d.w.)	Median diameter (μm)	Volume median diameter (μm)
S	–	66.8 ± 0.9	28.2 ± 0.3	29.8 ± 0.3	0.21 ± 0.11	n.d.[b]	n.d.[b]
SPS	100.0	50.9 ± 2.0	32.5 ± 0.1	43.4 ± 0.2	0.11 ± 0.04	3.0	133
SPS-Res	62.0 ± 0.9	29.4 ± 1.0	52.8 ± 0.9	69.8 ± 1.1	0.23 ± 0.03	2.9	89
SPS hydrolysate[a]	42.2 ± 0.4	–	–	–	–	–	–
SPS-DL	53.7 ± 2.4	93.9 ± 2.7	4.3 ± 0.4	4.3 ± 0.4	0.15 ± 0.01	2.8	115
SPS-Res-DL	16.8 ± 0.5	79.8 ± 5.9	15.2 ± 3.2	15.2 ± 3.2	0.50 ± 0.08	2.2	235

The primary enzymatic hydrolysis was carried out with enzyme dosages of 30 FPU/g cellulose Celluclast and 1000 nkat/g cellulose Novozym 188, at 5% consistency at 45 °C, pH 5 for 2 days; the lignin was removed by chlorite delignification. The yield is calculated to the dry weight of steam-pretreated spruce, the composition data are expressed as % of dry weight.

[a] The hydrolysate from the hydrolysis of steam-pretreated spruce with Celluclast (30 FPU /g cellulose) and Novozym 188 (1000 nkat/g cellulose).

[b] n.d. not determined.

Table 3
The sugar composition of polysaccharides of unpretreated native spruce (S), steam-pretreated spruce (SPS), delignified steam-pretreated spruce (SPS-DL), enzymatically hydrolyzed steam-pretreated spruce residue (SPS-Res), enzymatically hydrolyzed and delignified steam-pretreated spruce residue (SPS-Res-DL) and Avicel samples.

Sample	Glucose (% of PS)	Mannose (% of PS)	Xylose (% of PS)	Galactose (% of PS)	Arabinose (% of PS)
S	64.9	21.0	9.0	3.7	2.0
SPS	100	<0.39[b]	<0.39[b]	<0.39[b]	<0.39[b]
SPS-Res	96.7	2.2	1.1	<0.68[b]	<0.68[b]
SPS hydrolysate[a]	99.6	0.21	0.16	0.05	<0.03[b]
SPS-DL	99.4	0.25	0.34	<0.21[b]	<0.21[b]
SPS-Res-DL	99.5	<0.26[b]	0.51	<0.26[b]	<0.26[b]
Avicel	97.6	1.33	1.30	<0.10[b]	<0.10[b]

The sugar composition of polysaccharides is expressed as % of polysaccharides in the acid hydrolysates and was measured by HPLC.

[a] The hydrolysate from the hydrolysis of steam-pretreated spruce with Celluclast (30 FPU/g cellulose) and Novozym 188 (1000 nkat/g cellulose).

[b] Below detection limit.

Most of the galactoglucomannans were solubilized during the steam pretreatment, as reported earlier [50]; the hemicellulose content decreased to below 1% of the total polysaccharide content. During the steam pretreatment, galactomannans were evidently solubilized more efficiently than arabinoxylans as the ratio of mannose to xylose decreased from 2.3 to 0.7. The degree of substitution of xylan by arabinose in the native spruce was somewhat higher (arabinose:xylose ratio about 2.2:10) than that reported in the literature (1.3:10).

The particle size of the SPS slightly decreased during its delignification. The decrease in the median diameter due to the delignification was 6.7%, resulting in a median particle size of 2.8 μm. The volume median diameter followed the same pattern: the particles making up the bulk of the material decreased 14% in size, from 133 to 115 μm. Despite the size reduction, the delignification did not result in an extensive fragmentation of the original particles, meaning that the median particle size remained well in the same range. Thus, these materials are well suitable for comparative hydrolysis tests.

3.2. Hydrolysis of non-delignified and delignified steam-pretreated spruce

To gain deeper understanding on the limiting factors decreasing the rate of the hydrolysis, the enzymatic hydrolysis of the non-delignified and delignified steam-pretreated spruce feedstocks was first carried out with the various enzyme mixtures combining commercial (Celluclast and Novozym 188) and purified *T. reesei* and *A. niger* enzymes (Table 1). The purified enzymes were used to allow a comparison of the hydrolysis of cellulose and hemicellulose. The enzymes were chosen based on their capability to hydrolyze only cellulose, cellulose and glucomannan or also xylan. The purified enzymes were dosed based on the protein content. The CBH, EG and filter paper activities of the commercial and purified enzyme preparations were compared based on the dosage per activity or protein (Table 4). The dosage of the commercial enzyme preparation (Celluclast) was 10 FPU/g of cellulose and when supplemented with β-glucosidase, the activity was increased to 14.7 FPU/g of cellulose. The complete mixture of purified enzymes represented a slightly lower activity level at 11.7 FPU/g of cellulose. The enzyme spectra were reflected in the activity units, as expected.

The results reveal that the chemical composition of the two samples (SPS and SPS-DL) resulted in significant differences in the hydrolyzability due to various restricting factors in the hydrolysis. Only about 40% of the total carbohydrates of the SPS could be hydrolyzed with the commercial enzyme mixture with the higher dosage (Table 5, Fig. 1A). During the hydrolysis of 48 h, the pure enzyme mixtures, CBH-EG-XYL and CBH-EG, hydrolyzed the lignin-containing substrate approximately to the same degree, 36.9 and 34.6%, respectively (Table 5). Obviously due to the low amount of available xylan in the substrate, the xylanase supplementation did only slightly improve the hydrolysis yield. A slightly lower sugar yield was obtained with the halved amount of the commercial enzyme. As could be expected, the CBH enzymes alone resulted in a significantly lower yield, 22.2% of the theoretical. Thus, the hydrolysis levels of the SPS containing lignin were very close to each other with the two commercial, the CBH-EG-XYL and the CBH-EG preparations using protein dosages of the same level.

The hydrolysis of the SPS-DL, however, showed more striking differences with the enzymes studied. The hydrolysis of the relatively pure cellulose was clearly more affected by the composition of the enzyme mixture (Fig. 1B). The pure enzyme mixtures were clearly deficient of the minor cellulase activities needed for efficient synergistic action. The SPS-DL could be hydrolyzed with the higher dosage of the commercial enzyme almost totally (95.9% of theoretical). With the lower dosage, Cel-Nov 5 FPU, the hydrolysis was still better than that with the pure enzymes, CBH-EG-XYL and CBH-EG; *i.e.* 73.5, 63.9 and 56.9%, respectively (Table 5). The hydrolysis of

Table 4
Enzyme activities of the enzyme preparations used per gram of cellulose in the hydrolysis experiments.

Enzyme mixtures	CBH-activity (nkat/g cellulose)	EG-activity (nkat/g cellulose)	FP-activity (FPU/g cellulose)
Cel-Nov 10 FPU	516	3510	14.7
Cel-Nov 5 FPU	243	1920	9.8
CBH + EG + XYL	351	2740	11.7
CBH + EG	384	2670	12.0
CBH	459	0	3.9

Table 5
Carbohydrate composition of hydrolysates from steam pretreated (SPS) and delignified steam-pretreated spruce (SPS-DL) samples with various enzyme mixtures, sugars analyzed by GC, hydrolysis time 2 days, results expressed as % of total carbohydrates (as monomeric sugars).

Sample	Enzyme mixture	Glucose (% of PS)	Mannose (% of PS)	Xylose (% of PS)	Galactose (% of PS)	Arabinose (% of PS)
SPS	Cel-Nov 10 FPU	40.3	0.18	0.18	0.12	0.15
	Cel-Nov 5 FPU	34.1	0.15	Traces	Traces	b.d.[a]
	CBH + EG + XYL	36.9	Traces	b.d.[a]	b.d.[a]	b.d.[a]
	CBH + EG	34.6	Traces	b.d.[a]	b.d.[a]	b.d.[a]
	CBH	22.2	Traces	b.d.[a]	b.d.[a]	b.d.[a]
SPS-DL	Cel-Nov 10 FPU	95.9	0.15	0.34	Traces	Traces
	Cel-Nov 5 FPU	73.5	0.14	0.27	Traces	b.d.[a]
	CBH + EG + XYL	63.9	Traces	Traces	b.d.[a]	b.d.[a]
	CBH + EG	56.9	Traces	b.d.[a]	b.d.[a]	b.d.[a]
	CBH	35.7	Traces	b.d.[a]	b.d.[a]	b.d.[a]

[a] Below detection limit (0.02 g/l, corresponding to 0.1% of total carbohydrate or theoretical yield).

the delignified sample was thus about twice as efficient as without delignification using the commercial enzyme. Somewhat surprisingly, the effect of the complete set of cellulolytic and accessory enzymes in both of the commercial enzyme preparations was superior on the delignified sample. It thus seems that, in the latter case of delignified substrate (Fig. 1B), the hydrolysis is limited by cellulosic structure and crystallinity while in the former case (Fig. 1A) other, *i.e.* lignin- and hemicellulose-based, restrictions dominate.

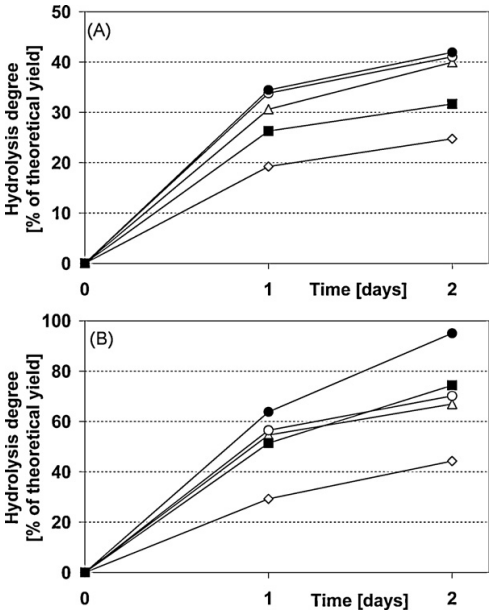

Fig. 1. Hydrolysis of (A) steam-pretreated spruce and (B) delignified steam-pretreated spruce samples with Celluclast (10 FPU/g cellulose) and Novozym 188 (100 nkat/g cellulose) (●), Celluclast (5 FPU/g cellulose) and Novozym 188 (100 nkat/g cellulose) (■) or with the CBH-EG-XYL (○); the CBH-EG (△) and the CBH (◊) purified enzyme mixtures; pH 5, 45 °C, 1% cellulose content. The degree of hydrolysis is based on reducing sugars formed in the hydrolysis.

Surprisingly, the addition of xylanase improved the hydrolysis of SPS-DL significantly compared to that of SPS, despite the low amount of xylan present in the samples. It is noteworthy that in the presence of 0.34% xylan, the additional xylanase could cause a 12% increase in the cellulose hydrolysis degree (*i.e.* from 56.9 to 63.9%), without an increase in the total FP-activity of the enzyme mixture (Table 4). This implicates the important role of the location of xylan in the cellulose hydrolysis of lignocellulosic substrates.

The hydrolysis degree was analyzed on the basis of reducing sugars (Fig. 1) and the hydrolysis products by GC (Table 5). As expected, the major sugar in the hydrolysates was glucose whereas in most cases, the amount of hemicellulose sugars released did not reach the detection limit (*i.e.* about 0.1% of total carbohydrates). The slightly lower amount of identified monomeric sugars, as compared to the total amount of reducing sugars obtained, can be explained by the presence of hemicellulose-derived oligosaccharides due to lack of specific exo-enzymes (*e.g.* β-xylosidase, β-mannosidase) in the CBH-EG-XYL, CBH-EG and CBH preparations. The hemicellulose residues may also be linked to lignin fragments as lignin–carbohydrate complexes, not being hydrolyzed into monomers.

3.3. Hydrolysis of Avicel

To compare further the hydrolysis of pure cellulose with the various enzyme preparations, Avicel was hydrolyzed under the same conditions as the spruce samples (Fig. 2). In this case, both commercial enzymes resulted in the highest conversion, and the purified enzyme preparations in slightly lower hydrolysis yields (Table 6). Surprisingly, the hydrolysis of Avicel was considerably improved by the xylanase supplementation to the enzyme preparation. In spite of the purity of Avicel (chemically pure cellulose, alpha-cellulose from coniferous wood), there were traces of xylan (1.3% xylose) and glucomannan (1.33% mannose) left in the substrate (Table 3). Previously Avicel was shown to contain 0.6% xylan and 1.0% mannan [51]. Berlin et al. showed that a high xylanase activity increased the glucose yield of steam-pretreated Douglas fir even though the substrate contained only 0.6% xylan, as compared with a total carbohydrate content of 51.1%, proving the need for accessory enzymes in the hydrolysis [52]. Consistent with this,

Table 6
Carbohydrate composition of hydrolysates from Avicel with various enzyme mixtures, sugars analyzed by GC, hydrolysis time 2 days, results expressed as % of total carbohydrates (as monomeric sugars).

Sample	Enzyme mixture	Glucose (% of PS)	Mannose (% of PS)	Xylose (% of PS)	Galactose (% of PS)	Arabinose (% of PS)
Avicel	Cel-Nov 10 FPU	75.9	1.04	3.50	0.14	Traces
	Cel-Nov 5 FPU	66.7	0.43	1.05	Traces	Traces
	CBH + EG + XYL	58.0	Traces	0.31	b.d.[a]	b.d.[a]
	CBH + EG	44.1	Traces	0.16	b.d.[a]	b.d.[a]
	CBH	31.3	Traces	Traces	b.d.[a]	b.d.[a]

[a] Below detection limit (0.02 g/l, corresponding to 0.1% of total carbohydrate or theoretical yield).

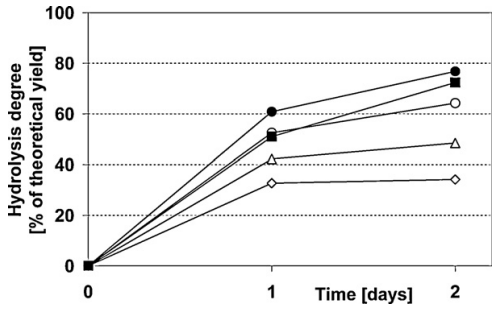

Fig. 2. Hydrolysis of Avicel with Celluclast (10 FPU/g cellulose), Novozym 188 (100 nkat/g cellulose) (●), Celluclast (5 FPU/g cellulose), Novozym 188 (100 nkat/g cellulose) (■) or with the CBH-EG-XYL (○); the CBH-EG (△) and the CBH (◊) purified enzyme mixtures; pH 5, 45 °C, 1% cellulose content. The degree of hydrolysis is based on reducing sugars formed in the hydrolysis.

the effect of xylanase could be clearly observed as an increase in the amount of released glucose (from 44.1 to 58.0% of the total carbohydrates), parallel to the increased release of xylose in the hydrolysates (from 12.3 to 23.8% of total xylan content).

3.4. Composition and particle size of non-delignified and delignified steam-pretreated spruce enzymatic residues

In order to further investigate the limiting factors decreasing the rate of the hydrolysis, a residue from enzymatic hydrolysis was produced with a higher dosage of commercial enzymes. This residue was also further delignified. The composition and the particle size of the enzymatic residue, as well as the delignified enzymatic residue were determined (Tables 2 and 3).

The composition of the enzymatic spruce residue from the primary hydrolysis showed that 64.2% of the polysaccharide content of SPS had been hydrolyzed leading to enrichment of the sample in lignin (Table 2). The lignin content including the extractives increased thus to about 70% of dry weight. During the fairly extensive enzymatic hydrolysis, the sugar composition of the residual spruce samples changed slightly, resulting in a relative increase in the amount of hemicellulose-derived sugars (Table 3).

During the delignification of SPS-Res, especially the mannose content decreased below the detection limit (to less than 10% of the original), while the xylose content was approximately halved. Although the delignification was effective and 96% of the lignin was removed, the lignin content in the delignified sample decreased only to 15.2% of the dry weight, because of the low polysaccharide content in the residue (Table 2). The composition data also suggest that glucomannan was mainly present in lignin–carbohydrate complexes in the SPS which is in accordance with previous observations [12]. Delignification is generally a harsh method, during which hemicellulosic sugars can be removed along with lignin. Chlorite delignification, however, has been shown to be more specific and to remove less hemicellulose than the other delignification methods, such as alkali extraction [28]. According to the results shown in Tables 2 and 3, the delignification removed most of the hemicellulose sugars present in SPS-Res. Partial removal of hemicellulose may also have been caused by breaking the lignin–carbohydrate complexes, reported to be present in softwood [12].

The enzymatic hydrolysis resulted in no significant change in the distribution of the particles below 20 μm. The median diameter decreased only from 3.0 to 2.9 μm during the enzymatic hydrolysis of the SPS. On the contrary, the enzymatic hydrolysis caused a decline of the volume median diameter by 33%. On the other hand, the data from the delignification of the enzymatic residue seemed to show a surprisingly different pattern. The mean diameter decreased 33% from 2.9 to 2.2 μm due to the lignin removal, while the volume mean diameter increased 2.6-fold. The size decrease due to lignin removal of the smaller particles could explain why the number of small particles increases with the relative increase of the mass of the larger particles, which might be more resistant towards enzymatic hydrolysis and lignin removal both.

3.5. Hydrolysis of non-delignified and delignified steam-pretreated spruce enzymatic residue

The non-delignified and delignified enzymatic residues of SPS were further enzymatically hydrolyzed in a secondary

Table 7
Carbohydrate composition of hydrolysates from steam-pretreated spruce enzymatic residue (SPS-Res) and delignified steam-pretreated spruce enzymatic residue (SPS-Res-DL) with various enzyme mixtures, sugars analyzed by GC, hydrolysis time 2 days, results expressed as % of total carbohydrates (as monomeric sugars).

