

Cellulase-lignin interactions in the enzymatic hydrolysis of lignocellulose

Jenni Rahikainen



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Cellulase-lignin interactions in the enzymatic hydrolysis of lignocellulose

Sellulaasi-ligniini-vuorovaikutukset lignoselluloosan entsyymaattisessa hydrolyysissä.
Jenni Rahikainen. Espoo 2013. VTT Science 41. 90 p. + app. 44 p.

Abstract

Lignin, a major non-carbohydrate polymer in lignocellulosic plant biomass, restricts the action of hydrolytic enzymes in the enzymatic hydrolysis of lignocellulosic feedstocks. Non-productive enzyme adsorption onto lignin is a major inhibitory mechanism, which results in decreased hydrolysis rates and yields and difficulties in enzyme recycling. The mechanisms of non-productive binding are poorly understood; therefore, in this thesis, enzyme-lignin interactions were studied using isolated lignins from steam pretreated and non-treated spruce and wheat straw as well as monocomponent cellulases with different modular structures and temperature stabilities.

The origin of the isolated lignin had an undisputable effect on non-productive binding. Ultrathin lignin films, prepared from steam pretreated and non-treated lignin preparations, were employed in QCM adsorption studies in which *Trichoderma reesei* Cel7A (*TrCel7A*) was found to bind more onto lignin isolated from steam pretreated biomass than onto lignin isolated from non-treated lignocellulosic biomass. Botanical differences in lignin chemistry had only a minor effect on non-productive binding when enzyme binding to non-treated wheat straw and spruce lignin was compared.

Increase in temperature was found to increase the inhibitory effect arising from non-productive enzyme binding to lignin. Different enzymes were shown to have a characteristic temperature at which the inhibition emerged. Thermostable enzymes were the most lignin-tolerant at high temperatures, suggesting that in addition to the surface properties of an enzyme, non-productive binding onto lignin may be influenced by stability of the enzyme structure. In addition, for lignin-bound *T. reesei* cellulases, increase in temperature resulted in loss of catalytic activity and tighter binding, suggesting that at high temperature enzyme binding to lignin was probably coupled to conformational changes in the protein folding.

With *TrCel7A*, carbohydrate-binding module (CBM) was found to increase non-productive adsorption to lignin. The *Talaromyces emersonii* Cel7A catalytic module was linked to a CBM from *TrCel7A*, giving rise to a fusion enzyme *TeCel7A*-CBM1. Despite a similar CBM, *TeCel7A*-CBM adsorbed significantly less to lignin than *TrCel7A*, indicating that the catalytic module (*TeCel7A*) had a strong contribution to the low binding. Probably, the contribution of CBM or catalytic core module in non-productive binding varies between different enzymes, and the role of the CBM is not always dominant.

To date, very little attention has been paid to the role of electrostatic interactions in lignin-binding. In this work, binding of *Melanocarpus albomyces* Cel45A endoglucanase onto lignin was found to be very dependent on pH, suggesting that electrostatic interactions were involved in the binding. At high pH, significantly less non-productive binding occurred, probably due to increasing electrostatic repulsion between negatively charged enzymes and lignin. Modification of the charged chemical groups in enzymes or lignin may be a viable strategy to reduce non-productive enzyme binding in the hydrolysis of lignocellulosic substrates.

Keywords lignocellulose, enzymatic hydrolysis, non-productive binding, lignin, cellulase

Sellulaasi-ligniini-vuorovaikutukset lignoselluloosan entsyymaattisessa hydrolyysissä

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Tiivistelmä

Kasvien lignoselluloosa on vaihtoehtoinen uusiutuva raaka-aine liikennepolttoainesten sekä erilaisten kemikaalien tuotantoon. Lignoselluloosan biokemiallisella prosessoinnilla pyritään hajottamaan biomassan rakennepolysakkaridit, selluloosa ja hemiselluloosa, entsyymaattisesti liukoiksi sokereiksi, joista pystytään esimerkiksi mikrobien avulla tuottamaan haluttuja yhdisteitä. Lignoselluloosa koostuu pääosin rakennepolysakkarideista (selluloosa ja hemiselluloosa) sekä ligniinistä, joka on aromaattinen polymeeri. Ligniinin läsnäolo estää rakennepolysakkarideja hajottavien entsyymien toimintaa useilla mekanismeilla, joista entsyymien epäspesifi sitoutuminen ligniiniin on eräs tärkeimmistä. Entsyymien sitoutuminen ligniiniin heikentää niiden toimintaa sekä rajoittaa entsyymien kierrätettävyyttä. Molekyyllitason mekanismit, jotka mahdollistavat entsyymien sitoutumisen ligniiniin, tunnetaan heikosti. Tämän väitöskirjan tavoitteena oli tutkia entsyymi-ligniini-vuorovaikutuksia käyttäen hyödyksi eristettyjä ligniininäytteitä sekä rakenteeltaan ja lämpöstabiili-suudeltaan erilaisia sellulaasi-entsyymejä. Ligniininäytteet eristettiin joko höyry-esikäsitellystä tai käsittelemättömästä kuusesta tai vehnän korjuutähteestä.