Sample	Enzyme mixture	Glucose (% of PS)	Mannose (% of PS)	Xylose (% of PS)
SPS-Res	Cel-Nov 10 FPU	46.4	0.13	0.17
	Cel-Nov 5 FPU	30.2	0.13	Traces
	CBH + EG + XYL	29.0	Traces	b.d.[a]
	CBH + EG	28.4	Traces	b.d.[a]
	CBH	21.7	Traces	b.d.[a]
SPS-Res-DL	Cel-Nov 10 FPU	99.0	0.12	0.65
	Cel-Nov 5 FPU	77.6	0.14	0.53
	CBH + EG + XYL	62.9	Traces	0.17
	CBH + EG	66.2	Traces	Traces
	CBH	32.9	b.d.[a]	b.d.[a]

Galactose and arabinose sugars did not reach the detection limit thus have been omitted from the table.
[a] Below detection limit (0.02 g/l, corresponding to 0.1% of total carbohydrate or theoretical yield).

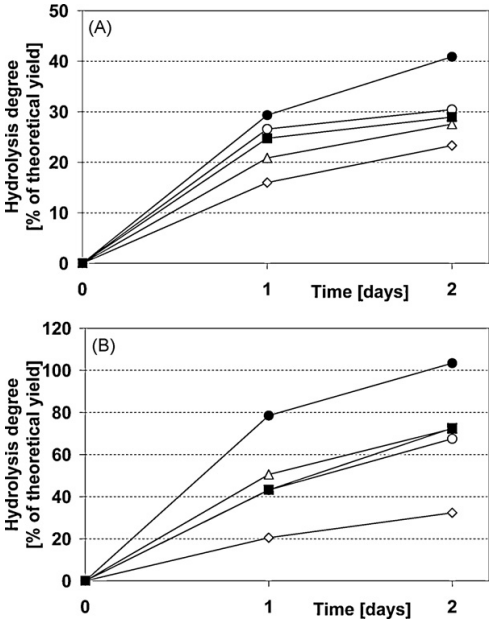

Fig. 3. Hydrolysis of (A) enzymatically hydrolyzed steam-pretreated spruce residue and (B) delignified enzymatically hydrolyzed steam-pretreated spruce residue. Hydrolysis with Celluclast (10 FPU/g cellulose), Novozym 188 (100 nkat/g cellulose) (●), Celluclast (5 FPU/g cellulose), Novozym 188 (100 nkat/g cellulose) (■) or with the CBH-EG-XYL (○); the CBH-EG (△) and the CBH (◊) purified enzyme mixtures; pH 5, 45 °C, 1% cellulose. The degree of hydrolysis is based on reducing sugars formed in the hydrolysis.

hydrolysis, under the same conditions as in the hydrolysis of SPS and SPS-DL (Fig. 3A and B). The composition of the hydrolysates implicated the same trend as in the former hydrolysis experiments. This is consistent with previous hydrolysis results performed on steam-pretreated spruce by commercial [30,53] and purified enzymes [54], as well as on Avicel [55].

The lignin-containing SPS-Res could be hydrolyzed with the higher and lower commercial enzyme preparations approximately to the same level, *i.e.* up to 46.4 and 30.2% of the theoretical, (Table 7, Fig. 3A) as the original SPS, *i.e.* 40.3 and 34.1% (Table 5, Fig. 1A), respectively. However, the purified CBH-EG-XYL, CBH-EG and CBH mixtures were somewhat less efficient resulting in hydrolysis degrees of 29.0, 28.4 and 21.7%, respectively (Table 7, Fig. 3A). The purified enzymes were obviously barred by the higher lignin content in the enzymatic residue decreasing the hydrolysis level as compared with the original SPS substrate. The difference between the hydrolysis levels of the purified enzymes was not significant and the supplementation with the various purified enzymes seemed again to play minor role in the hydrolysis of the lignin rich enzymatic residue. On the other hand, hydrolysis yields obtained on the delignified enzymatic residue were again higher than on the non-delignified residue.

The hydrolysis of SPS-Res-DL was again more influenced by all cellulase components present in the commercial enzyme, pointing out the dominant role of the recalcitrance of the cellulosic structure in the lignin-free substrate. The hydrolysis levels of 62.9, 66.2 and 32.9% could be reached with the purified enzyme mixtures, respectively (Table 7). Somewhat surprisingly, about the same hydrolysis levels could be reached on both delignified samples (*i.e.* SPS-DL and SPS-Res-DL) (Figs. 1B and 3B). This proved not only that the delignification was successful, but also that the residual carbohydrate structure had no limiting effect on the hydrolysis. The same phenomenon was observed previously on Avicel hydrolyzed with commercial cellulases by Yang et al. where the cellulose hydrolysis rate, after protein removal, was shown to be constant until 70% of conversion when restarting the hydrolysis of Avicel residues [55].

The sugar composition of the hydrolysates corresponded to the average composition of the samples (Tables 3 and 7). Relatively less mannose (and xylose) was released in the hydrolysis of the lignin-containing samples as compared with the overall composition of the sample. Furthermore, in all cases, the hydrolyses with the CBH-EG mixture and the CBH-EG-XYL mixture confirmed the low amount of xylan present in the samples.

3.6. Mass balance of spruce fractions

The overall mass balances after various treatments and hydrolysis experiments are summarized in Table 2. The composition of spruce has also previously been shown to change significantly during the pretreatment and various enzymatic and chemical treatments [56]. The relative amounts and compositions of the various fractions changed significantly during the treatments. It can be seen that delignification did not affect significantly the polysaccharide content of the SPS as 99% of the sugar remained in the solid fraction, whereas 93% of the lignin was solubilized. On the other hand, delignification of the SPS-Res dissolved 26% of the polysaccharides, besides removing 92% of lignin and 73% of dry matter of SPS-Res. This may give evidence that the enzymatic residue contained relatively more carbohydrates linked to lignin.

Changes in the sugar composition of the main hemicelluloses in various stages clearly reveal that even minor carbohydrate residues, *i.e.* galactoglucomannan and xylan, may restrict the hydrolysis. However, in SPS, cellulose was the major carbohydrate in all stages.

4. Conclusions

The enzyme patterns used revealed that, somewhat surprisingly, the commercial enzyme preparation used, containing the main cellulolytic components, was not essentially better in the presence of lignin than the pure enzymes used, despite the limited amount of enzyme components present. The results suggest that lignin was the major component restricting the enzymatic hydrolysis of steam-pretreated softwood. In particular, in the absence of lignin, the hydrolysis of cellulose itself was the major limiting factor, supporting this hypothesis. The limiting role of lignin may also be affected by lignin carbohydrate complexes.

Attempts were taken to follow the relative amounts of hemicellulose side groups with special reference to the lignin carbohydrate linkages and their changes during the treatments. Despite the fairly low amount of hemicellulose sugars in the sample, xylan seemed to restrict the hydrolysis of cellulose in the various pretreated and delignified spruce samples, but most clearly in the fairly pure commercial cellulose preparation, Avicel, which also originates from softwood. The hydrolysis results of Avicel revealed significant hemicellulose impurities affecting the hydrolysis in the commonly used cellulose model substrate. The delignified spruce substrate was clearly more pure with respect to the xylan content. The fact that a relatively small release of xylose could increase the cellulose hydrolysis indicated that not only the relative amount of hemicellulose but also the location of these carbohydrates with respect to cellulose (or lignin) plays an important role in the efficiency of the hydrolysis.

Somewhat surprisingly, neither enzymatic hydrolysis nor lignin removal decreased significantly the particle size, and did not destroy the basic skeletal structure of the substrate.

The non-productive adsorption of enzymes has been observed to decrease the hydrolysis rate. In this work, it was found that the hydrolysis of the washed residual samples continued in the secondary hydrolysis at about the same rate as in the primary hydrolysis, in spite of the bound/adsorbed enzymes. Expectedly, the hydrolysis yield in the secondary hydrolysis of the delignified samples was at the same level as in the primary hydrolysis, due to removal of the bound enzymes along with lignin in the chlorite extraction of lignin. No enrichment of carbohydrates restricting the hydrolysis of pretreated spruce could be detected.

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References

[1] Wyman CE. Biomass ethanol: technical progress, opportunities, and commercial challenges. Annu Rev Energy Environ 1999;24:189–226.
[2] de Vries BJM, van Vuuren DP, Hoogwijk MM. Renewable energy sources: their global potential for the first-half of the 21st century at a global level: an integrated approach. Energy Policy 2007;35:2590–610.
[3] Galbe M, Zacchi G. A review of the production of ethanol from softwood. Appl Microbiol Biotechnol 2002;59:618–28.
[4] Sun Y, Cheng J. Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour Technol 2002;83:1–11.
[5] Betts WB, Dart RK, Ball AS, Pedlar SL. Biosynthesis and structure of lignocellulose. In: Betts WB, editor. Biodegradation: natural and synthetic materials. Berlin, Germany: Springer-Verlag; 1991. p. 139–55.
[6] Bertaud B, Holmbom B. Chemical composition of earlywood and latewood in Norway spruce heartwood, sapwood and transition zone wood. Wood Sci Technol 2004;38:245–56.
[7] Sjöström E. Wood polysaccharides: wood chemistry, fundamentals, applications. San Diego: Academic Press Inc; 1993. pp. 1–20, 51–70.
[8] Timell TE. Recent progress in the chemistry of wood hemicelluloses. Wood Sci Technol 1967;1:45–70.
[9] Willför S, Sundberg A, Hemming J, Holmbom H. Polysaccharides in some industrially important softwood species. Wood Sci Technol 2005;39:245–58.
[10] Lundqvist J, Teleman A, Junel L, Zacchi G, Dahlman O, Tjerneld F, et al. Isolation and characterization of galactoglucomannan from spruce (*Picea abies*). Carbohydr Polym 2002;48:29–39.
[11] Minor JL. Chemical linkage of polysaccharides to residual lignin in loblolly pine kraft pulps. J Wood Chem Technol 1986;6:185–201.
[12] Laine C, Tamminen T, Hortling B. Carbohydrate structures in residual lignin–carbohydrate complexes of spruce and pine pulp. Holzforschung 2004;58:611–21.
[13] Meier H. Isolation and characterisation of an acetylated glucomannan from pine (*Pinus silvestris* L.). Acta Chem Scand 1961;15:1381–5.
[14] Lawoko M, Henriksson G, Gellerstedt G. Characterisation of lignin–carbohydrate complexes (LCCs) of spruce wood (*Picea abies* L.) isolated with two methods. Holzforschung 2006;60:156–61.
[15] Eriksson Ö, Goring DAI, Lindgren BO. Structural studies on the chemical bonds between lignins and carbohydrates in spruce wood. Wood Sci Technol 1980;14:267–79.
[16] Choi JW, Choi D-H, Faix O. Characterization of lignin–carbohydrate linkages in the residual lignins isolated from chemical pulps of spruce (*Picea abies*) and beech wood (*Fagus sylvatica*). J Wood Sci 2007;53:309–13.
[17] Watanabe T. Analysis of native bonds between lignin and carbohydrate by specific chemical reactions. In: Koshijima T, Watanabe T, editors. Association between lignin and carbohydrates in wood and other plant tissues. Heidelberg, Germany: Springer-Verlag; 2003. p. 91–130.
[18] Mosier N, Wyman Ch, Dale B, Elander R, Lee YY, Holtzapple M, et al. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour Technol 2005;96:673–86.
[19] Söderström J, Galbe M, Zacchi G. Effect of washing on yield in one- and two-step steam pretreatment of softwood for production of ethanol. Biotechnol Prog 2004;20:744–9.
[20] McMillan JD. Pretreatment of lignocellulosic biomass. In: Himmel ME, Baker JO, Overend RP, editors. Enzymatic conversion of biomass for fuels production. Washington, DC: American Chemical Society; 1994. p. 292–324.
[21] Morjanoff PJ, Gray PP. Optimization of steam explosion as method for increasing susceptibility of sugarcane bagasse to enzymatic saccharification. Biotechnol Bioeng 1987;29:733–41.
[22] Shevchenko SM, Beatson RP, Saddler J. The nature of lignin from steam explosion/enzymatic hydrolysis of softwood. Appl Biochem Biotechnol 1999;77–79:867–76.
[23] Ramos LP. The chemistry involved in the steam treatment of lignocellulosic materials. Quim Nova 2003;26:863–71.
[24] Tanahashi M, Takada S, Aoki T, Goto T, Higuchi T, Hanai S. Characterization of explosion wood. 1. Structure and physical properties. Wood Res 1983;69:36–51.
[25] Li J, Henriksson G, Gellerstedt G. Lignin depolymerisation/repolymerization and its critical role for delignification of aspen wood by steam explosion. Bioresour Technol 2007;98:3061–8.
[26] Donaldson LA, Wong KKY, Mackie KL. Ultrastructure of steam-exploded wood. Wood Sci Technol 1988;22:103–14.
[27] Kristensen JB, Thygesen LG, Felby C, Jørgensen H, Elder T, Holtzapple MT. Cell-wall structural changes in wheat straw pretreated for bioethanol production. Biotechnol Biofuels, 2008; 1. DOI 101186/1754-6834-1-5. Retrieved from: http://www.biotechnologyforbiofuels.com/content/pdf/1754-6834-1-5.pdf.
[28] Höije A, Gröndahl M, Tømmeraas K, Gatenholm P. Isolation and characterization of physicochemical and material properties of arabinoxylans from barley husks. Carbohydr Polym 2005;61:266–75.
[29] Yokoyama T, Kadla JF, Chang H-M. Microanalytical method for the characterization of fiber components and morphology of woody plants. J Agric Food Chem 2002;50:1040–4.
[30] Gregg DJ, Saddler JN. Factors affecting cellulose hydrolysis and the potential of enzyme recycle to enhance the efficiency of an integrated wood to ethanol process. Biotechnol Bioeng 1996;51:375–83.
[31] Grohmann K, Torget R, Himmel ME. Optimization of dilute acid pretreatment of biomass. Biotechnol Bioeng Symp 1985;15:59–80. Wiley, 1986.
[32] Grethlein HE. The effect of pore size distribution on the rate of enzymatic hydrolysis of cellulosic substrates. Bio/Technol 1985;3:155–60.
[33] Saddler JN, Brownell HH, Clermont LP, Levitin N. Enzymatic hydrolysis of cellulose and various pretreated wood fractions. Biotechnol Bioeng 1982;24:1389–402.
[34] Brownell HH, Saddler JN. Steam pretreatment of lignocellulosic material for enhanced enzymatic hydrolysis. Biotechnol Bioeng 1987;29:228–35.
[35] Puls J, Schuseil J. Chemistry of hemicellulose: relationship between hemicellulose structure and enzymes required for hydrolysis. In: Coughlan MP, Hazlewood GP, editors. Hemicellulose and hemicellulases. London: Portland Press; 1993. p. 1–27.
[36] Alkasrawi M, Rudolf A, Lidén G, Zacchi G. Influence of strain and cultivation procedure on the performance of simultaneous saccharification and fermentation of steam pretreated spruce. Enzyme Microb Technol 2006;38:279–86.
[37] Chemical Analysis and Testing Task, Laboratory Analytical Procedures 001-005. http://cobweb.ecn.purdue.edu/~lorre/16/research/.
[38] Blakeney AB, Harris PJ, Henry RJ, Stone BA. A simple and rapid preparation of alditol acetates for monosaccharide analysis. Carbohydr Res 1983;113:291–9.
[39] Tenkanen M, Siika-aho M. An α-glucuronidase of *Schizophyllum commune* acting on polymeric xylan. J Biotechnol 2000;75:149–61.
[40] Suurnäkki A, Tenkanen M, Siika-aho M, Niku-Paavola M-L, Viikari L, Buchert J. *Trichoderma reesei* cellulases and their core domains in the hydrolysis and modification of chemical pulp. Cellulose 2000;7:189–209.
[41] Sipos B, Benkő Z, Dienes D, Réczey K, Viikari L, Siika-aho M. Characterisation of specific activities and hydrolytic properties of cell-wall degrading enzymes produced by *Trichoderma reesei* Rut C30 on different carbon sources. Appl Biochem Biotechnol; in press. DOI 10.1007/s12010-009r-r8824-4.
[42] Tenkanen M, Puls J, Poutanen K. Two major xylanases of *Trichoderma reesei*. Enzyme Microb Technol 1992;14:566–74.
[43] Biely P, Vršanská M, Claeyssens M. The endo-1,4-β-glucanase I from *Trichoderma reesei*. Action on β-1,4-oligomers and polymers derived from D-glucose and D-xylose. Eur J Biochem 1991;200:157–63.
[44] Karlsson J, Siika-aho M, Tenkanen M, Tjerneld F. Enzymatic properties of the low molecular mass endoglucanases Cel12A (EG III) and Cel45A (EG V) of *Trichoderma reesei*. J Biotechnol 2002;99:63–78.
[45] van Tilbeurgh H, Claeyssens M, de Bruyne CK. The use of 4-methylumbelliferyl and other chromophoric glycosides in the study of cellulolytic enzymes. FEBS Lett 1982;149:152–6.
[46] van Tilbeurgh H, Loontiens FG, de Bruyne CK, Claeyssens M. Fluorogenic and chromogenic glycosides as substrates and ligands of carbohydrases. Methods Enzymol 1988;160:45–59.
[47] Bailey MJ, Nevalainen KMH. Induction, isolation and testing of stable *Trichoderma reesei* mutants with improved production of solubilising cellulose. Enzyme Microb Technol 1981;3:153–7.
[48] Bailey MJ, Biely P, Poutanen K. Interlaboratory testing of methods for assay of xylanase activity. J Biotechnol 1991;23:257–70.
[49] IUPAC (International Union of Pure and Applied Chemistry). Measurement of cellulose activity. Pure Appl Chem 1987; 59:257-68.
[50] Palm M, Zacchi G. Extraction of hemicellulosic oligosaccharides from spruce using microwave oven or steam treatment. Biomacromolecules 2003;4:617–23.
[51] Sloneker JH. Determination of cellulose and apparent hemicellulose in plant tissue by gas-liquid chromatography. Anal Biochem 1971;43:539–46.
[52] Berlin A, Gilkes N, Kilburn D, Bura R, Markov A, Skomarovsky A, et al. Evaluation of novel fungal cellulase preparations for ability to hydrolyze softwood substrates—evidence for the role of accessory enzymes. Enzyme Microb Technol 2005;37:175–84.