Eristetyn ligniinin alkuperällä oli selvä vaikutus sellulaasien sitoutumiseen, kun sitoutumista tutkittiin eri ligniininäytteistä valmistetuilla ohuilla ligniinalvoilla käyttäen QCM-tekniikkaa. *Trichoderma reesei* -sellobiohydrolaasi (Cel7A) sitoutui huomattavasti enemmän ligniiniin, joka oli eristetty esikäsitellystä biomassasta kuin ligniiniin, joka eristettiin käsittelemättömästä materiaalista. Vehnän ja kuusen ligniinien kemiallisella erolla oli huomattavasti heikompi vaikutus entsyymien sitoutumiseen.

Lämpötilan nousu lisäsi selkeästi ligniinin haitallista vaikutusta entsyymien toimintaan, mutta haitan suuruus riippui tutkittavasta entsyymistä. Lämpötila, jossa ligniinistä johtuva inhibitio huomattiin, oli kullekin tutkitulle sellulaasientsyymille yksilöllinen. Lämpöstabiilit sellobiohydrolaasientsyymit kestivät paremmin ligniiniä korkeissa lämpötiloissa verrattuna *T. reesei* Cel7A -sellobiohydrolaasiin. Näin ollen entsyymiproteiinin pinnan ominaisuudet sekä proteiinin rakenteen stabiilisuus saattavat molemmat vaikuttaa entsyymi-ligniini-vuorovaikutukseen. Lisäksi lämpötilan huomattiin vaikuttavan merkittävästi ligniiniin sitoutuneiden *T. reesei*-entsyymien toimintaan. Korkeassa lämpötilassa sitoutuneet entsyymit menettivät katalyyttistä aktiivisuuttaan sekä sitoutuivat voimakkaammin ligniinin pintaan. Näiden huomioiden perusteella voidaan olettaa, että ligniiniin sitoutuneiden entsyymien rakenne purkautuu korkeassa lämpötilassa.

T. reesei Cel7A -sellobiohydrolaasin hiilihydraatteihin sitoutuva moduuli (CBM) lisäsi entsyymien adsorptiota ligniiniin. Kun sama CBM liitettiin toiseen, *Talaromy-*

ces emersonii -homeesta peräisin olevaan sellobiohydraasiin, fuusioentsyymi sitoutui huomattavasti vähemmän ligniiniin kuin *T. reesei* -homeen Cel7A sellobiohydraasi. Näin ollen katalyyttisen domeenin vaikutus entsyymi-ligniini-vuorovaikutuksessa voi olla hyvinkin merkittävä, ja aiemmasta tutkimuksesta poiketen voidaan todeta, että CBM:n merkitys entsyymi-ligniini-vuorovaikutuksessa ei ole aina määräävä.

Tutkimus elektrostaattisten vuorovaikutusten roolista entsyymi-ligniini-vuorovaikutusten yhteydessä on ollut vähäistä. Työssä huomattiin, että *Melanocarpus albomyces* Cel45A -endoglukanaasin sitoutuminen ligniiniin on pH-riippuvaista: korkeassa pH:ssa vähemmän entsyymiä sitoutui ligniiniin. pH-Riippuvuuden perusteella elektrostaattiset vuorovaikutukset vaikuttanevat entsyymien sitoutumiseen, ja sitoutumisen väheneminen korkeassa pH:ssa saattaa johtua lisääntyvästä repulsiosta negatiivisesti varautuneiden entsyymien sekä ligniinin välillä. Negatiivisesti varautuvien kemiallisten ryhmien lisääminen entsyymi- tai ligniinirakenteeseen saattaa olla keino vähentää sitoutumista teollisissa prosesseissa.