[53] Desai SG, Converse AO. Substrate reactivity as a function of the extent of reaction in the enzymatic hydrolysis of lignocellulose. Biotechnol Bioeng 1997;56:650–5.
[54] Eriksson T, Karlsson J, Tjerneld F. A model explaining declining rate in hydrolysis of lignocellulose substrates with cellobiohydrolase I (Cel7A) and endoglucanase I (Cel7B) of *Trichoderma reesei*. Appl Biochem Biotechnol 2001;101:41–60.
[55] Yang B, Willies DM, Wyman CE. Changes in the enzymatic hydrolysis rate of Avicel cellulose with conversion. Biotechnol Bioeng 2006;94:1122–8.
[56] Rudolf A, Alkasrawi M, Zacchi G, Lidén G. A comparison between batch and fed-batch simultaneous saccharification and fermentation of steam pretreated spruce. Enzyme Microb Technol 2005;37:195–204.

PAPER III

Adsorption of monocomponent enzymes in enzyme mixture analyzed quantitatively during hydrolysis of lignocellulose substrates

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Adsorption of monocomponent enzymes in enzyme mixture analyzed quantitatively during hydrolysis of lignocellulose substrates

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ABSTRACT

The adsorption of purified *Trichoderma reesei* cellulases (TrCel7A, TrCel6A and TrCel5A) and xylanase TrXyn11 and *Aspergillus niger* β-glucosidase AnCel3A was studied in enzyme mixture during hydrolysis of two pretreated lignocellulosic materials, steam pretreated and catalytically delignified spruce, along with microcrystalline cellulose (Avicel). The enzyme mixture was compiled to resemble the composition of commercial cellulase preparations. The hydrolysis was carried out at 35 °C to mimic the temperature of the simultaneous saccharification and fermentation (SSF). Enzyme adsorption was followed by analyzing the activity and the protein amount of the individual free enzymes in the hydrolysis supernatant. Most enzymes adsorbed quickly at early stages of the hydrolysis and remained bound throughout the hydrolysis, although the conversion reached was fairly high. Only with the catalytically oxidized spruce samples, the bound enzymes started to be released as the hydrolysis degree reached 80%. The results based on enzyme activities and protein assay were in good accordance.

1. Introduction

Replacing fossil fuels with alternative energy sources, such as renewable bioenergy is an important target in most countries today. Biomass is the only raw material which can be used for the production of renewable liquid fuels. Cellulose conversion to sugar monomers on a commercial scale is, however, still hampered by technical and economic obstacles. Efficient, optimised pretreatment technologies and more efficient, economically competitive enzymatic hydrolysis are the most commonly addressed approaches to overcome the recalcitrancy of biomass (Himmel et al., 2007). The high costs of enzyme production along with the high enzymatic dosages necessary to hydrolyse pretreated biomass are often considered the major bottlenecks on the path to a commercial lignocellulosic ethanol industry (Margeot et al., 2009). Hence, obtaining a better understanding on the enzyme adsorption–desorption during the hydrolysis could contribute to diminishing the enzyme loading or recycling the enzymes, which are some of the key factors to achieve economically feasible production of lignocellulose derived ethanol.

Adsorption of enzymes on the substrate during the hydrolysis is a desired phenomenon: the productive binding of the proteins on the surface of the substrate has been shown to aid the two-phase enzyme–substrate interaction (Klyosov et al., 1986; Tomme et al., 1988). However, non-productive binding of the hydrolytic enzymes on the lignocellulosic substrates also occurs: the enzymes either remain blocked in the dead ends of the substrate structure, or bind non-productively to cellulose (Jalak and Väljamäe, 2010) or to lignin (Palonen et al., 2004). So far, it has not been possible to differentiate between these two types of binding on complex substrates. In addition to the non-productive binding, enzyme deactivation and various rate limitations, such as decrease in substrate reactivity and inaccessibility caused by lignin, slow down the saccharification.

Adsorption of commercial enzyme preparations and purified monocomponent enzymes from *Trichoderma reesei* has been studied on lignocellulosic substrates as a function of various physical parameters, such as time, temperature, pH and ionic strength for the last 30 years (Kyriacou et al., 1988; Tu et al., 2009). Most of these studies agree that the adsorption equilibrium occurs already within the first 10–20 min of incubation at pH 4.8–5.6, irrespective of temperature (4 or 40–50 °C), enzyme dosage, purity of the enzyme preparations or lignin content of the substrate (Nidetzky et al., 1994; Medve et al., 1998). According to Karlsson et al. (1999), however, the adsorption equilibrium of purified TrCel7A and TrCel5A was reached on steam pretreated willow only within 2 h. Increasing the temperature from 4–5 °C to 40–50 °C has been shown to result in an increased adsorption both on cellulose and lignocellulose (Kyriacou et al., 1988; Karlsson et al., 1999). When further raising the temperature, adsorption may increase due to heat denaturation. Increasing the pH leads to decreased adsorption

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due to changes in the charge distribution of the enzyme (Kyriacou et al., 1988). On the other hand, altering the ionic strength by NaCl (up to 1 M) has been shown to result in different adsorption patterns of pure Cel7A and EG's on cellulose (Kyriacou et al., 1988). However, the ionic strength did not seem to significantly affect the overall adsorption of commercial cellulases on the lignin residue (Tu et al., 2009).

The presence of cellulose-binding module (CBM) has been found to have a profound role in the adsorption of cellulases on lignocelluloses. The cleavage of CBM results in a strongly reduced enzyme activity toward insoluble cellulose while the enzyme activity on soluble substrates is fully retained (Tomme et al., 1988; Nidetzky et al., 1994). Similarly, the absence of CBM leads to decreased adsorption on lignin (Palonen et al., 2004).

During hydrolysis, adsorption of cellulases has been studied using both commercial and purified enzyme preparations in order to understand the mechanism of the enzyme actions. Two main phenomena take place in parallel: a quick initial adsorption followed by a slow adsorption–desorption of the enzyme components. This effect is eventually overcome by denaturation and precipitation of the enzyme proteins. The practical aim has been to enhance enzyme desorption at the end of the hydrolysis to recover the enzymes. This could be achieved essentially by three means: by development of advanced pretreatment technologies to improve the accessibility of substrates (Yang and Wyman, 2008), by application of surfactants to prevent non-productive binding of enzymes (Ooshima et al., 1986) and by discovery of high mobility enzymes with lower affinity to the lignin-rich hydrolysis residues (Berlin et al., 2005).

Pretreatment of lignocelluloses changes the surface properties of substrates and affects enzyme adsorption capacities during hydrolysis. Removal of lignin has been shown to have an important effect on decreasing the irreversible enzyme adsorption on the lignocellulosic substrates, *i.e.* on lignin (Boussaid and Saddler, 1999; Lu et al., 2002). On pretreated softwood with a relatively high lignin content, cellulases show a quick initial adsorption followed by continuously increasing adsorption (Boussaid and Saddler, 1999; Lu et al., 2002). On the other hand, a quick release of the enzymes occurs on delignified substrates and on Avicel after the initial adsorption (Boussaid and Saddler, 1999; Lu et al., 2002; Tu et al., 2007). In most cases, the adsorption remains at a constant level, however, continuous desorption of the enzyme proteins has also been observed on delignified substrates (Boussaid and Saddler, 1999; Tu et al., 2007).

Adsorption of pure cellulases during hydrolysis has been less extensively studied both on cellulosic model substrates and on lignocellulosic substrates. In the early phase of the hydrolysis of Avicel and steam pretreated willow at 40 °C, the adsorption of TrCel7A and TrCel5A seemed to remain constant, irrespective of added alone or in equimolar mixture (Medve et al., 1998; Karlsson et al., 1999). On the other hand, the adsorption of TrCel7A, TrCel6A, TrCel7B and TrCel5A steadily increased during the hydrolysis of the filter paper substrate (Nidetzky and Claeyssens, 1994). The studies showed that the ratio of the adsorbed individual enzymes was very close to their originally loaded ratio throughout the hydrolysis. The binding of TrCel7A was only slightly reduced by the presence of TrCel5A while the binding of TrCel5A was strongly reduced by the presence of the other enzyme (Medve et al., 1998). A linear relationship between the conversion rate and the total amount of bound enzymes was observed when TrCel7A and TrCel5A were added together (Medve et al., 1998; Karlsson et al., 1999).

To prevent non-productive binding of cellulases to residual lignin, surfactants have been added to the hydrolysis prior to the enzyme addition. The presence of surfactants leads to enhanced cellulose conversion and higher amount of free enzymes in the supernatant (Eriksson et al., 2002; Sipos et al., 2010). Among these, non-ionic surfactants have proved to be the most effective due to their hydrophobic interaction with lignin, affecting positively enzyme–substrate interactions and leading to higher cellulose conversion (Eriksson et al., 2002).

Although binding of enzymes during the hydrolysis of cellulose and lignocellulosic materials containing lignin has been extensively studied with commercial preparations and individual enzymes, only few results using enzyme mixtures composed of purified enzymes have been published (Nidetzky and Claeyssens, 1994; Medve et al., 1998). Furthermore, the commercial enzyme mixtures used during hydrolysis have been mostly characterised by their overall cellulase activity (filter paper activity) and total protein content. Only in some cases attempts were made to follow the amount of the individual enzymes (Tu et al., 2007; Sipos et al., 2010).

In this paper, the adsorption of five different monocomponent enzymes composing a mixture during the hydrolysis of three structurally different substrates, *i.e.* steam pretreated spruce, catalytically oxidised spruce and microcrystalline cellulose (Avicel) was studied. The enzyme mixture contained the purified cellobiohydrolases TrCel7A and TrCel6A, endoglucanase TrCel5A and xylanase TrXyn11 from *T. reesei*, and the β-glucosidase AnCel3A from *Aspergillus niger*. The adsorption of the individual enzymes was followed during hydrolysis by two different methods: by measuring the individual enzyme activities and by quantifying the protein amount on SDS–PAGE, revealing distinct differences in the adsorption behaviour of the enzymes on the different substrates.

2. Methods

2.1. Raw materials and preparation of substrates

Two differentially pretreated lignocellulosic softwood raw materials, *i.e.* steam pretreated (SPS) and catalytically delignified spruce (COS) and commercial microcrystalline cellulose (Avicel, SERVA) were used as substrates in the experiments. SPS was kindly provided by Lund University, Sweden. In the steam pretreatment, the spruce chips were impregnated with 3% SO_2, steam pretreated at 215 °C for 5 min and the solid fraction was washed with hot water. In COS preparation, the spruce chips were delignified with a novel catalytic oxidation method modified from the method of Korpi et al. (2004) using Na_2CO_3 solution (0.5 mol/kg wood) and copper–1,10-phenanthroline complex (containing $CuSO_4$ and ligand in molar ratio 1:2) as catalyst at a loading of 33.3 mmol phenanthroline and 16.8 mmol $CuSO_4$ per kg wood. The delignification was carried out for 500 g dry weight of spruce chips at 10 L total volume, at 10 bar oxygen pressure, at 140 °C for 4 h. The catalytic oxidation resulted in a fibre fraction and soluble fraction which were separated by filtration. The solid fraction, after washing, was used as substrate in the experiments. The microcrystalline cellulose (Avicel) was used as reference.

2.2. Compositional analysis of the samples

2.2.1. Carbohydrates

The monosaccharide composition of the raw material samples was determined after a two-step hydrolysis with sulphuric acid according to the NREL-procedure (NREL LAP's) on Dionex ICS-3000 gradient HPLC system (Dionex ICS-3000, Sunnyvale, CA) using CarboPac PA-1 column and 1 mL/min eluent flow and 30 °C column temperature (Tenkanen and Siika-aho, 2000). The composition of carbohydrates is expressed as anhydro units, *i.e.* corresponding to polymeric form as weight percentage of the total dry weight (Table 1). Carbohydrates liberated in enzymatic hydrolysis experiments were analyzed by HPLC as described above. The anal-

Table 1
Composition of the raw materials.

Sample	Carbohydrate (% of d.w.)	Glucan (% of d.w.)	Mannan (% of d.w.)	Xylan (% of d.w.)	Arabinan (% of d.w.)	Galactan (% of d.w.)	Lignin (% of d.w.)	Extractives (% of d.w.)	Ash (% of d.w.)
SPS	51.5	51.0	0.23	0.21	<0.1	<0.1	32.5	10.9	0.11
COS-FF	96.1	85.3	6.94	3.42	0.31	0.11	4.1	0.4	1.27
Avicel	94.0	91.3	1.45	1.23	<0.1	<0.1	n.d.	n.d.	<0.05

n.d., not determined.

ysis of linear cello- and xylooligomers was performed from the final hydrolysates on the same HPLC system, applying different gradient profile described by Tenkanen et al. (1997).

2.2.2. Lignin and extractives

Klason-lignin and acid-soluble lignin in the SPS and COS was determined after acetone extraction according to the NREL methods with slight modification (NREL LAP's), as previously described (Várnai et al., 2010). The lignin was calculated as the sum of the Klason- and acid-soluble lignin. Lignin content was not determined from Avicel since it is considered pure cellulose.

2.2.3. Ash

The remaining ash of dried samples combusted in furnace at 575 °C was measured according to the NREL-method (NREL LAP's) (Table 1).

2.3. Enzymes

The cellobiohydrolases Cel7A (TrCel7A, CBH I, EC 3.2.1.91.) and Cel6A (TrCel6A, CBH II, EC 3.2.1.91.), as well as endoglucanase Cel5A (TrCel5A, EG II, EC 3.2.1.4.) from *T. reesei* were purified according to Suurnäkki et al. (2000); Xyn11 (TrXyn11, xylanase II, EC 3.2.1.8.) from *T. reesei* was purified according to Tenkanen et al. (1992) and β-glucosidase Cel3A (AnCel3A, EC 3.2.1.21.) from *A. niger* according to Sipos et al. (2009). The enzymes used were pure as judged by SDS–PAGE.

2.4. Enzymatic hydrolysis

In the enzymatic hydrolysis experiments, the samples were hydrolysed using a mixture of purified enzyme preparations at a total protein loading of 25 mg/g of cellulose. The enzyme mixture consisted of cellulases, *i.e.* 60% TrCel7A, 20% TrCel6A and 20% TrCel5A (14.7, 4.9 and 4.9 mg protein/g cellulose, respectively) supplemented with β-glucosidase AnCel3A at a dosage of 100 nkat/g cellulose (0.050 mg protein/g cellulose) and xylanase TrXyn11 at 5000 nkat/g cellulose (approximately 0.50 mg protein/g cellulose). The hydrolysis was carried out in duplicates at 2% cellulose consistency in 50 mM sodium–citrate buffer (pH 5.0) in 1 mL total volume, at 35 °C, mixing with magnetic stirrer bars at 250 rpm for up to 144 h. Individual samples at each time point were chilled on ice for 5 min. The hydrolysis supernatants were collected after centrifugation for 5 min at 3000 rpm at 4 °C. Before the enzyme quantification by activity assays and SDS–PAGE, the samples were stored frozen at −20 °C. For the carbohydrate analysis, the enzymes were inactivated by boiling for 10 min after sampling immediately. To obtain samples representing initial adsorption on the substrates (0-h samples), the substrates were incubated with the enzyme mixture in duplicates at 2% cellulose consistency in 50 mM sodium–citrate buffer (pH 5.0) in 1 mL total volume, at 4 °C for 30 min. To ensure proper mixing, the Eppendorf tubes were inverted by rotation at 20 rpm during incubation. The supernatants were separated by centrifugation at 4 °C for 5 min at 13,000 rpm.

2.5. Enzyme activity measurements

The enzyme activities were measured as follows: the cellobiohydrolase I activity using 4-methylumbelliferyl-β-D-lactoside (MUL) as substrate according to van Tilbeurgh et al. (1988); the endoglucanase II activity using 4-methylumbelliferyl-β-D-cellotrioside (MUG_3) according to Macarrón et al. (1993); the xylanase activity using birch xylan according to Bailey et al. (1991); as well as the β-glucosidase activity using *p*-nitrophenyl-β-glucopyranoside (*p*NPG) according to Bailey and Nevalainen (1981).

2.6. SDS–PAGE

The gel electrophoresis of the hydrolysates was carried out with BIORAD Criterion Stain Free Imager system (McDonald et al., 2008). The samples were mixed in a ratio of 3:1 with SDS-solution and boiled for 5 min, then loaded onto 4–20% Tris–HCl 1.0 mm Criterion Precast Gel and run in 25 mM Tris/192 mM glycine/0.1 M SDS buffer with 200 V and 100 mA for 55 min, using BIORAD Precision Plus standard. The evaluation of the image consisted of a 5-min gel activation step and of an exposure stage, the time of which could be adjusted to produce the desired intensity of the bands. During the activation, the gel was radiated with UV-light, which modifies the tryptophan residues contained in proteins to fluorescent trihalo compounds. In the next step, the fluorescence was scanned for a certain exposure time chosen between 0.001 and 10 s. In general, 0.1–0.2 s exposure time was used to detect the bands containing 1–3 μg protein. By increasing the exposure time, the sensitivity of the measurement could be increased roughly up to 100 times.

The detection and the quantitative evaluation of the bands were carried out with Image Lab software. The original enzyme mixture was used as control. Residual band intensities in the hydrolysis supernatants were calculated as percent of original intensity in the enzyme mixture. The gels are presented as recorded by the Image Lab software choosing the image colours according to the Coomassie staining.