Avainsanat lignocellulose, enzymatic hydrolysis, non-productive binding, lignin, cellulase

Preface

This thesis work was carried out during the years 2009–2013 at the VTT Technical Research Center of Finland. VTT is among the pioneering institutes in studying cellulose-degrading enzymes, and therefore it has been a great pleasure to be part of the continuum and contribute to a field which aims at meaningful goals: environmentally sound processing technologies and efficient and sustainable use of renewable resources. This study was mostly funded by the Graduate School for Biomass Refining (Academy of Finland) and by three EU-projects from the 7th framework programme: EU-DISCO, EU-HYPE and EU-NEMO.

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Academic dissertation

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List of publications

This thesis is based on the following original publications, which are referred to in the text as I–IV. The publications are reproduced with kind permission from the publishers.

- I Rahikainen, J., Mikander, S., Marjamaa, K., Tamminen, T., Lappas, A., Viikari, L., Kruus, K., 2011. Inhibition of enzymatic hydrolysis by residual lignins from softwood – study of enzyme binding and inactivation on lignin-rich surface. *Biotechnology and Bioengineering* 108, 2823–2834.
- II Rahikainen, J.L., Martin-Sampedro, R., Heikkinen, H., Rovio, S., Marjamaa, K., Tamminen, T., Rojas, O.J., Kruus, K., 2013. Inhibitory effect of lignin during cellulose bioconversion: the effect of lignin chemistry on non-productive enzyme adsorption. *Bioresource Technology* 133, 270–278.
- III Rahikainen, J.L., Evans, J.D., Mikander, S., Kalliola, A., Puranen, T., Tamminen, T., Marjamaa, K., Kruus, K., 2013. Cellulase-lignin interactions – The role of carbohydrate-binding module and pH in non-productive binding. *Enzyme and Microbial Technology*, in press.
- IV Rahikainen, J.L., Moilanen, U., Nurmi-Rantala, S., Lappas, A., Koivula, A., Viikari, L., Kruus, K., 2013. Effect of temperature on lignin-derived inhibition studied with three structurally different cellobiohydrolases. *Bioresource Technology* 146, 118–125.

Author's contributions

- I. The author planned the work together with the supervisors. The author carried out the experimental work, except for the fluorescent microscopy, BET surface area analysis and liquid chromatography analysis of carbohydrates. The author interpreted the data and had the main responsibility for writing the publication under the supervision of Kristiina Kruus.
- II. The author planned the work together with the supervisors. The author carried out the EMAL lignin isolations, enzyme purification and hydrolysis experiments. The QCM-runs and lignin-film preparations were carried out together with Dr. Raquel Martin-Sampedro. The author had the main responsibility for writing the publication under the supervision of Kristiina Kruus.
- III. The author planned the work together with Kristiina Kruus and Kaisa Marjamaa. The author carried out part of the enzyme radiolabeling and characterisation and supervised the adsorption studies. The author had the main responsibility for writing the publication under the supervision of Kristiina Kruus.
- IV. The author planned the work together with Kristiina Kruus and Anu Koivula. The author carried out the experimental work, except for the BET surface area analysis, liquid chromatography analysis of carbohydrates and the hydrolysis of technical substrates. The author had the main responsibility for writing the publication under the supervision of Kristiina Kruus.

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List of abbreviations

AFM	atomic force microscopy
CBM	carbohydrate-binding module
DP	degree of polymerisation
EC	enzyme commission
EMAL	enzymatic mild-acidolysis lignin
EnzHR	enzymatic hydrolysis residue
GH	glycosyl hydrolase
GHG	greenhouse gas
<i>MaCel45A</i>	<i>Melanocarpus albomyces</i> endoglucanase Cel45A
MCC	microcrystalline cellulose
MWL	milled wood lignin
QCM	quartz crystal microbalance
SE	steam explosion
T_m	melting temperature
<i>TeCel7A</i>	<i>Talaromyces emersonii</i> cellobiohydrolase Cel7A
<i>TrCel7A</i>	<i>Trichoderma reesei</i> cellobiohydrolase Cel7A
wt-%	weight-%

1. Introduction

Global energy demand is forecasted to increase by more than one third by 2035, mainly due to increasing standard of living in China, India and the Middle East (International Energy Agency, 2012). In 2010, 81 % of the world's primary energy demand was met with non-renewable resources: coal, oil and natural gas (International Energy Agency, 2012). Increasing energy demand and our great dependence on fossil resources are considered problematic both from environmental and societal aspects. Combustion of non-renewable resources generates greenhouse gas (GHG) emissions that contribute to global warming. Global warming has raised serious environmental concerns due its great impact on ecosystems all over the world. For example, climate change is predicted to lead to the extinction of numerous species (Thomas et al., 2004). Furthermore, uneven geographical distribution of fossil energy reserves in the world is a societal risk for countries that are highly dependent on imported oil, coal and natural gas. Improvements in energy efficiency and increased utilisation of renewable energy resources, such as plant biomass, are key measures to alleviate the concerns arising from our current dependence on fossil fuels (International Energy Agency, 2012).