3. Results and discussion

3.1. Composition of raw materials

Two of the raw materials were almost pure cellulose: the catalytically delignified softwood cellulose (COS) and Avicel. The carbohydrate content of the COS was above 96%, and thus most of the lignin had been removed from the substrate (Table 1). The monosaccharide composition revealed that, unlike during the steam explosion, a significant amount of hemicelluloses, in particular mannan (6.94%) and xylan (3.42%), remained in the solid material after the oxidative pretreatment. In Avicel, the major monosaccharide detected was glucose (ca. 91%) and only minor amounts of hemicelluloses could be detected (<3%). The monosaccharide analysis indicated that SPS contained ca. 50% of cellulose and ca. 30% of lignin. In addition, SPS contained a high fraction of acetone soluble extractives.

3.2. Enzymatic hydrolysis

All three substrates, SPS, Avicel and COS, were fairly efficiently hydrolysed at conditions resembling the simultaneous saccharification and fermentation (SSF) (35 °C, pH 5) using the mixture of *T. reesei* cellulases (Cel7A, Cel6A and Cel5A) and xylanase (Xyn11) and the *A. niger* β-glucosidase (Cel3A). The cellulase mixture used in the hydrolysis (Cel7A 60%, Cel6A 20% and Cel5A 20%) was designed to resemble the average composition of a typical *T. reesei* broth. Cel7B (endoglucanase I) was omitted from the enzyme mixture partly because it has hydrolytic activity on xylan (Biely et al., 1991), and partly because of the similar molecular weight to that of Cel6A, which would have made the separation of the enzymes by SDS–PAGE analysis difficult.

The carbohydrate analysis indicated that after 3 days, up to 64%, 76% and 87% and after 6 days, up to 79%, 96% and 98% of the glucan content were hydrolysed to glucose monomers from the SPS, Avicel and COS substrates, respectively (Fig. 1). As expected, lignin removal in the oxidative pretreatment enhanced the hydrolysis more extensively than the lignin preserving steam explosion. In addition, the catalytic oxidation seemed to increase the reactivity of the substrate, resulting in a higher hydrolysis rate than that of Avicel. This could also be due to the fact that the catalytically oxidized cellulose was never dried while Avicel is a dried product.

Hydrolysis of xylan and cellulose occurred in parallel (Fig. 1). At the end of the hydrolysis (after 144 h), 11%, 42% and 21% of the xylan was detected as xylose monomers in the hydrolysates of SPS, Avicel and COS, and further 12%, 26% and 16% of the total xylan content was present as linear xylo-oligomers. Despite the lower hemicellulose content of the substrate, xylan in Avicel was liberated to a higher extent than from COS.

3.3. Enzyme adsorption during hydrolysis

The adsorption of enzymes during the hydrolysis was analyzed by measuring the free enzyme activities (Fig. 2) and protein concentrations (Table 2–4) in the hydrolysis supernatants. In the enzyme activity measurements, chromophoric substrates (MUL, MUG_3 and *p*NPG) were used in order to measure individual enzyme activities without interference by the elevated sugar concentrations in the hydrolysis supernatants. The activity of TrCel6A (CBH II) could not be measured due to lack of available specific substrate for the activity determination. For xylanase activity measurement, no chromogenic substrate was available and the enzyme activities were quantified using birch xylan as substrate. Since the assay is based on the quantification of reducing sugars, the measured activity was interfered by the high glucose background in the enzymatic hydrolysate especially in the late phase of the hydrolysis. Otherwise, interference of the background sugars did not cause problems when using suitable blanks. To confirm that the proteins were adsorbed to the residual substrate rather than remained in an inactive form in the supernatant, the free enzyme proteins were also quantified in the hydrolysis supernatants using the Criterion Stain Free SDS–PAGE system and Image Lab software (Figs. 3–5 and Tables 2–4).

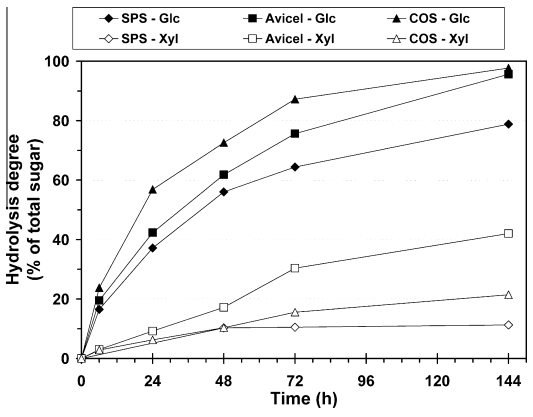

Fig. 1. Hydrolysis degree of glucan (full symbols) and xylan (open symbols) as percent of total polymer as free glucose or xylose during hydrolysis of (A) SPS (diamond), (B) COS (square) and (C) Avicel (triangle) based on HPLC analysis. Hydrolysis with mixture of purified enzymes at 35 °C.

3.4. Adsorption of cellulases

Both enzyme activity assays and SDS–PAGE analysis indicated that on SPS, both TrCel7A and TrCel5A were bound extensively: only 1.1% and 0.6% of the original activity corresponding to 1.2% and 0.9% residual protein, respectively, could be detected from the supernatants after the early stages of the hydrolysis (Fig. 2A and B and Table 2). The SDS–PAGE analysis indicated that the adsorption of TrCel6A followed the same trend (Fig. 3). The concentrations of the free enzyme activity and free protein decreased further as the hydrolysis proceeded. Even though 80% of the total polysaccharides were hydrolysed, more than 99% of the original enzyme activities were still bound on the lignin containing hydrolysis residue. The initial adsorption results are well in accordance with the results of Palonen et al. (2004) where 98% of TrCel7A of the loaded protein (39 mg per gram of SPS) was bound at 4 °C, whereas slightly lower, about 90% adsorption of TrCel5A of the loaded protein (30 mg per gram of SPS) was observed. The measured higher adsorptions can be explained by the lower loading of enzymes in this work, *i.e.* 14.7 mg TrCel7A and 4.9 mg TrCel5A per gram SPS, as it is suggested that the enzyme adsorption follows the Langmuir isotherm and the enzymes adsorb to relatively lower extent at higher enzyme loadings (Karlsson et al., 1999).

In the hydrolysis of Avicel, TrCel7A and TrCel5A were less bound on Avicel during the initial phase of the hydrolysis than on SPS, indicated by both activity assays and protein quantification (Fig. 2A and B and Fig. 4). Obviously, this is due to the absence of lignin, and eventually due to the higher degree of hydrolysis. TrCel5A seemed to adsorb less to the original Avicel substrate than TrCel7A but was later equally adsorbed to the residual substrate. During the first 48 h, the amount of free TrCel7A and TrCel5A decreased to below 3% of original. By the end of the hydrolysis (144 h), over 99% of the original enzyme had disappeared from the supernatant. The SDS–PAGE analysis indicated that also TrCel6A adsorbed relatively less to Avicel as compared to TrCel7A during the first hours (0–6 h) of the hydrolysis (Table 3). During the course of the hydrolysis, Cel7A and Cel6A were, however, adsorbed to the same extent. The adsorption results on SPS and Avicel are consistent with previous results showing that most of the adsorbed enzymes remained bound on the residual substrate even after high conversion of pretreated softwood or Avicel (Lu et al., 2002; Tu et al., 2007). The degree of adsorption was, however, much higher, over 95% in present experiments, most probably due to the lower enzyme loading (equalling to about 10 FPU/g cellulose instead of 20 or 40 FPU/g cellulose) and due to the lack of other proteins which are present in the commercial enzyme preparations.

During the hydrolysis of COS, both enzyme assays and SDS–PAGE analysis indicated that after the initial phase of hydrolysis (up to 6 h), ca. 98% of TrCel7A and TrCel5A were bound to the COS substrate (Fig. 2A and B and Table 4). However, after the initial fast adsorption, the enzymes began to desorb and after 48 h when the hydrolysis degree approached 73% of total glucan content, the activity of desorbed enzymes increased continuously with the

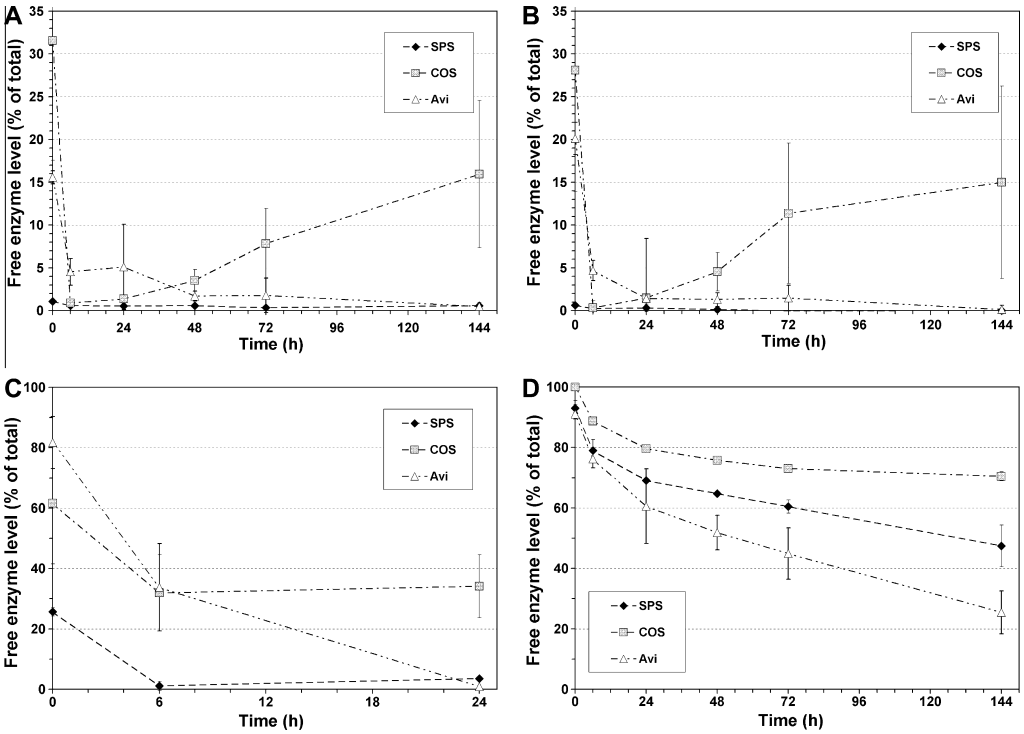

Fig. 2. Free enzyme activities of (A) TrCel7A, (B) TrCel5A, (C) TrXyn11 and (D) AnCel3A in supernatant during hydrolysis of SPS, COS and Avicel.

Table 2
Free proteins in SPS hydrolysates quantified from the SDS–PAGE as % of total dosed to the hydrolysis.

Time (h)	TrCel7A (% of total)	TrCel6A (% of total)	TrCel5A (% of total)	TrXyn11 (% of total)	AnCel3A (% of total)
0	1.2	1.1	0.9	18.6	102.7
6	0.7	1.0	b.d.[a]	traces	93.0
24	0.7	1.3	b.d.[a]	b.d.[a]	91.1
48	0.8	1.0	b.d.[a]	b.d.[a]	83.2
72	b.d.	0.7	b.d.[a]	b.d.[a]	79.4
144	b.d.	b.d.[a]	b.d.[a]	b.d.[a]	65.8

[a] Below detection limit.

Table 3
Free proteins in Avicel hydrolysates quantified from the SDS–PAGE as% of total dosed to the hydrolysis.

Time (h)	TrCel7A (% of total)	TrCel6A (% of total)	TrCel5A (% of total)	TrXyn11 (% of total)	AnCel3A (% of total)
0	11.2	28.4	18.1	84.5	96.6
6	6.7	8.9	3.7	27.1	84.4
24	3.0	3.3	0.9	1.7	71.9
48	2.4	2.5	0.8	0.3	63.2
72	3.8	3.6	2.1	0.5	74.9
144	0.8	1.3	b.d.[a]	b.d.[a]	49.6

[a] Below detection limit.

hydrolysis degree. The ratio of adsorbed TrCel7A and TrCel5A seemed to remain constant equalling to the originally loaded ratio (in the present case 3:1) during the whole hydrolysis, which is in accordance with the results by Medve et al. (1998) and Karlsson et al. (1999) on Avicel and steam pretreated willow at 40 °C, respectively. Similar to TrCel7A and TrCel5A, a quick initial adsorption of TrCel6A after the start of hydrolysis as well as desorption of all the three cellulolytic enzymes during the course of hydrolysis was revealed by the SDS–PAGE analysis (Fig. 5). On the other hand, as compared with TrCel7A, a lower amount of TrCel6A remained bound on the residual substrate throughout the hydrolysis. Our results are analogous to those reported by Boussaid and Saddler (1999) showing desorption of both *T. reesei* cellobiohydrolases and endoglucanases into solution during the hydrolysis of Douglas fir kraft pulp. Interestingly, during the later stage of hydrolysis the measured amount of free TrCel7A enzyme quantified by enzyme assays was constantly lower than the amount of TrCel7A protein measured by SDS–PAGE analysis. The oligosaccharide analysis revealed that 58, 58 and 18 μM cellobiose was present in the final supernatants of SPS, Avicel and COS hydrolysis, respectively, in the activity assay. These concentrations are at the same range as the cellobiose inhibition constant, 19 ± 4 μM, determined by Vouti-

Table 4
Free proteins in COS-FF hydrolysates quantified from the SDS–PAGE as% of total dosed to the hydrolysis.

Time (h)	TrCel7A (% of total)	TrCel6A (% of total)	TrCel5A (% of total)	TrXyn11 (% of total)	AnCel3A (% of total)
0	21.5	40.4	29.7	58.2	94.3
6	2.1	11.5	1.7	31.0	105.3
24	2.5	8.9	1.9	19.8	100.4
48	7.7	20.5	6.3	33.5	104.0
72	17.5	36.2	17.4	34.7	103.4
144	20.6	34.1	17.4	9.4	84.2

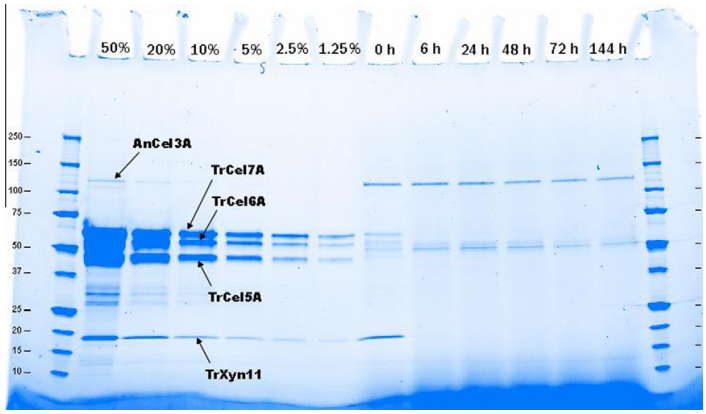

Fig. 3. SDS–PAGE of SPS hydrolysis supernatants: Lane 1 and 14: BIORAD Precision Plus Standard, Lane 2–7: standard curve: 50%, 20%, 10%, 5%, 2.5% and 1.25% of total protein loaded for band quantification; Lane 8–13: Hydrolysates after 0, 6, 24, 48, 72 and 144 h of hydrolysis. (Enzyme reference at 0 h (100%) equals to 14.7 mg/g TrCel7A, 4.9 mg/g TrCel6A, 4.9 mg/g TrCel5A, 0.5 mg/g TrXyn11, 0.05 mg/g AnCel3A dosed based on cellulose content).

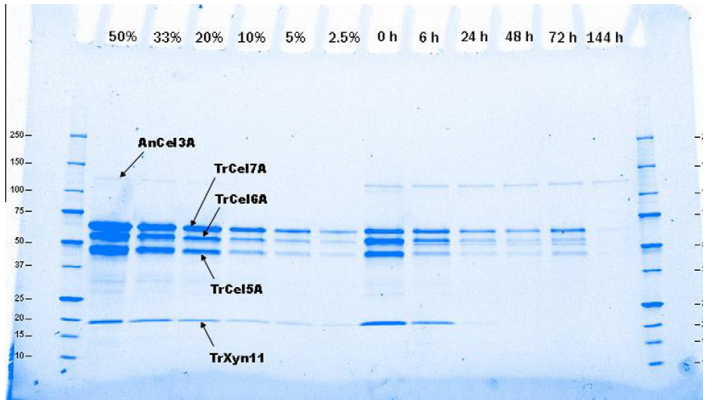

Fig. 4. SDS–PAGE of Avicel hydrolysis supernatants: Lane 1 and 14: BIORAD Precision Plus Standard, Lane 2–7: Standard curve: 50%, 33%, 20%, 10%, 5% and 2.5% of total protein loaded for band quantification; Lane 8–13: Hydrolysates after 0, 6, 24, 48, 72 and 144 h of hydrolysis. (Enzyme reference at 0 h (100%) equals to 14.7 mg/g TrCel7A, 4.9 mg/g TrCel6A, 4.9 mg/g TrCel5A, 0.5 mg/g TrXyn11, 0.05 mg/g AnCel3A dosed based on cellulose content).

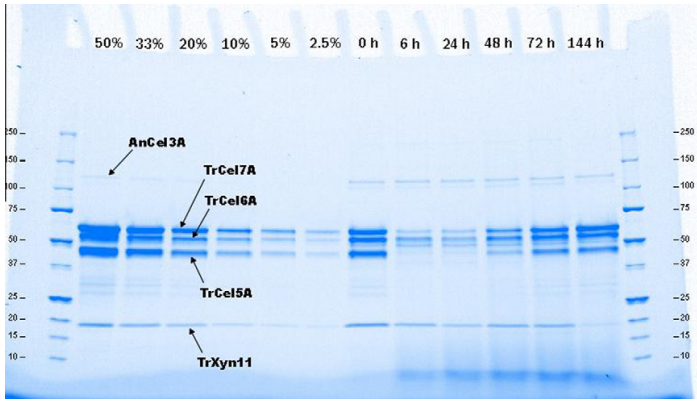

Fig. 5. SDS–PAGE of COS hydrolysis supernatants: Lane 1 and 14: BIORAD Precision Plus Standard, Lane 2–7: standard curve: 50%, 33%, 20%, 10%, 5% and 2.5% of total protein loaded for band quantification; Lane 8–13: Hydrolysates after 0, 6, 24, 48, 72 and 144 h of hydrolysis. (Enzyme reference at 0 h (100%) equals to 14.7 mg/g TrCel7A, 4.9 mg/g TrCel6A, 4.9 mg/g TrCel5A, 0.5 mg/g TrXyn11, 0.05 mg/g AnCel3A dosed based on cellulose content).

lainen et al. (2008). This could explain the slightly lower enzyme activities as compared to the protein amount measured.