Today the transport sector consumes more than half of the annually produced oil and only 2 % of the global fuel demand is met by refining renewable feedstocks into transportation fuels (International Energy Agency, 2012). The European Union, the United States and Brazil have binding regulations for blending biomass-based fuel compounds with gasoline or diesel. However, the currently exploited "first generation" biofuel feedstocks include sugar cane, corn starch and palm oil that may also be used in food production. Violation of the food chain threatens food security and may increase food prices (Solomon, 2010). Furthermore, the GHG emissions arising from "first generation" biofuel production might be significantly underestimated by some widely used life cycle assessment methodologies (Soimakallio & Koponen, 2011).

Lignocellulose is the most abundant renewable biomass resource on Earth and for the past 80 years it has been acknowledged as a potential feedstock for the production of fuels and chemicals (Himmel et al., 2007). The majority of plant biomass, including stems and leaves, is composed of lignocellulose. Lignocellulose is called "the second generation" feedstock for fuel and chemical production to emphasize the difference to the edible "first generation" feedstocks. Lignocellu-

lose is a complex and tightly organised matrix of three main polymers, cellulose, hemicellulose and lignin. Historically, lignocellulose recalcitrance has hindered its utilisation as a feedstock in fuel and chemical production; however, the current drivers as well as technological development have renewed interest in lignocellulose (Himmel et al., 2007). Lignocellulose processing is envisioned to occur analogously to oil refining, meaning that the feedstock is efficiently utilised for the production of fuels, chemicals and energy in a concept called biorefining (Foust et al., 2008). At the moment (April 2013), a database of the International Energy Agency lists 13 commercial-scale factories that use lignocellulose as a feedstock for liquid fuel production. The 13 facilities are either operational, under construction or planned (<http://demoplants.bioenergy2020.eu/>).

The biochemical processing route of lignocellulosic biomass aims at enzymatic depolymerisation of cellulose and hemicellulose to monomeric sugars that may be further converted to various desired chemical products, such as ethanol, butanol and alkanes by exploiting microbial metabolism (Fortman et al., 2008) or chemical conversion. A simplified process description of biochemical lignocellulose conversion is shown in Fig. 1. Pretreatment based on heat, chemicals or mechanical grinding is a prerequisite for enzymatic depolymerisation of the cell wall carbohydrates in lignocellulosic biomass. Different types of steam pretreatments and treatments with dilute acids or bases are widely exploited in opening up the tightly packed structure of lignocellulose (Mosier et al., 2005). Several process configurations have been suggested for the saccharification of lignocellulosic polysaccharides and subsequent fermentation of the monosaccharides to desired chemicals. The different process configurations, such as separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and co-fermentation (SSCF) and consolidated bioprocessing (CBP) differ in their degree of integration (Lynd et al., 1999).

Lignocellulose may also be processed by thermochemical means, such as pyrolysis and gasification. Wright & Brown (2007) conducted an economical comparison of biochemical and thermochemical conversion routes for lignocellulose and concluded that both approaches were equally viable with the present state of technology.

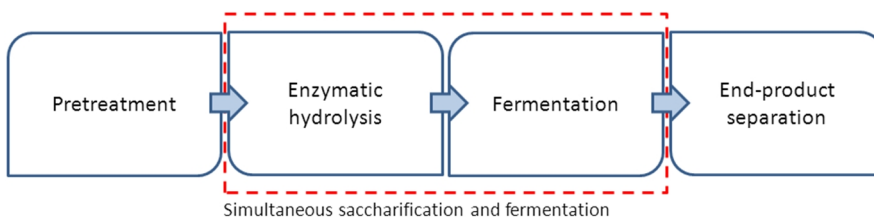


Figure 1. Simplified process scheme of biochemical processing of lignocellulosic feedstocks. Combining enzymatic hydrolysis and fermentation into one reactor would lead to a process configuration with simultaneous saccharification and fermentation (SSF).

1.1 Lignocellulose

1.1.1 Lignocellulosic feedstocks for fuel and chemical production

Agriculture, forestry, households and the industries refining agricultural or forestry products generate lignocellulosic side streams or wastes that are often poorly utilised or refined only to low-cost products, typically combusted to energy. Therefore, the potential of various lignocellulosic feedstocks in fuel and chemical production has been evaluated using e.g. residues from agriculture and forestry, waste streams from the pulp and paper industry (Kemppainen et al., 2012) and bagasse from the sugarcane industry (Dias et al., 2012). In addition, dedicated energy crops, such as perennial grasses, coppice willow and poplar are also potential feedstocks for fuel and chemical production (Somerville et al., 2010).