3.5. Adsorption of β-glucosidase

In general, the AnCel3A was less adsorbed to the surface of the SPS, Avicel and COS than the *T. reesei* cellulases. This could be at least partially due to the fact that AnCel3A does not have a CBM. By the end of the hydrolysis (144 h), the free activity of AnCel3A β-glucosidase decreased continuously on all three substrates, SPS, Avicel and COS, to 47.4%, 25.5% and 70.5% of the original, respectively (Fig. 2D). Based also on the SDS–PAGE analysis, the amount of free β-glucosidase was shown to decrease on SPS and Avicel while on COS no such diminishing trend of free protein was observed (Table 2–4). At the end of the hydrolysis, the lowest amount of free AnCel3A β-glucosidase was measured in Avicel hydrolyzates. The surface of SPS, rich in lignin, was thus obviously more repulsive towards the β-glucosidase than the crystalline Avicel surface. On the other hand, in case of the other low-lignin containing substrate, COS, the highest β-glucosidase activity could be retained at the end of the hydrolysis. The reason may be the less ordered, easily accessible and hydrolysable structure of this substrate and possibly also the altered surface characteristics due to the oxidative pretreatment. For the AnCel3A, a clear difference in measured free activity and protein was seen, *i.e.* the free protein was mostly higher than the free activity. This can be explained by a strong end-product inhibition due to the released glucose during the activity assay on pNPG: 1.9, 2.1 and 2.1 mM glucose concentration from SPS, Avicel and COS hydrolysates, respectively. However, some enzyme inactivation during the hydrolysis experiment might also have occurred.

3.6. Adsorption of xylanase

The adsorption of xylanase TrXyn11 seemed to depend more on the lignin content than on the xylan content of the substrates. During the initial phase of the hydrolysis, both enzyme activity assay and SDS–PAGE analysis indicated that the SPS, containing the lowest xylan amount, adsorbed the xylanase more pronouncedly (74.3% of original activity) than the highest xylan-containing COS (38.4% of the original activity) or the low xylan containing Avicel (18.3% of the original activity) (Fig. 2C). Xylanase was adsorbed rather rapidly after the hydrolysis of SPS started, and after 6 h less than 1% of the original enzyme remained in the supernatant (Table 2). The analysis of the Avicel supernatants also revealed that xylanase adsorbed less to Avicel within the first hours of the hydrolysis than the cellulases TrCel7A, TrCel6A and TrCel5A (Table 3). The xylanase used does not have a xylan- or cellulose-binding module. Hence, as could be expected, xylanase was initially adsorbed on the substrates to a much lower extent than TrCel7A, TrCel6A or TrCel5A.

On both Avicel and COS, the xylanase activities were only measured during the first 24 h of the hydrolysis because the high glucose background interfered with the enzyme assay. The adsorption at the later stage of hydrolysis was followed by the SDS–PAGE protein quantification. The quantitative SDS–PAGE also confirmed that, analogously to the cellulolytic enzymes, over 98% of xylanase was adsorbed and remained bound on SPS and Avicel until the end of hydrolysis (Tables 2 and 3). This suggests that xylanase was adsorbed not only on xylan but on glucan and lignin as well. On the contrary, in the case of COS desorption of xylanase TrXyn11 was detected during the course of hydrolysis, similar to *T. reesei* cellulases (Fig. 5). Despite the higher xylan content and lower hydrolysis degree of xylan than that of Avicel, TrXyn11 seemed to have less affinity to COS. The binding mechanism of this enzyme to the low- or non-xylan containing samples is obscure and remains to be further studied. It can be anticipated that the enzyme remains bound on poorly accessible residual xylans or is structurally blocked to non-hydrolysable sites, eventually connected with cellulose.

4. Conclusions

The main individual cellulolytic enzyme components studied; Cel7A, Cel6A and Cel5A remained mostly bound throughout the hydrolysis of two different types of substrates; Avicel and steam pretreated spruce. A higher part of the enzymes could be desorbed only from the oxidatively delignified spruce. Of the cellobiohydrolases, TrCel6A remained clearly less bound than TrCel7A. The binding behaviour of xylanase was obscure and could not be explained by the lignin or xylan contents in the substrate. The quantitative SDS–PAGE results correlated well with the enzyme activity measurements and confirmed that most desorbed, free enzymes, including the β-glucosidase, were also active.

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References

Bailey, M.J., Biely, P., Poutanen, K., 1991. Interlaboratory testing of methods for assay of xylanase activity. J. Biotechnol. 23, 257–270.
Bailey, M.J., Nevalainen, K.M.H., 1981. Induction, isolation and testing of stable *Trichoderma reesei* mutants with improved production of solubilising cellulose. Enzyme Microb. Technol. 3, 153–157.
Berlin, A., Gilkes, N., Kurabi, A., Bura, R., Tu, M., Kilburn, D., Saddler, J., 2005. Weak lignin-binding enzymes: a novel approach to improve activity of cellulases for hydrolysis of lignocellulosics. Appl. Biochem. Biotechnol. 121–124, 163–170.
Biely, P., Vršanská, M., Claeyssens, M., 1991. The endo-1,4-β-glucanase I from *Trichoderma reesei*. Action on β-1,4-oligomers and polymers derived from D-glucose and D-xylose. Eur. J. Biochem 200, 157–163.
Boussaid, A., Saddler, J.N., 1999. Adsorption and activity profiles of cellulases during hydrolysis of two Douglas fir pulps. Enzyme Microb. Technol. 24, 138–143.
Eriksson, T., Börjesson, J., Tjerneld, F., 2002. Mechanism of surfactant effect in enzymatic hydrolysis of lignocellulose. Enzyme Microb. Technol. 31, 353–364.
Himmel, M.E., Ding, S.Y., Johnson, D.K., Adney, W.S., Nimlos, M.R., Brady, J.W., Foust, T.D., 2007. Biomass recalcitrance. engineering plants and enzymes for biofuels production. Science 315, 804–807.
Jalak, J., Väljamäe, P., 2010. Mechanism of initial rapid rate retardation in cellobiohydrolase catalyzed cellulose hydrolysis. Biotechnol. Bioeng. 106, 871–883.
Karlsson, J., Medve, J., Tjerneld, F., 1999. Hydrolysis of steam-pretreated lignocellulose. Synergism and adsorption for cellobiohydrolase I and endoglucanase II of *Trichoderma reesei*. Appl. Biochem. Biotechnol. 82, 243–258.
Klyosov, A.A., Mitkevich, O.V., Sinitsyn, A.P., 1986. Role of the activity and adsorption of cellulases in the efficiency of the enzymatic hydrolysis of amorphous and crystalline cellulose. Biochemistry 25, 540–542.
Korpi, H., Lahtinen, P., Sippola, V., Krause, O., Leskelä, M., Repo, T., 2004. An efficient method to investigate metal–ligand combinations for oxygen bleaching. Appl. Catal., A 268, 199–206.
Kyriacou, A., Neufeld, R.J., MacKenzie, C.R., 1988. Effect of physical parameters on the adsorption characteristics of fractionated *Trichoderma reesei* cellulase components. Enzyme Microb. Technol. 10, 675–681.
Lu, Y., Yang, B., Gregg, D., Saddler, J.N., Mansfield, S.D., 2002. Cellulase adsorption and an evaluation of enzyme recycle during hydrolysis of steam-exploded softwood residues. Appl. Biochem. Biotechnol. 98–100, 641–654.
Macarrón, R., Acebal, C., Castillón, M.P., Domínguez, J.M., de la Mata, I., Pettersson, G., Tomme, P., Claeyssens, M., 1993. Mode of action of endoglucanase III from *Trichoderma reesei*. Biochem. J. 289, 867–873.
Margeot, A., Hahn-Hagerdal, B., Edlund, M., Slade, R., Monot, F., 2009. New improvements for lignocellulosic ethanol. Curr. Opin. Biotechnol. 20, 372–380.
McDonald, K., Choe, J., Elbaggari, A., Zhu, M., Alburo, A., 2008. Tech note 5782. Bio-Rd Laboratories, Inc. Retrieved from: <http://www3.bio-rad.com/cmc_upload/Literature/220635/Bulletin_5782B.pdf>.
Medve, J., Karlsson, J., Lee, D., Tjerneld, F., 1998. Hydrolysis of microcrystalline cellulose by cellobiohydrolase I and endoglucanase II from *Trichoderma reesei*:

adsorption, sugar production pattern, and synergism of the enzymes. Biotechnol. Bioeng. 59, 621–634.
Nidetzky, B., Claeyssens, M., 1994. Specific quantification of *Trichoderma reesei* cellulases in reconstituted mixtures and its application to cellulase–cellulose binding studies. Biotechnol. Bioeng. 44, 961–966.
Nidetzky, B., Steiner, W., Claeyssens, M., 1994. Cellulose hydrolysis by the cellulases from *Trichoderma reesei*: adsorptions of two cellobiohydrolases, two endocellulases and their core proteins on filter paper and their relation to hydrolysis. Biochem. J. 303, 817–823.
NREL Chemical Analysis and Testing Task, Laboratory Analytical Procedures (LAP's) 001-005. Available from: <http://cobweb.ecn.purdue.edu/~lorre/16/research/>.
Ooshima, H., Sakata, M., Harano, Y., 1986. Enhancement of enzymatic hydrolysis of cellulose by surfactant. Biotechnol. Bioeng. 28, 1727–1734.
Palonen, H., Tjerneld, F., Zacchi, G., Tenkanen, M., 2004. Adsorption of *Trichoderma reesei* CBH I and EG II and their catalytic domains on steam pretreated softwood and isolated lignin. J. Biotechnol. 107, 65–72.
Sipos, B., Benkő, Z., Dienes, D., Réczey, K., Viikari, L., Siika-aho, M., 2009. Characterisation of specific activities and hydrolytic properties of cell-wall degrading enzymes produced by *Trichoderma reesei* Rut C30 on different carbon sources. Appl. Biochem. Biotechnol. DOI: 10.1007/s12010-009-8824-4 Retrieved from: http://www.springerlink.com/content/c4nl6x81l7461j04/fulltext.pdf.
Sipos, B., Dienes, D., Schleicher, Á., Perazzini, R., Crestini, C., Siika-aho, M., Réczey, K., 2010. Hydrolysis efficiency and enzyme adsorption on steam-pretreated spruce in the presence of poly (ethylene glycol). Enzyme Microb. Technol. 47, 84–90.
Suurnäkki, A., Tenkanen, M., Siika-aho, M., Niku-Paavola, M.-L., Viikari, L., Buchert, J., 2000. *Trichoderma reesei* cellulases and their core domains in the hydrolysis and modification of chemical pulp. Cellulose 7, 189–209.
Tenkanen, M., Puls, J., Poutanen, K., 1992. Two major xylanases of *Trichoderma reesei*. Enzyme Microb. Technol. 14, 566–574.
Tenkanen, M., Makkonen, M., Perttula, M., Viikari, L., Teleman, A., 1997. Action of *Trichoderma reesei* mannanase on galactoglucomannan in pine kraft pulp. J. Biotechnol. 57, 191–204.
Tenkanen, M., Siika-aho, M., 2000. An α-glucuronidase of *Schizophyllum commune* acting on polymeric xylan. J. Biotechnol. 75, 149–161.
Tomme, P., van Tilbeurgh, H., Pettersson, G., van Damme, J., Vandekerckhove, J., Knowles, J., Teeri, T., Claeyssens, M., 1988. Studies of the cellulolytic system of *Trichoderma reesei* QM 9414. Analysis of domain function in two cellobiohydrolases by limited proteolysis. Eur. J. Biochem. 170, 575–581.
Tu, M., Chandra, R.P., Saddler, J.N., 2007. Evaluating the distribution of cellulases and the recycling of free cellulases during the hydrolysis of lignocellulosic substrates. Biotechnol. Progr. 23, 398–406.
Tu, M., Zhang, X., Paice, M., MacFarlane, P., Saddler, J.N., 2009. The potential of enzyme recycling during the hydrolysis of a mixed softwood feedstock. Bioresour. Technol. 100, 6407–6415.
van Tilbeurgh, H., Loontiens, F.G., de Bruyne, C.K., Claeyssens, M., 1988. Fluorogenic and chromogenic glycosides as substrates and ligands of carbohydrases. Methods Enzymol. 160, 45–59.
Várnai, A., Siika-aho, M., Viikari, L., 2010. Restriction of the enzymatic hydrolysis of steam-pretreated spruce by lignin and hemicellulose. Enzyme Microb. Technol. 46, 185–193.
Voutilainen, S.P., Puranen, T., Siika-aho, M., Lappalainen, A., Alapuranen, M., Kallio, J., Hooman, S., Viikari, L., Vehmaanperä, J., Koivula, A., 2008. Cloning, expression, and characterization of novel thermostable family 7 cellobiohydrolases. Biotechnol. Bioeng. 101, 515–528.
Yang, B., Wyman, C.E., 2008. Pretreatment: the key to unlocking low-cost cellulosic ethanol. Biofuels, Bioprod. Bioref. 2, 26–40.

PAPER IV

Synergistic action of xylanase and mannanase improves the total hydrolysis of softwood

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Synergistic action of xylanase and mannanase improves the total hydrolysis of softwood

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ABSTRACT

The impact of xylan and glucomannan hydrolysis on cellulose hydrolysis was studied on five pretreated softwood substrates with different xylan and glucomannan contents, both varying from 0.2% to 6.9%, using mixtures of purified enzymes.

The supplementation of pure cellulase mixture with non-specific endoglucanase TrCel7B and xylanase TrXyn11 enhanced the hydrolysis of all substrates, except the steam pretreated spruce, by more than 50%. The addition of endo-β-mannanase increased the overall hydrolysis yield by 20–25%, liberating significantly more glucose than theoretically present in glucomannan.

When supplemented together, xylanolytic and mannanolytic enzymes acted synergistically with cellulases. Moreover, a linear correlation was observed between the hydrolysis of polysaccharides, irrespective of the composition, indicating that glucomannan and xylan form a complex network of polysaccharides around the cellulosic fibres extending throughout the lignocellulosic matrix. Both hemicellulolytic enzymes are crucial as accessory enzymes when designing efficient mixtures for the total hydrolysis of lignocellulosic substrates containing both hemicelluloses.

1. Introduction

Lignocellulosic biomass has a great potential as renewable energy source. The enzymatic hydrolysis to monomeric sugars is, however, still hindered by obstacles caused by the complex and inhomogeneous structure of the lignocellulosic substrates (Somerville et al., 2004; Himmel et al., 2007). In softwood, not only lignin but also hemicellulose may prevent the hydrolysis of cellulose in the absence of accessory enzymes (Berlin et al., 2005). The location of hemicelluloses, xylans and galactoglucomannans, has been proposed to be closely associated with cellulose bundles and lignin (Gübitz et al., 1998; Somerville et al., 2004).

The main hemicelluloses in softwood are galactoglucomannans (GGM) (Timell, 1967; Willför et al., 2005). GGM's consist of β-1,4 linked backbone of β-D-glucopyranose and β-D-mannopyranose units, substituted at the C-6 by α-D-galactopyranose units. For the complete hydrolysis of mannan-type hemicelluloses, a wide array of enzymes is required (Stålbrand, 2003; Moreira and Filho, 2008). Amongst these, the main enzyme is endo-1,4-β-mannanase (EC.3.2.1.78), which cleaves the GGM main chain to oligosaccharides producing a mannose residue on the reducing end, hence facilitating the solubilisation of GGM's (Tenkanen et al., 1997). The action of both *Aspergillus niger* and *Trichoderma reesei* endomannanases is restricted by the galactosyl side groups, hence their cleavage by α-galactosidase is needed for the complete hydrolysis of the polymer (McCleary and Matheson, 1983; Tenkanen et al., 1997). Oligosaccharides are hydrolysed to monomers by β-mannosidase and β-glucosidase, hydrolysing mannose and glucose residues, respectively, at the non-reducing-end. Softwood glucomannan is acetylated and acetyl-glucomannan-esterase catalyses the deacetylation of the acetyl substituents of the glucomannan backbone.

The second most important hemicelluloses in softwood are arabinoglucuronoxylans (xylan for short) (Timell, 1967; Willför et al., 2005). Xylans consist of β-1,4 linked backbone of β-D-xylopyranose units, substituted at C-2 and C-3 with α-L-arabinofuranose and at C-2 with (4-O-methyl-)α-D-glucopyranuronic acid residues. Xylan is solubilised by xylanases cleaving the xylan backbone to oligomers. Besides endo-1,4-β-xylanase, several accessory enzymes are needed for the complete hydrolysis of the polymer, *i.e.* to cleavage of the side groups in the oligomers and further hydrolysis to monomers. These include α-arabinosidases and α-glucuronidases, as well as β-xylosidases. Unlike annual plants, softwood xylans are not esterified by acetyl groups.

Xylanolytic and mannanolytic activities can also originate from unspecific cellulases possessing hemicellulolytic side activities. Some endoglucanases (EG's) have been found to be active towards

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hemicellulose model substrates (Biely et al., 1991; Lawoko et al., 2000; Karlsson et al., 2002; Vlasenko et al., 2010). Especially, the family Cel5 (EGII) endoglucanases have been shown to be active on β-1,4-linked mannan and carob galactomannan, and the Cel7B (EGI) on birchwood xylan and wheat arabinoxylan (Vlasenko et al., 2010). The endoglucanase TrCel7B (EGI) from *T. reesei* has been shown to hydrolyse birch wood xylan and all four *T. reesei* EG's showed activity towards konjak glucomannan, whereas none of them has been shown to act on locust bean mannan (Biely et al., 1991; Lawoko et al., 2000; Karlsson et al., 2002).