Fuel and chemical production from lignocellulosic biomass is dependent on a continuous supply of raw material with low enough feedstock cost. For agricultural residues the feedstock costs include the grower payment and the feedstock supply system costs that result from harvesting, collecting, storing, handling and transporting. Transportation costs are critical for the economic viability and therefore, location of the biorefining facility is highly important for its profitability (Foust et al., 2008). Brazil is among the first countries where lignocellulosic feedstocks will be used for industrial scale ethanol production due to the abundance and constant supply of sugarcane bagasse, a lignocellulosic residue of the sugarcane industry.

1.1.2 Structural features of lignocellulose

Lignocellulosic biomass is a complex matrix of three main biopolymers: cellulose, hemicellulose and lignin. Despite the common building blocks, plant species differ in the relative amounts as well as in the chemical structures of these main polymers. Variation also occurs between plant tissues. For example, parenchyma and vascular tissues in wheat straw differ greatly in lignin content. Furthermore, different layers of plant cell walls, such as primary and secondary cell walls in wood, differ in the relative amounts of the cell wall components (Sjöström, 1993). The structure of lignocellulose is therefore strongly dependent on its origin. A general presentation of polymer deposition in a lignified secondary cell wall is shown in Fig. 2.

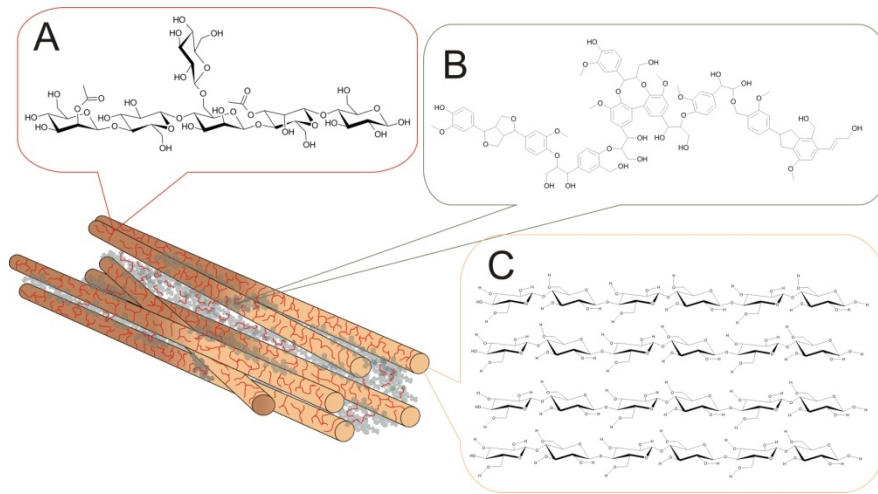


Figure 2. Schematic presentation of lignocellulose composition. Partial chemical structures of A) hemicellulose (*O*-acetyl galactoglucomannan from softwood (Willför et al., 2008)), B) softwood lignin (Crestini et al., 2011) and C) parallel cellulose chains in a crystal cellulose fibril are presented.

1.1.2.1 Lignin and lignin-carbohydrate complexes

Lignin is an aromatic cell wall polymer accounting typically for 26–32 % and 20–25 % of total mass in softwoods and hardwoods, respectively (Sjöström, 1993). Lignin content in agricultural residues varies substantially depending on the species. In corn stover and wheat straw, lignin accounts typically for 15–21 % and 5–17 % of dry weight, respectively (Buranov & Mazza, 2008). Evolutionarily, lignin was introduced to the cell walls when plants colonised land: cell wall lignification enabled water transport through conducting cells, gave the compressive strength necessary to support the weight of the plant on land and protected against microbial decay (Weng & Chapple, 2010).

Lignin biosynthesis occurs through radical coupling of three phenylpropanoid precursors called monolignols that differ in their methoxyl group content in the phenolic ring (Fig. 3). The monolignols, *p*-coumaryl alcohol, sinapyl alcohol and coniferyl alcohol give rise to the aromatic units *p*-hydroxyphenyl (H), syringyl (S) and guaiacyl (G), respectively, in the lignin polymer. Softwood lignin is mainly composed of G-units, whereas both S and G-units are abundant in hardwoods. Lignin in agriculturally important grasses, such as in wheat or maize, is composed of all three aromatic units H, S and G, with varying proportions (Buranov & Mazza, 2008).

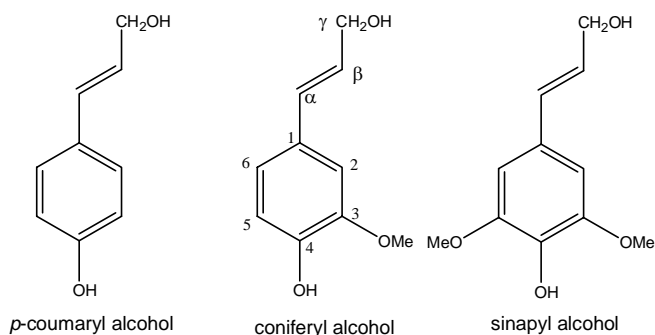


Figure 3. Phenylpropanoid precursors of polymeric lignin.

The polymeric structure of lignin is complex because of the great number of possible linkage types between the aromatic units and the random occurrence of the different linkage types. Traditional lignin models describe a cross-linked or branched polymer (Fengel & Wegener, 1984), although a recent study by (Crestini et al., 2011) demonstrated that isolated softwood lignin consists mainly of 6–12 phenolic units long linear oligomers (Fig. 4). The exact structure of native lignin remains an open question because lignin characterisation *in situ* lacks powerful analytical techniques, and lignin isolation prior to chemical characterisation always alters the polymeric structure.

The aromatic moieties in lignin are linked together via various types of linkages, β -O-4 ether linkage being the most abundant type accounting for 30–40 % of total linkages in softwood and 40–50 % in hardwood lignin (Brunow & Lundquist, 2010). Other abundant linkage types in wood lignin include β -5, β - β' , 5-5' and 4-O-5' type linkages as well as dibenzodioxocin (DBDO) structures that are potential branching points for lignin polymers (Brunow & Lundquist, 2010). A model of softwood milled wood lignin (MWL) molecules with designated linkages is shown in Fig. 4. Characteristic for wheat straw lignin is the high amount of esterified units in the structure. Crestini & Argyropoulos (1997) reported that isolated wheat straw lignin was found to contain on average 12 esterified units per 100 phenylpropane units. These are typically various cinnamic acids esterified to the gamma position in the phenolic unit (see Fig. 3 for position assignments). In the case of wheat straw, 77 % of the ester-bound chemical moieties were *p*-coumaric acids (Crestini & Argyropoulos, 1997).

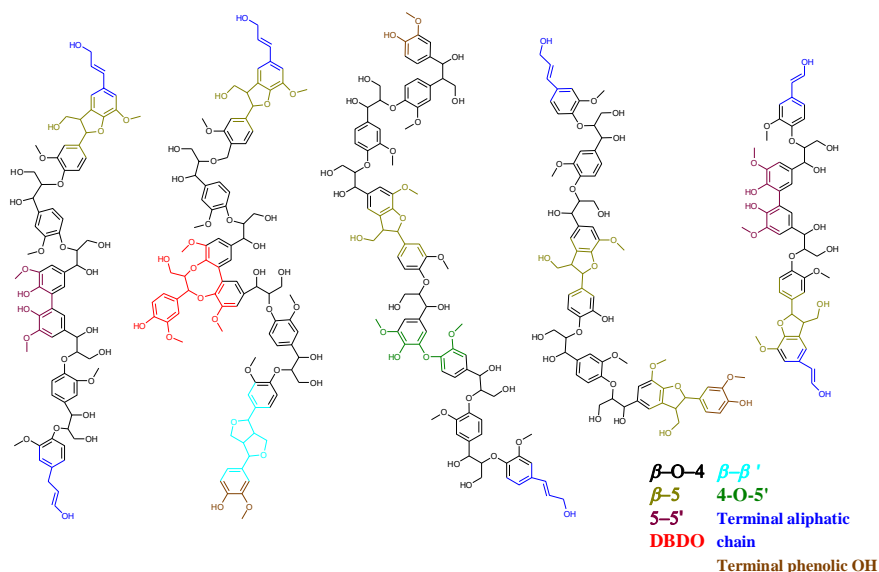


Figure 4. Softwood milled wood lignin (MWL) structures with linkage types and terminal groups designated with different colours. Figure modified from Crestini et al. (2011).