In softwood, the content of glucomannan increases steadily from the outer parts to the inner parts whereas xylan seems to be more concentrated in the outer parts of the cell wall. After alkaline chemical delignification of softwood fibres, no significant relocation of hemicelluloses has been observed (Meier, 1985; Suurnäkki et al., 1996). However, acetyl groups of hemicellulose are totally or partially released in alkaline pulping or pretreatment conditions, as well as during thermochemical pretreatments at high temperatures. The action of xylanases and mannanases on lignocellulosic substrates has been most extensively studied to improve the bleachability of kraft pulps in the pulp and paper industry (Suurnäkki et al., 1997). The partial hydrolysis of hemicelluloses has been shown to increase the extractability of lignin from the fibres due to uncoverage of lignin on fibre surfaces. Lignin has also been found to be connected covalently to hemicellulose residues forming lignin carbohydrate complexes (LCC's) (Eriksson et al., 1980).

Hemicellulases have been found to facilitate the total hydrolysis of biomass (Berlin et al., 2005; Himmel et al., 2007). Several recent studies aimed at the optimisation of commercial or purified enzyme mixtures to improve the economic viability of the biotechnical conversion of crops to ethanol (Berlin et al., 2007; Banerjee et al., 2010). Besides cellulases acting on cellulose itself, auxiliary enzymes, especially hemicellulases, are needed to expose the cellulose fibres in the plant cell wall matrix, making the substrate more accessible to hydrolysis. The presence of xylanases has been shown to increase the hydrolysability of cellulose, *i.e.* the glucose yield from softwood, hardwood and agricultural crops (Berlin et al., 2005, 2007; Kumar and Wyman, 2009; Banerjee et al., 2010). The presence of mannanase in the enzyme mixture was shown to increase the hydrolysability of distillers' spent grain but not that of pretreated corn stover (Banerjee et al., 2010). However, no studies have recognised the role of glucomannan hydrolysis by either glucanases or mannanases to improve the total hydrolysis of cellulose in softwood substrates.

Synergistic action of two or more enzymes occurs if the amount of the overall hydrolysis products obtained by the enzymes acting together is higher than the sum of the hydrolysis products by the enzymes acting individually. Synergism may occur during hydrolysis of one polymeric substrate or of several interlinked or structurally associated polymers by the relevant enzymes. Thus, depending on the substrates, intra- or intermolecular synergisms may be distinguished. So far, most of the synergistic studies on fungal enzymes have focused on intramolecular synergism, *i.e.* exo-exo and endo-exo synergism, studied mainly on homopolymers such as cellulose (Nidetzky et al., 1993), or on synergism observed on heteropolymers such as hemicelluloses between backbone and side-group cleaving enzymes (Selig et al., 2008). Less studied, intermolecular synergism takes place during hydrolysis of structurally different, interlinked polymers such as cellulose and hemicelluloses, which form a complex network (Tenkanen et al., 1999; Selig et al., 2008). This synergism could also be described as multipolymer or "peeling" synergism where cellulose and hemicelluloses are simultaneously or sequentially peeled off by cellulases and hemicellulases, exposing new substrate which becomes available to hydrolysis. In these cases, the total hydrolysis of biomass is synergistically increased by several types of carbohydrases acting together (Selig et al., 2008).

In this study, the impact of hemicelluloses on the hydrolysis of five pretreated softwood substrates with different hemicellulose and lignin compositions was studied. The hydrolysis of hemicelluloses was studied with different tailored enzyme mixtures composed of the *T. reesei* cellulases TrCel7A (CBH I), TrCel6A (CBH II) and TrCel5A (EG II), complemented with TrCel7B (EG I) endoglucanase, TrXyn11 β-xylanase and TrMan5A endo-β-mannanase. The effect of the hydrolysis of mannan and xylan on the hydrolysis of cellulose was thoroughly investigated.

2. Methods

2.1. Raw materials and preparation of substrates

Five softwood raw materials of different origins: steam pretreated (SPS), catalytically delignified spruce (COS), commercial microcrystalline cellulose (Avicel, SERVA), softwood chemical pulp (SCP) and nanofibrillated cellulose (NFC) were used as substrates in the experiments. SPS was kindly provided by Guido Zacchi from Lund University, Sweden. Spruce chips were impregnated with 3% SO_2 and steam exploded at 215 °C for 5 min; the solid fraction was washed with hot water. To obtain the COS substrate, spruce chips were catalytically oxidised in the presence of copper-phenatroline catalyst and the solid fraction was washed after filtration (Hakola et al., 2010). Elemental chlorine free (ECF) bleached softwood chemical pulp (SCP) was obtained from a Finnish pulp mill (UPM-Kymmene Oy, Pietarsaari). The same pulp was used as the starting material for production of nanofibrillated cellulose. Prior to fibrillation, the never-dried kraft pulp was subjected to prerefining in a laboratory scale refiner at a consistency of 4% (Voith labrefiner LR-1, Voith) using a nominal energy consumption of 300 kWh/t. Thereafter, the pulp slurry was diluted to the consistency of 2% and fibrillation was carried out in three steps with a high-pressure fluidizer (Microfluidizer M700, Microfluidics Corp.). In order to induce efficient dispersion, the pulp was first passed once through a z-shaped chamber (500 μm) at an operating pressure of 300 bars. The fibrillation of fibres was then induced in several successive passes through two differently sized chamber pairs in series using a pressure of 1850 bars. During processing, the pulp turned into a milky, gel-like material (zero level viscosity 0.2 MPas), which had lost its original fibre structure (Fig. 1). The final material was not fully homogenous and fibrillar structures of lateral widths ranging from tens of nanometers up to few hundreds nanometers were observed in the SEM image (Fig. 1).

2.2. Compositional analysis of the samples

The carbohydrate composition of the raw materials was determined in a two-step sulphuric acid hydrolysis according to the NREL-procedure (LAP-001-005). The sugars were separated and quantified on Dionex ICS-3000 gradient HPLC system (Dionex ICS-3000, Sunnyvale, CA) using CarboPac PA-1 column (Dionex Sunnyvale, USA), 1 mL/min NaOH/water gradient eluent flow and 30 °C column temperature as described by Pakarinen et al. (in press). The carbohydrate composition is expressed in polymeric form as weight percentage of the total dry weight (Table 1). Lignin content was determined as described previously (Várnai et al., 2010).

The sugars released in enzymatic hydrolysis experiments were analysed by reducing sugar assay according to Miller (1959) or by HPLC as described above. The sugar oligomers liberated in enzymatic hydrolysis were further hydrolysed by dilute acid hydrolysis (LAP-001-005) and quantified as monomer sugars by HPLC as described above.

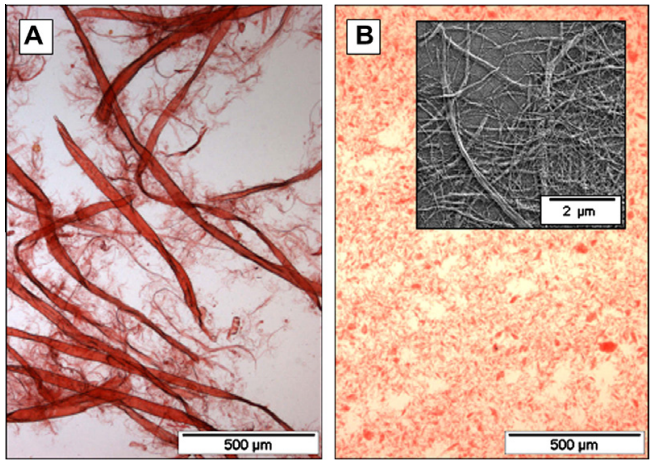

Fig. 1. Light microscopic images of softwood chemical pulp (A) before (SCP) and (B) after (NFC) mechanical fibrillation. Scale bars: 500 μm. The inset of Fig. 1B shows the SEM image of the nanofibrillated cellulose. Scale bar: 2 μm.

Table 1
Composition of the raw materials.

Sample	Lignin (% of d.w.)	Carbohydrate (% of d.w.)	Glucan (% of d.w.)	Mannan (% of d.w.)	Xylan (% of d.w.)	Arabinan (% of d.w.)	Galactan (% of d.w.)
SPS	32.5 ± 0.1	51.5 ± 0.5	51.1 ± 0.5	0.23 ± 0.00	0.21 ± 0.04	<0.1	<0.1
COS	4.1 ± 1.4	96.1 ± 0.7	85.3 ± 0.5	6.94 ± 0.15	3.42 ± 0.02	0.31 ± 0.00	0.11 ± 0.03
AVI	<1	94.0 ± 0.4	91.3 ± 0.4	1.45 ± 0.02	1.23 ± 0.02	<0.1	<0.1
SCP	<1	83.1 ± 1.4	69.1 ± 1.2	6.27 ± 0.08	6.87 ± 0.09	0.64 ± 0.00	0.27 ± 0.00
NFC	<1	86.5 ± 1.3	72.5 ± 1.2	6.24 ± 0.03	6.83 ± 0.04	0.63 ± 0.00	0.27 ± 0.00

2.3. Enzymes

The four *T. reesei* cellulases: the cellobiohydrolases Cel7A (TrCel7A, formerly CBH I, EC 3.2.1.91.) and Cel6A (TrCel6A, formerly CBH II, EC 3.2.1.91.), the endoglucanases Cel7B (TrCel7B, formerly EG I, EC 3.2.1.4.) and Cel5A (TrCel5A, EG II, EC 3.2.1.4.) were purified according to Suurnäkki et al. (2000). The two *T. reesei* hemicellulases; the xylanase II (pI 9, TrXyn11, EC 3.2.1.8.) and the mannanase (pI 5.4, TrMan5A, EC 3.2.1.78.) were purified according to Tenkanen et al. (1992) and Stålbrand et al. (1993), respectively, and the *A. niger* β-glucosidase Cel3A (AnCel3A, EC 3.2.1.21.) was purified according to Sipos et al. (2009). The enzymes used were pure as judged by SDS–PAGE.

Xylanase activity was measured using birch xylan as substrate according to Bailey et al. (1991).

2.4. Enzymatic hydrolysis

In the enzymatic hydrolysis experiments, enzyme mixtures were used in various combinations. The enzyme mixtures contained purified cellulases loaded at a total protein dosage of 10 mg/g total polysaccharide. The basic cellulase mixture was composed of the three cellulases TrCel7A, TrCel6A and TrCel5A, at a ratio (on protein basis) of 60%, 20% and 20%, respectively, denoted as C. In the other enzyme mixtures, half of the TrCel5A endoglucanase was replaced by TrCel7B endoglucanase (EG I), denoted as CE. Both mixtures were complemented with either xylanase (CX and CEX) or mannanase (CM and CEM), at a loading of 0.5 and 2.5 mg protein/g total polysaccharide, respectively, resulting in 10.5 and 12.5 mg total protein/g total polysaccharides, respectively. A complete mix was prepared with complementing both xylanase and mannanase to the CE mix (CEXM) at the same loading as mentioned above (*i.e.* 13.0 mg total protein/g total polysaccharides). All mixtures were supplemented with β-glucosidase at a dosage of 500 nkat/g polysaccharide (0.25 mg protein/g polysaccharide).

The hydrolysis experiments were carried out in triplicates with 1 w/w% polysaccharide content in 50 mM sodium-citrate buffer (pH 5.0) in 2.5 ml total volume, at 45 °C in water bath, mixing with magnetic stirrer bars at 250 rpm for up to 96 h. The hydrolysis was terminated by boiling for 10 min to inactivate the enzymes. The supernatants were separated by centrifugation for 5 min at 3000 rpm at 4 °C. The samples were stored frozen at −20 °C for further analyses. Hydrolysis references included substrate and enzyme blanks; substrate blanks were obtained by incubating the substrates for the same time without enzymes and enzyme blanks by boiling the enzymes without substrates.

3. Results and discussion

3.1. Characterisation of raw materials

Five softwood samples were chosen with significantly different lignin and hemicellulose contents to enable a representative comparison of the effects of xylan and mannan removal on the hydrolysis of cellulose using the mixtures of purified enzymes.

The SPS contained very low amounts of mannan (0.23% of d.w.) and xylan (0.21% of d.w.) whereas the commercially available cellulosic substrate Avicel contained relatively higher amounts of mannan and xylan, 1.45% and 1.23% of d.w., respectively. In the three other delignified substrates (COS, SCP and NFC), the mannan content was the highest, around 6–7% of d.w. The xylan content was almost as high as 7% of d.w. in SCP and NFC while COS contained 3.4% of d.w. xylan. The composition of SCP and NFC were similar, due to the fact that NFC was produced from SCP without weight loss.

The SPS differed from the other substrates with respect to the high lignin content, *i.e.* 32.5% of d.w., while the COS contained 4.1% lignin of d.w. The lignin content was less than 1% in Avicel, chemical pulp and nanocellulose.

3.2. Hemicellulolytic activities of the enzyme preparations

Of these cellulases, TrCel7A and TrCel6A have not been reported to possess hemicellulolytic side activity. Both TrCel7A and TrCel6A liberated insignificant amount of xylose and mannose monomers from bleached softwood kraft pulp (Suurnäkki et al., 2000). TrCel5A and TrCel7B have minor hydrolytic activity against (galacto)glucomannans as they have previously been shown to be active towards konjak glucomannan (Karlsson et al., 2002) and to liberate some mannose from bleached softwood kraft pulp (Suurnäkki et al., 2000). TrCel7B has also been reported to be active against beech and birchwood xylans, as well as against xylans from annual plants (Biely et al., 1991; Vlasenko et al., 2010).

The xylanase activity of the enzyme mixtures was measured against birch wood xylan. The cellulase mixture (C) showed insignificant xylanase activity (below detection limit), whereas when complemented with either TrXyn11 (CX), TrCel7B (CE) or both TrXyn11 and TrCel7B (CEX), the xylanase activity applied in the hydrolysis was 4500, 500 and 5100 nkat/g cellulose, respectively. Although the cellulase mixture (C) showed no xylanase activity towards birch wood xylan, xylo-oligomers were detected in the hydrolysates with the basic cellulase mixture (Tables 2–6). The release of soluble xylan or xylo-oligomers by cellobiohydrolases has been observed earlier on softwood substrates (Oksanen et al., 2000; Suurnäkki et al. 2000). A reason could be that some soluble xylan or xylo-oligomers were entrapped within cellulose matrices and were released during hydrolysis. Similarly, a significant amount of manno-oligomers was also released by the cellulase mixture (C) from each substrate (Tables 2–6). This could also be explained by the liberation of entrapped soluble mannans and mannooligomers or by the activity of TrCel5A endoglucanase against galactoglucomannan.

In the hydrolysis experiments, due to the lack of β-xylosidase and β-mannosidase, very low amounts of xylan and mannan were solubilised as monomers. The xylose monomers released during the hydrolysis of NFC and SCP were about 10–16% of the total xylan hydrolysis products liberated, irrespective of the addition of the xylanolytic enzymes (Tables 2 and 3). Mannose released as monomers remained below 0.2% of total polysaccharides (4% of solubilised mannan) in the hydrolysates of all substrates when no mannanase was added (Tables 2–6). When supplementing with mannanase, more than 13% of the solubilised glucomannan was converted to mannose which could be due to the hydrolysis by β-glucosidase of the oligomers produced by endo-β-mannanase. Hence, analysis after dilute acid hydrolysis of the supernatants was necessary to reveal the xylo- and manno-oligomers present in the hydrolysates (Tables 2–6).

3.3. Hydrolysis of NFC

Nanocellulose (NFC) was chosen as substrate for the first experiments due to its equal content (6–7%) of both hemicelluloses and the defined nanofibrillar structure (Fig. 1B). NFC was hydrolysed with all the enzyme combinations to investigate the effects of the addition of hemicellulases on the hydrolysis of hemicelluloses and of cellulose. The aim was to examine if the removal of either xylan or glucomannan would improve the hydrolysis of the other hemicellulose, in addition to the hydrolysis of cellulose.

3.4. Effect of hemicellulolytic enzymes on the hydrolysis of cellulose

Addition of xylanolytic enzymes clearly enhanced the hydrolysability of NFC. Despite the similar xylan and mannan contents (ca. 7% of d.w.) in the NFC, hydrolysis of xylan by either TrCel7B (CE) or TrXyn11 (CX) had a more significant effect on the improvement of cellulose hydrolysis than hydrolysis of mannan by mannanase (CM) (Fig. 2A and B). This is in agreement with previous observations on pine kraft pulp (Tenkanen et al., 1999). Addition of either TrCel7B (EG I) or TrXyn11 increased the hydrolysis yield of NFC after 12 h from 31% to 65% and 68% of the dry weight, respectively, while addition of TrMan5A increased the yield only to 39%. Well in accordance with this, the amount of glucose liberated during the hydrolysis was 33.2, 61.3, 63.6 and 38.0 mg glucan/100 mg total polysaccharide with the enzyme mixtures C, CE, CX and CM, respectively (Table 2). This difference in the increase of the hydrolysis of cellulose between xylanolytic enzymes and mannanase could possibly originate in the hydrolytic ability of TrCel5A towards glucomannan.

When supplemented to the cellulase mixture C, TrCel7B and TrXyn11 had a very similar effect on the hydrolysis, although TrCel7B showed only one ninth of the activity of TrXyn11 on birch wood xylan model substrate (Table 2). Obviously, even the lower activity amount used of the TrCel7B was enough to hydrolyse the xylan due to its efficiency on fibre bound xylan substrates. The hydrolysis products released from xylan, 5.38 and 5.79 mg xylan/

Table 2
Hydrolysis products of NFC after 12 h measured by HPLC. (The amount of solubilised glucan equalled before and after dilute acid hydrolysis.).