Numerous studies have suggested lignin to be covalently linked to the cell wall carbohydrates through lignin-carbohydrate complexes (LCC) in woody plants and grasses (Brunow & Lundquist, 2010). Generally the linkages are thought to form between hemicellulose and lignin (Salanti et al., 2012) or pectin and lignin (Meshitsuka et al., 1982). Phenyl glycoside, ester and benzyl ether types of lignin-carbohydrate linkages have been identified from softwood MWL using liquid state NMR methods (Balakshin et al., 2007). Herbaceous crops also contain LCCs in which carbohydrates and lignin are linked through ferulic acid moieties (Iiyama et al., 1994).

Isolation of lignin is challenging due to its close association with the other cell wall polymers, poor solubility in any commonly used solvents and the tendency of lignin to degrade or react upon isolation (Guerra et al., 2006). Nevertheless, isolation is often necessary for research purposes. Two approaches can be employed in lignin isolation. Complete enzymatic or acid hydrolysis of the structural polysaccharides leads to the isolation of a lignin-rich hydrolysis residue (Palonen et al., 2004). On the other hand, lignin may be dissolved out from the cell wall matrix. The downside of both approaches is that good yield and high purity require lignocellulose pretreatment (e.g. milling) prior to isolation, which is likely to alter the chemical structure of lignin. A comparison of different isolation procedures with their advantages and disadvantages is presented in Table 1.

For structural studies, lignin is generally dissolved from the lignocellulosic matrix after extensive milling using dioxane-water azeotrope (Björkman, 1956). This lignin is called milled wood lignin (MWL) and it is generally considered to represent native lignin, although extraction yields are low (< 50 %), the polymeric structure may have undergone chemical changes during isolation and the material is always contaminated with polysaccharides to some extent (Sjöström, 1993).

Table 1. Lignin isolation for research purposes. Advantages and disadvantages of two approaches.

Process	Isolation steps	Advantages	Disadvantages	References
Carbohydrate hydrolysis for a lignin-rich residue	<ul style="list-style-type: none"> • Pretreatment of the lignocellulosic material • Degradation of the cell wall carbohydrates using enzymes or acid 	<ul style="list-style-type: none"> • Represents close to total lignin in the sample • Retains the molecular orientation (important in certain studies) • Enzymatic treatment is not likely to alter lignin structure 	<ul style="list-style-type: none"> • Contaminations from cellulose and hemicellulose, extractives, inorganic compounds, enzyme proteins • Acid treatment likely to alter the chemical structure of lignin • Pretreatment alters lignin structure 	(Berlin et al., 2006; Palonen et al., 2004)
Lignin solubilisation (e.g. MWL)	<ul style="list-style-type: none"> • Removal of extractives • Pretreatment of the lignocellulosic material by milling • Enzymatic degradation of carbohydrates (not always applied) • Dissolution of lignin from the powdered sample, generally in dioxane-water or acidified dioxane-water • Extraction yields may be increased by subjecting the powdered sample to enzymatic hydrolysis prior to extraction 	<ul style="list-style-type: none"> • Lignin analytics is easier due to good solubility in various solvents • High purity can be obtained 	<ul style="list-style-type: none"> • Represents only a minor fraction of total lignin in the material • Loss of molecular orientation upon dissolution → self-aggregation in buffer solutions and probably also upon drying • Pretreatment alters lignin structure 	(Björkman, 1956; Wu & Argyropoulos, 2003)

1.1.2.2 Cell wall carbohydrates, cellulose and non-cellulosic polysaccharides

Cellulosic and non-cellulosic polysaccharides (hemicelluloses, pectin) in lignocellulosic feedstocks are targeted for enzymatic hydrolysis in biochemical processing. Cellulose is the main constituent of wood, accounting typically for 40–45 wt-% (Sjöström, 1993). In herbaceous crops the cellulose content varies approximately between 30 and 50 wt-% depending on the species (Buranov & Mazza, 2008).

Cellulose is a homopolymer of glucose with an organised 3-dimensional structure. The primary cellulose chains are composed of glucose units that are connected through β 1–4 glycosidic bonds (Fig. 2C). The average degree of polymerisation (DP) of cellulose is up to 14 000 in secondary cell walls and 6 000 in primary cell walls (Harris & Stone, 2008). Within the cellulose chain, every glucose unit is 180° rotated with respect to its neighbouring molecule and therefore the actual repeating chemical unit in the chain is cellobiose. The cellulose chains pack together in parallel orientation and the hydroxyl groups in glucose molecules give rise to an intra- and interchain hydrogen-bonding network leading to a partially crystalline, 3-dimensional microfibril (elemental fibril) structure. The exact microfibril structure is still debated and it is considered possible that microfibrils of different sizes exist in higher plants (Guerriero et al., 2010). In different studies, microfibrils are reported to be formed of 16, 18 or 36 cellulose chains that give rise to microfibrils of 2–4 nm in diameter (Guerriero et al., 2010). Microfibrils associate with each other, forming larger fibrils (macrofibrils) with varying diameters (Fig. 2) (Donaldson, 2007). In native cellulose two crystalline forms I α and I β are present (Atalla & van der Hart, 1984), whereas different chemical treatments are able to alter the crystalline structure.