Enzyme mixture	After dilute acid hydrolysis[a]					Before dilute acid hydrolysis[b]			
	Glucan mg/100 mg PS[c]	Xylan mg/100 mg PS[c]	Arabinan mg/100 mg PS[c]	Mannan mg/100 mg PS[c]	Galactan mg/100 mg PS[c]	Xylan mg/100 mg PS[c]	Arabinan mg/100 mg PS[c]	Mannan mg/100 mg PS[c]	Galactan mg/100 mg PS[c]
C	33.2	0.79	0.11	2.61	0.07	0.12	b.d.[d]	0.08	b.d.[d]
CM	38.0	1.36	0.14	4.22	0.19	0.14	b.d.[d]	0.73	b.d.[d]
CX	63.6	5.79	0.58	4.18	0.18	0.91	b.d.[d]	b.d.[d]	b.d.[d]
CE	61.3	5.38	0.57	3.94	0.17	0.61	b.d.[d]	b.d.[d]	b.d.[d]
CEM	74.5	6.62	0.66	6.47	0.24	0.63	b.d.[d]	1.01	b.d.[d]
CEX	64.7	6.44	0.63	4.61	0.21	0.98	b.d.[d]	b.d.[d]	b.d.[d]
CEXM	87.2	7.01	0.69	6.80	0.23	0.92	b.d.[d]	0.82	b.d.[d]
Total carbohydrates[e]	83.9	7.89	0.73	7.21	0.31				

[a] The hydrolysates after dilute acid hydrolysis (oligosaccharides hydrolysed to monomers).
[b] The hydrolysates before dilute acid hydrolysis (only monomeric sugars analysed).
[c] mg Anhydrosugar released/100 mg total polysaccharide.
[d] Below detection limit.
[e] Maximum sugar amount calculated from the compositional analysis after acid hydrolysis.

Table 3
Hydrolysis products of SCP after 12 h measured by HPLC. (The amount of solubilised glucan equalled before and after dilute acid hydrolysis.).

Enzyme mixture	After dilute acid hydrolysis[a]					Before dilute acid hydrolysis[b]			
	Glucan mg/100 mg PS[c]	Xylan mg/100 mg PS[c]	Arabinan mg/100 mg PS[c]	Mannan mg/100 mg PS[c]	Galactan mg/100 mg PS[c]	Xylan mg/100 mg PS[c]	Arabinan mg/100 mg PS[c]	Mannan mg/100 mg PS[c]	Galactan mg/100 mg PS[c]
C	34.1	1.07	0.11	2.39	0.14	0.11	b.d.[d]	b.d.[d]	b.d.[d]
CE	53.7	5.28	0.51	3.20	0.16	0.61	b.d.[d]	b.d.[d]	b.d.[d]
CEX	64.8	6.38	0.63	3.98	0.18	1.04	0.05	b.d.[d]	b.d.[d]
CEXM	78.3	6.86	0.64	6.63	0.25	1.12	b.d.[d]	1.04	b.d.[d]
Total carbohydrates[e]	83.1	8.26	0.77	7.54	0.32				

[a] The hydrolysates after dilute acid hydrolysis (oligosaccharides hydrolysed to monomers).
[b] The hydrolysates before dilute acid hydrolysis (only monomeric sugars analysed).
[c] mg Anhydrosugar released/100 mg total polysaccharide.
[d] Below detection limit.
[e] Maximum sugar amount calculated from the compositional analysis after acid hydrolysis.

Table 4
Hydrolysis products of SPS after 96 h measured by HPLC. (The amount of solubilised glucan equalled before and after dilute acid hydrolysis. The level of arabinose and galactose were below detection in the hydrolysates.).

Enzyme mixture	After dilute acid hydrolysis[a]			Before dilute acid hydrolysis[b]	
	Glucan	Xylan	Mannan	Xylan	Mannan
	mg/100 mg PS[c]	mg/100 mg PS[c]	mg/100 mg PS[c]	mg/100 mg PS[c]	mg/100 mg PS[c]
C	48.4	0.02	0.18	b.d.[d]	b.d.[d]
CE	50.1	0.06	0.19	0.03	b.d.[d]
CEX	49.9	0.04	0.17	0.04	b.d.[d]
CEXM	51.9	0.06	0.17	0.04	b.d.[d]
Total carbohydrates[e]	99.1	0.41	0.45		

[a] The hydrolysates after dilute acid hydrolysis (oligosaccharides hydrolysed to monomers).
[b] The hydrolysates before dilute acid hydrolysis (only monomeric sugars analysed).
[c] mg Anhydrosugar released/100 mg total polysaccharide.
[d] Below detection limit.
[e] Maximum sugar amount calculated from the compositional analysis after acid hydrolysis.

Table 5
Hydrolysis products of AVI after 96 h measured by HPLC. (The amount of solubilised glucan equalled before and after dilute acid hydrolysis. The level of arabinose and galactose were below detection in the hydrolysates.).

Enzyme mixture	After dilute acid hydrolysis[a]			Before dilute acid hydrolysis[b]	
	Glucan mg/100 mg PS[c]	Xylan mg/100 mg PS[c]	Mannan mg/100 mg PS[c]	Xylan mg/100 mg PS[c]	Mannan mg/100 mg PS[c]
C	54.8	0.33	0.65	0.15	b.d.[d]
CE	76.3	0.96	1.10	0.59	b.d.[d]
CEX	76.6	0.93	1.04	0.71	b.d.[d]
CEXM	94.4	1.16	1.39	0.80	0.20
Total carbohydrates[e]	97.2	1.31	1.54		

[a] The hydrolysates after dilute acid hydrolysis (oligosaccharides hydrolysed to monomers).
[b] The hydrolysates before dilute acid hydrolysis (only monomeric sugars analysed).
[c] mg Anhydrosugar released/100 mg total polysaccharide.
[d] Below detection limit.
[e] Maximum sugar amount calculated from the compositional analysis after acid hydrolysis.

100 mg total PS by the mixtures CE and CX, respectively, were well comparable.

Nanocellulose was hydrolysed with pairs of enzyme mixtures including and excluding mannanase (CM – C, CEM – CE, CEXM – CEX). The hydrolysis of mannan improved the overall degree of hydrolysis by 20–25% irrespective of the hydrolysis of xylan (Fig. 2A and B). The hydrolysis yield increased from 65% to 81% and from 78% to 95% of d.w. by addition of mannanase to CE and CEX, respectively, and from 31% to 39% of d.w. with C and CM.

Mannanase addition increased the cellulose hydrolysis by 14%, 20% and 23% after 12 h when supplemented with C, CE or CEX, respectively (Table 2). In each case, the increase in the amount of released glucose by the addition of mannanase exceeded the theoretical amount of glucose present in GGM, *i.e.* 2.40 mg glucan/100 mg total PS calculated with a glucose-to-mannose ratio of 1:3 (Tables 1 and 2). The results indicate that glucose was released not only from glucomannan but also from cellulose.

The HPLC results revealed that a higher overall solubilisation of mannan occurred when xylan was also hydrolysed (CM vs. CEM or CEXM) (Table 2). Without addition of xylanolytic enzymes, 4.22 mg mannose/100 mg total PS could be detected (CM). When TrCel7B and both TrCel7B and TrXyn11 were included, the amount of released mannose was 53–61% higher, *i.e.* 6.47 and 6.80 mg/100 mg total PS, respectively, after 12 h of hydrolysis. Similarly, addition

Table 6
Hydrolysis products of COS after 48 h measured by HPLC. (The amount of solubilised glucan equalled before and after dilute acid hydrolysis.).

Enzyme mixture	After dilute acid hydrolysis[a]					Before dilute acid hydrolysis[b]			
	Glucan mg/100 mg PS[c]	Xylan mg/100 mg PS[c]	Arabinan mg/100 mg PS[c]	Mannan mg/100 mg PS[c]	Galactan mg/100 mg PS[c]	Xylan mg/100 mg PS[c]	Arabinan mg/100 mg PS[c]	Mannan mg/100 mg PS[c]	Galactan mg/100 mg PS[c]
C	46.4	1.65	0.14	4.18	0.05	0.10	b.d.[d]	0.17	b.d.[d]
CE	62.1	3.01	0.25	5.36	0.07	0.33	b.d.[d]	b.d.[d]	b.d.[d]
CEX	66.5	2.86	0.24	5.74	0.08	0.53	b.d.[d]	0.12	b.d.[d]
CEXM	77.4	3.34	0.27	6.93	0.09	0.57	b.d.[d]	1.32	b.d.[d]
Total carbohydrates[e]	88.8	3.56	0.32	7.22	0.11				

[a] The hydrolysates after dilute acid hydrolysis (oligosaccharides hydrolysed to monomers).
[b] The hydrolysates before dilute acid hydrolysis (only monomeric sugars analysed).
[c] mg anhydrosugar released/100 mg total polysaccharide.
[d] Below detection limit.
[e] Maximum sugar amount calculated from the compositional analysis after acid hydrolysis.

of TrMan5A facilitated the hydrolysis of xylan. The amount of xylose increased from 6.44 to 7.01 mg/100 total PS when mannanase was added to the xylanolytic enzymes (CEX vs. CEXM) and from 5.38 to 6.62 mg in the absence of xylanase (CE vs. CEM). These results indicate that xylan and glucomannan networks are closely associated with each other and partially cover each other.

3.5. Synergism between cellulases and hemicellulolytic enzymes

Analogous to previous findings by Tenkanen et al. (1999), the combined hydrolysis of mannan and xylan improved synergistically the hydrolysis of cellulose. The xylanolytic enzymes alone (CEX) increased the release of glucose by 31.5 mg (95%), and the mannanase alone (CM) by 4.8 mg (14%), whereas when supplemented together by 54.0 mg/100 mg total PS (163%) (Table 2), indicating notable multipolymer synergism in the action of these enzymes. This suggests that both xylan and glucomannan may be closely associated with the cellulose fibrils and form a joint network.

There seemed also to be some synergism between the TrCel7B and TrMan5A in the hydrolysis of glucomannan, whereas no synergism between TrCel7B and TrXyn11 was observed in the hydrolysis of xylan (Table 2). The basic cellulase mixture solubilised 2.61 mg mannan/100 mg total PS, which was increased to 3.94, 4.22 and 6.47 mg mannan/100 mg total PS (*i.e.* by 51%, 62% and 148%) by supplementation with TrCel7B and TrMan5A individually and with TrCel7B and TrMan5A together, respectively (Table 2). This observation suggests that TrCel7B and TrMan5A, as well as TrCel5A; all act on glucomannan but with hydrolysis patterns resulting in different hydrolysis products.

3.6. Comparison of the hydrolysis of NFC and SCP

The NFC and SCP were chemically almost identical and differed from each other only with respect to the dimensions of fibrils (Table 1, Fig. 1). Due to the separation of elementary fibrils, nanocellulose had higher surface area and was expected to show a clearly better accessibility in enzymatic hydrolysis than SCP. However, the differences were surprisingly small, probably because the low enzyme dosage used did not enable the full exploitation of the available binding sites.

Comparing the hydrolysis of NFC and SCP with CE mixture suggests that the location and micro/nanostructure of the fibrils also have an impact on the hydrolysis of xylan and concomitantly on the hydrolysis of cellulose. The cellulase mixtures without hemicellulolytic enzymes (C) and with both xylanolytic enzymes (CEX) were able to hydrolyse both cellulose and xylan to a similar extent in NFC and SCP (Tables 2 and 3). Instead, addition of TrCel7B alone (CE) improved the capacity of the enzyme mixture to hydrolyse cellulose, resulting in a more extensive hydrolysis of cellulose in the NFC, 61.3 mg glucan/100 mg total PS released, as compared to 53.7 mg of SCP, whereas an equal amount of xylan was released: 5.38 and 5.28 mg, respectively. As the cellulose hydrolysis by the cellulase mixture lacking xylanolytic activity did not increase after the extensive fibrillation of chemical pulp, this increase could be attributed to partial hydrolysis of the available xylan by TrCel7B at different locations in the two substrates. This observation implies that xylans surrounding the cellulose microfibrils in NFC could become more accessible due to the fibrillation of the softwood chemical pulp. Addition of xylanase together with TrCel7B, increased further the hydrolysis yield of both NFC and SCP, suggesting that the xylan content (7% of d.w.) in these substrates was too high to be efficiently hydrolysed by only the xylanolytic endoglucanase Cel7B.

The fibrillation of the substrate did not improve the solubilisation of xylan by the hemicellulolytic enzymes, although it slightly facilitated the liberation of GGM from NFC, as compared to SCP (Tables 2 and 3), suggesting that GGM might have become more accessible due to fibrillation.

With the most complete enzyme mixture (CEXM), the hydrolysis of cellulose of NFC gave a higher yield than of SCP, 87.2 and 78.3 mg glucan/100 mg total PS, respectively. The ratio of the sugars liberated from the two substrates was equal, implying that the remaining, most resistant structures are the same in both substrates.

3.7. Hydrolysis of COS, AVI and SPS

To further investigate the effects of xylanase and mannanase, the hydrolysis of a range of softwood substrates with various hemicellulose levels was studied with four selected enzyme

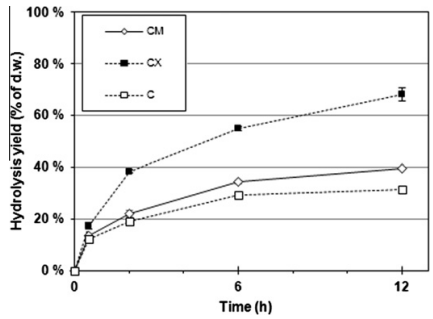

Fig. 2A. Hydrolysis of NFC with enzyme mixtures C, CM and CX. All enzyme mixtures contained the basic cellulase mixture (C), containing TrCel7A, TrCel6A, TrCel5A and β-glucosidase, complemented with either mannanase (CM) or xylanase (CX). Hydrolysis yield as reducing sugars (% of d.w.).

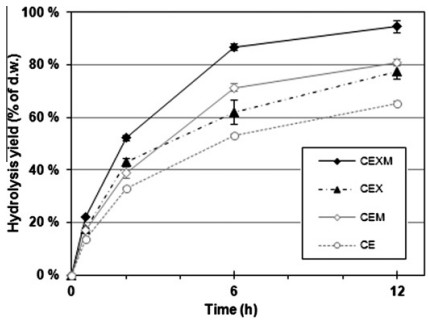

Fig. 2B. Hydrolysis of NFC with enzyme mixtures CE, CEM, CEX and CEXM. All enzyme mixtures contained the basic cellulase mixture complemented with TrCel7B (CE), and with either mannanase (CEM), xylanase (CEX) or both (CEXM). Hydrolysis yield as reducing sugars (% of d.w.).

combinations: the cellulase mixture (C), the cellulase mixture complemented with TrCel7B (CE), with both xylanolytic enzymes (CEX) and with all three hemicellulolytic enzymes (CEXM). The overall hemicellulose content of the substrates COS, Avicel, and SPS varied between 0.44% and 10.78% of d.w. (Table 1).

The complementation of cellulase mixture with xylanolytic enzymes (CE and CEX) seemed to increase the hydrolysis yield of all substrates, except of SPS (Figs. 2 and 3). Addition of xylanase together with TrCel7B increased further the hydrolysis yield of only NFC and SCP. In case of COS, AVI and SPS, where the xylan content did not exceed 4% of the dry weight, addition of xylanase did not further increase the hydrolysis yield (CEX), as compared to the TrCel7B (CE). The addition of xylanase, however, facilitated a more extensive break-down of xylan as the amount of monomeric xylose increased in the supernatant (Tables 2–6).

The addition of TrCel7B increased the hydrolysis of cellulose of all substrates (Tables 2–6). On the other hand, xylanase addition to the CE mixture had no significant effect on the hydrolysis of cellulose of SPS or AVI, containing 0.21 and 1.23% xylan of d.w., respectively (Tables 4 and 5). Instead, supplementation of cellulases with TrXyn11 xylanase improved the cellulose hydrolysis of COS slightly, from 62.1 to 66.5 mg glucan/100 mg total PS, although the release of xylose was not increased (Table 6). This implies that on substrates with low xylan content, TrCel7B acts as efficiently as the actual endoxylanase TrXyn11.

Surprisingly, mannanase addition showed a remarkable impact on the total hydrolysis of all substrates (Figs. 2A, B and 3), even of SPS (Fig. 3D), although the steam pretreated substrate contained only 0.23% of d.w. mannan (equalling to approx. 0.30% GGM of d.w., with a Glc:Man ratio of 1:3). The yield of the other substrates containing 1–7% of d.w. mannan increased by 14–24% (Figs. 2 and 3A–C). The hydrolysis of cellulose of SPS increased by 4%, from 49.9 to 51.9 mg glucan/100 mg total PS, whereas those of AVI and COS by 23 and 16%, from 76.6 to 94.4 mg and from 66.5 to 77.4 mg/100 mg total PS, respectively, when mannanase was added (Tables 4–6).

Similar to previous findings on NFC, mannanase addition enhanced the hydrolysis of xylan in case of all substrates except SPS (Tables 3–6), suggesting that in these substrates hemicelluloses form a complex network where glucomannan and xylan are closely interlinked. SPS contained a very low amount of hemicellulose, 0.21% of d.w. xylan and 0.23% of d.w. mannan (Table 1), and only less than 15% of the xylan and 40% of the glucomannan could be hydrolysed with the CEXM enzyme mixture (Table 4). The residual hemicellulose in SPS was thus hardly accessible to the enzymes. This suggests that in SPS the residual hemicellulose might be partially covered by or linked to lignin or not evenly distributed throughout the lignocellulose matrix.

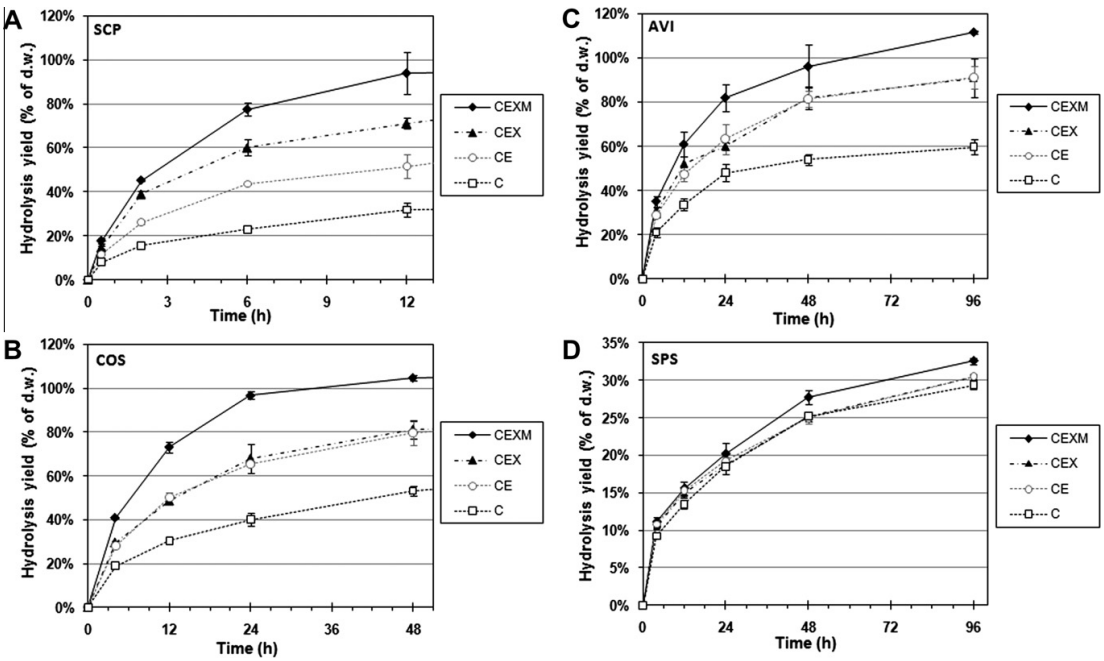

Fig. 3. Hydrolysis of (A) SCP, (B) COS, (C) AVI and (D) SPS with enzyme mixtures C, CE, CEX and CEXM. Hydrolysis yield as reducing sugars (% of d.w.).

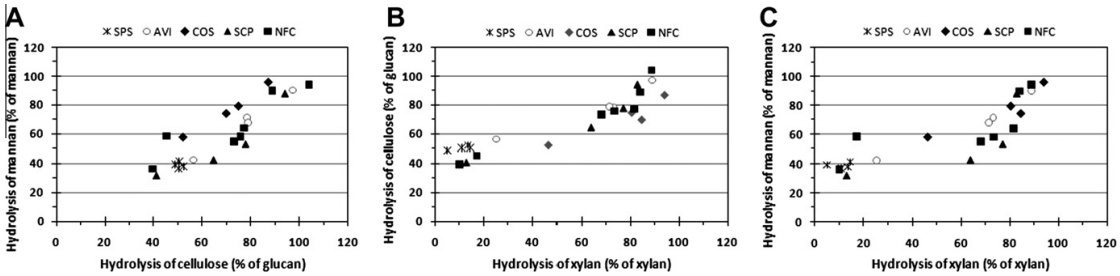

Fig. 4. Correlation between the hydrolysis yield of (A) glucomannan and cellulose, (B) cellulose and xylan, (C) glucomannan and xylan.

3.8. Correlation between the release of cellulose and hemicelluloses

Irrespective of the pretreatment and composition of softwood substrates, a clear correlation was observed between the hydrolysis of cellulose and of hemicelluloses (Fig. 4). Cellulose and glucomannan were hydrolysed to the same extent irrespective of the enzyme mixtures applied (Fig. 4A). Interestingly, cellulose and glucomannan were hydrolysed up to about 40–60% without major simultaneous hydrolysis of xylan (Fig. 4B and C). The differences observed between the solubilisation of xylan and glucomannan could be due to the activity of TrCel5A against galactoglucomannan, which was present in each enzyme mixture, or due to the location of xylan which may cover partially cellulose and glucomannan in the lignocellulose matrix.

When xylanolytic enzymes were supplemented, the release of hydrolysis products from xylan seemed to take place synergistically with the hydrolysis of both cellulose and GGM, and seemed to show a linear correlation. These results suggest that xylans and glucomannans both are evenly distributed around cellulose fibrils or located deeply within the lignocellulosic matrix, and that their concurrent hydrolysis is crucial in order to obtain a more extensive hydrolysis of cellulose and a higher overall yield from softwood materials.

The presence of hemicellulolytic enzymes is crucial in commercial enzyme preparations. In addition, non-specific endoglucanases present in the common commercial enzyme mixtures enhance significantly the hydrolysis of the lignocellulosic substrates. However, when new preparations are designed for various substrates, it is important to understand which enzyme components are crucial for an efficient hydrolysis. Identification of all necessary enzyme components is difficult when studies are performed using present commercial enzyme mixtures.

4. Conclusions

Irrespective of the hemicellulose content in the substrates, a linear correlation was observed between the solubilised cellulose and hemicelluloses. The hydrolysis of hemicelluloses facilitated not only the hydrolysis of cellulose but also of the other hemicellulose synergistically, suggesting that xylans and glucomannans form together a complex network around the cellulose bundles.

The addition of xylanolytic enzymes improved the cellulose hydrolysis to a higher extent than the mannanase when supplemented to pure cellulases alone. The presence of both types of hemicellulolytic enzymes was, however, found to be crucial to obtain high yields in the total hydrolysis of softwood derived raw materials.

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References

Bailey, M.J., Biely, P., Poutanen, K., 1991. Interlaboratory testing of methods for assay of xylanase activity. J. Biotechnol. 23, 257–270.

Banerjee, G., Car, S., Scott-Craig, J.S., Borrusch, M.S., Walton, J.D. 2010. Rapid optimization of enzyme mixtures for deconstruction of diverse pretreatment/biomass feedstock combinations. Biotechnol. Biofuels 3(22). doi:10.1186/1754-6834-3-22. Retrieved from: http://www.biotechnologyforbiofuels.com/content/pdf/1754-6834-3-22.pdf.

Berlin, A., Gilkes, N., Kilburn, D., Bura, R., Markov, A., Skomarovsky, A., Okunev, O., Gusakov, A., Maximenko, V., Gregg, D., Sinitsyn, A., Saddler, J., 2005. Evaluation of novel fungal cellulase preparations for ability to hydrolyze softwood substrates–evidence for the role of accessory enzymes. Enzyme Microb. Technol. 37, 175–184.

Berlin, A., Maximenko, V., Gilkes, N., Saddler, J., 2007. Optimization of enzyme complexes for lignocellulose hydrolysis. Biotechnol. Bioeng. 97, 287–296.

Biely, P., Vršanská, M., Claeyssens, M., 1991. The endo-1,4–glucanase I from *Trichoderma reesei*. Action on 1,4-oligomers and polymers derived from D-glucose and D-xylose. Eur. J. Biochem. 200, 157–163.

Eriksson, Ö., Goring, D.A.I., Lindgren, B.O., 1980. Structural studies on the chemical bonds between lignins and carbohydrates in spruce wood. Wood Sci. Technol. 14, 267–279.

Gübitz, G.M., Stebbing, D.W., Johansson, C.I., Saddler, J.N., 1998. Lignin-hemicellulose complexes restrict enzymic solubilization of mannan and xylan from dissolving pulp. Appl. Microbiol. Biotechnol. 50, 390–395.

Hakola, M., Kallioinen, A., Kemell, M., Lahtinen, P., Lankinen, E., Leskelä, M., Repo, T., Riekkola, T., Siika-Aho, M., Uusitalo, J., Vuorela, S., Von-Weymarn, N., 2010. Liberation of cellulose from the lignin cage: A catalytic pretreatment method for the production of cellulosic ethanol. Chem. Sus. Chem. 3, 1142–1145.

Himmel, M.E., Ding, S.Y., Johnson, D.K., Adney, W.S., Nimlos, M.R., Brady, J.W., Foust, T.D., 2007. Biomass recalcitrance. Engineering plants and enzymes for biofuels production. Science 315, 804–807.

Karlsson, J., Siika-aho, M., Tenkanen, M., Tjerneld, F., 2002. Enzymatic properties of the low molecular mass endoglucanases Cel12A (EG III) and Cel45A (EG V) of *Trichoderma reesei*. J. Biotechnol. 99, 63–78.

Kumar, R., Wyman, C.E., 2009. Effect of xylanase supplementation of cellulase on digestion of corn stover solids prepared by leading pretreatment technologies. Bioresour. Technol. 100 (2009), 4203–4213.

Lawoko, M., Nutt, A., Henriksson, H., Gellerstedt, G., Henriksson, G., 2000. Hemicellulase activity of aerobic fungal cellulases. Holzforschung 54, 497–500.

McCleary, B.V., Matheson, N.K., 1983. Action patterns and substrate-binding requirements of β-D-mannanase with mannosaccharides and mannan-type polysaccharides. Carbohydr. Res. 119, 191–219.

Meier, H., 1985. Localization of polysaccharides in wood cell walls. In: Higuchi, T. (Ed.), Biosynthesis and Biodegradation of Wood Components. Academic Press, Inc., Orlando, USA, pp. 43–50.

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31, 426–428.

Moreira, L.R.S., Filho, E.X.F., 2008. An overview of mannan structure and mannan-degrading enzyme systems. Appl. Microbiol. Biotechnol. 79, 165–178.

Nidetzky, B., Hayn, M., Macarron, R., Steiner, W., 1993. Synergism of *Trichoderma reesei* cellulases while degrading different celluloses. Biotechnol. Lett. 15, 71–76.

NREL Chemical Analysis and Testing Task, Laboratory Analytical Procedures 001-005. http://www.cobweb.ecn.purdue.edu/~lorre/16/research/.

Oksanen, T., Pere, J., Paavilainen, L., Buchert, J., Viikari, L., 2000. Treatment of recycled kraft pulps with *Trichoderma reesei* hemicellulases and cellulases. J. Biotechnol. 78, 39–48.

Pakarinen, A., Maijala, P., Stoddard, F.L., Santanen, A., Tuomainen, P., Kymäläinen, M., Viikari, L. in press. Evaluation of annual bioenergy crops in the boreal zone for biogas and ethanol production. Biomass Bioenerg. in press. doi:10.1016/j.biombioe.2011.04.022.

Selig, M.J., Knoshaug, E.P., Adney, W.S., Himmel, M.E., Decker, S.R., 2008. Synergistic enhancement of cellobiohydrolase performance on pretreated corn stover by addition of xylanase and esterase activities. Bioresour. Technol. 99 (11), 4997–5005.

Sipos, B., Benkő, Z., Dienes, D., Réczey, K., Viikari, L., Siika-aho, M. 2009. Characterisation of specific activities and hydrolytic properties of cell-wall degrading enzymes produced by *Trichoderma reesei* Rut C30 on different carbon sources. Appl. Biochem. Biotechnol. doi:10.1007/s12010-009-8824-4 Retrieved from: http://www.springerlink.com/content/c4nl6x81l7461j04/fulltext.pdf.

Somerville, C., Bauer, S., Brininstool, G., Facette, M., Hamann, T., Milne, J., Osborne, E., Paredez, A., Persson, S., Raab, T., Vorwerk, S., Youngs, H., 2004. Toward a systems approach to understanding plant cell walls. Science 306, 2206–2211.

Stålbrand, H., 2003. Enzymology of endo-1,4-β-mannanases. In: Whitaker, J.R., Voragen, A.J.G., Wong, D.W.S. (Eds.), Handbook of food enzymology. Marcel Dekker, Inc., New York, pp. 961–969.

Stålbrand, H., Siika-aho, M., Tenkanen, M., Viikari, L., 1993. Purification and characterization of two β-mannanases from *Trichoderma reesei*. J. Biotechnol. 29, 229–242.

Suurnäkki, A., Heijnesson, A., Buchert, J., Viikari, L., Westermarck, U., 1996. Chemical characterization of the surface layers of unbleached pine and birch kraft pulp fibres. J. Pulp Paper Sci 22 (2), J43–J47.

Suurnäkki, A., Tenkanen, M., Buchert, J., Viikari, L., 1997. Hemicellulases in the bleaching of chemical pulp. Adv. Biochem. Eng. Biot. 57, 261–287.

Suurnäkki, A., Tenkanen, M., Siika-aho, M., Niku-Paavola, M.-L., Viikari, L., Buchert, J., 2000. *Trichoderma reesei* cellulases and their core domains in the hydrolysis and modification of chemical pulp. Cellulose 7, 189–209.

Tenkanen, M., Puls, J., Poutanen, K., 1992. Two major xylanases of *Trichoderma reesei*. Enzyme Microb. Technol. 14, 566–574.

Tenkanen, M., Makkonen, M., Perttula, M., Viikari, L., Teleman, A., 1997. Action of *Trichoderma reesei* mannanase on galactoglucomannan in pine kraft pulp. J. Biotechnol. 57, 191–204.

Tenkanen, M., Tamminen, T., Hortling, B., 1999. Investigation of lignin-carbohydrate complexes in kraft pulps by selective enzymatic treatments. Appl. Microbiol. Biotechnol. 51, 241–248.

Timell, T.E., 1967. Recent progress in the chemistry of wood hemicelluloses. Wood Sci. Technol. 1, 45–70.

Várnai, A., Siika-aho, M., Viikari, L., 2010. Restriction of the enzymatic hydrolysis of steam-pretreated spruce by lignin and hemicellulose. Enzyme Microb. Technol. 46, 185–193.

Vlasenko, E., Schülein, M., Cherry, J., Xu, F., 2010. Substrate specificity of family 5, 6, 7, 9, 12, and 45 endoglucanases. Bioresour. Technol. 101, 2405–2411.

Willför, S., Sundberg, A., Hemming, J., Holmbom, B., 2005. Polysaccharides in some industrially important softwood species. Wood Sci. Technol. 39, 245–258.

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Title	**Improving enzymatic conversion of lignocellulose to platform sugars**
Author(s)	Anikó Várnai
Abstract	Increasing demand and uncertain availability of fossil fuels urge us to find alternative resources available in large quantities especially for the petrol-based transportation sector. Lignocellulosic biomass, available worldwide in plant cell walls, is a promising alternative feedstock. It can be depolymerised to sugar monomers, which provide potential raw material for sugar platform-based production of fuels and chemicals. However, the enzymatic saccharification of lignocellulose to platform sugars is hindered primarily by the complexity of lignocellulosic substrates as well as by the performance of the hydrolytic enzymes involved. This study focuses on various rate limiting factors such as the decrease in the reactivity and accessibility of the substrates which slow down the hydrolysis, on auxiliary enzymes needed for the efficient solubilisation of cellulose, as well as on the adsorption of enzymes. Consequently, solutions to these limitations were sought to improve the efficiency of biomass conversion processes. Following the morphological and structural changes in the substrate during hydrolysis revealed that the average crystal size and crystallinity of cellulose remained constant while particle size generally decreased (Paper I). In particular, cellulose microfibrils were proposed to be hydrolysed one-by-one in fibre aggregates by peeling off cellulose chains layer-by-layer from the outer crystals of microfibril aggregates. Microscopic observation showed that almost intact particles remained in the residue even after 60% conversion. Lignocellulose is a complex network of lignin and polysaccharides. Lignin was found to impede the hydrolysis of cellulose, and its extensive removal doubled the conversion yields of softwood (Paper II). On the other hand, accumulation of lignin during hydrolysis did not affect hydrolysability by commercial cellulase preparations. Residual hemicelluloses, especially glucomannan, were resistant to enzymatic hydrolysis but could be removed together with lignin during delignification. This suggests that especially glucomannans are bound to lignin as lignin-carbohydrate complexes. In addition, cellulose, xylan and glucomannan were shown to be structurally interlinked in softwood (Paper IV). The hydrolysis yield of these polysaccharides remained below 50% without the simultaneous hydrolysis of all polysaccharides. Synergism between the solubilisation of cellulose and hemicelluloses was found, and the release of glucose, xylose and mannose was in linear correlation. The adsorption and desorption of enzymes were followed during hydrolysis (Paper III). After a quick initial adsorption, slow desorption and re-adsorption of enzymes was observed in alkaline delignified spruce. On the other hand, unproductive adsorption to lignin as well as enzyme inactivation was predicted to play a primary role in the irreversible adsorption of cellulases in steam pretreated spruce or Avicel during hydrolysis when no desorption of cellulases could be detected. This study showed for the first time that increasing substrate concentration could compensate for the absence of carbohydrate binding modules (CBMs) in hydrolytic enzymes (Paper V). The performance of cellulases lacking CBMs was comparable to that of cellulases comprising CBM at 20% substrate concentration. At the same time, over 60% of the enzymes without CBMs could be recovered at the end of the hydrolysis. Thus, the major part of hydrolytic enzymes without CBMs could potentially be recovered in industrial high consistency processes.
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Improving enzymatic conversion of lignocellulose to platform sugars

Increasing demand and uncertain availability of fossil fuels urge us to find alternative resources available in large quantities especially for the petrol-based transportation sector. At present, first generation bioethanol and biodiesel are produced worldwide from cornstarch, sugarcane and rapeseed oil. However, fuels produced from these raw materials are not considered sustainable. Thus, recent efforts have been directed towards the use of sustainable raw materials, such as residues from forestry and agriculture as well as municipal wastes.

Lignocellulosic biomass, available worldwide in plant cell walls, is a promising alternative feedstock for the production of second generation biofuels. However, the enzymatic saccharification of lignocellulose to platform sugars is hindered primarily by the complexity of lignocellulosic substrates as well as by the performance of the hydrolytic enzymes involved. Therefore, this work focuses on various rate limiting factors such as the decrease in the reactivity and accessibility of the substrates which slow down the hydrolysis, on auxiliary enzymes needed for the efficient solubilisation of cellulose, as well as on the adsorption of enzymes. Consequently, solutions to these limitations were sought to improve the efficiency of biomass conversion processes.

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