Hemicelluloses are heteropolysaccharides that together with lignin form a matrix surrounding the network of cellulosic fibrils (Fig. 2). Both glycosidic and ester linkages are present in hemicellulosic structures. Hemicelluloses typically account for 20–30 % and 20–40 % of dry weight in wood and herbaceous crops, respectively (Buranov & Mazza 2008; Sjöström, 1993). Hemicelluloses have substantially lower DP (generally ca. 200) compared to cellulose (Sjöström, 1993); they are often branched and carry substitutions in their polymeric backbone. In comparison to cellulose, hemicelluloses are more susceptible to hydrolytic degradation. In softwoods, galactoglucomannan (Fig. 2A) is the most abundant type of hemicellulose (11–17 wt-%) and lower quantities of xylans (6–8 wt-%) are also present (Willför et al., 2005a). In hardwoods and herbaceous crops, xylans are the prevalent group of hemicelluloses. For example in hardwoods, xylans account typically for 15–25 wt-% (Willför et al., 2005b) although glucomannans are also present to a lower degree. Pectins, composed mainly of acidic sugars (galacturonic acids) are present in wood primary cell walls and middle lamella in low quantities (1.5–3 wt-%) (Willför et al., 2005a; Willför et al., 2005b).

1.2 Enzymes for lignocellulose degradation

Lignocellulose is the most abundant type of biomass on Earth and microbial degradation of lignocellulose is an essential link in the carbon cycle. Microbes with the

capability to degrade and utilise lignocellulosic feedstocks as their carbon source are mainly found from soil and decaying wood but also from the guts of ruminant animal species. Due to the complex structure of lignocellulose, synergistic action of various enzymes is needed for complete degradation of lignocellulose.

1.2.1 Cellulases

Cellulases are a group of enzymes responsible for cellulose degradation in nature. Cellulases are produced mainly by microorganisms (bacteria and fungi) (Lynd et al., 2002) but also by organisms representing the animal kingdom, including insects, molluscs, nematodes and protozoa (Watanabe & Tokuda, 2001).

Cellulases are hydrolases which catalyse the cleavage of β 1–4 glycosidic bonds in cellulose with concomitant addition of water to the cleavage point. Even though only one type of chemical bond is present in cellulose, enzymes with different modes of action are required for complete degradation of the recalcitrant and insoluble polymer. In nature, one organism may produce the enzymes needed for the complete degradation of cellulose, although synergistic action of many organisms has also been suggested (Wilson, 2011).

Generally, aerobic cellulose-degrading microorganisms secrete individual enzyme components (free-enzyme system), that act synergistically on cellulose. The most studied free-enzyme systems are those of aerobic and mesophilic fungi: *Trichoderma reesei* (Schmoll & Schuster, 2010), *Humicola insolens* (Schülein, 1997) and *Phanerochaete chrysosporium* (Broda et al., 1994). Industrial strains of *T. reesei* are highly efficient enzyme producers, which is a key reason why *T. reesei* cellulases still today dominate the cellulase markets. The genomic sequence of *T. reesei* was published in 2008 and it revealed the diversity of cell wall-degrading enzymes to be considerably lower in *T. reesei* compared to many other carbohydrate-degrading fungi (Martinez et al., 2008). In the *T. reesei* genome, only 26 genes are annotated as cellulases or hemicellulases, whereas for example in the *Magnaporthe grisea* genome the corresponding number of genes is 74 (Martinez et al., 2008).

In contrast to aerobic microorganisms, anaerobic cellulolytic bacteria generally produce a complexed multi-enzyme aggregate system called a cellulosome, which protrudes from the bacterial cell wall (Lynd et al., 2002). All known cellulosomes contain a large polypeptide (cohesin) which contains a cellulose-binding module and serves as an anchoring protein for the catalytic domains of cellulases and hemicellulases. The cellulosome of the anaerobic bacterium *Clostridium thermocellum* is the most studied complexed cellulase system (Bayer et al., 2004).

1.2.1.1 Action of free-enzyme systems in cellulose degradation

The classical model describing the action of free-enzyme systems involves three hydrolytic enzyme activities necessary for the complete degradation of crystalline cellulose: cellobiohydrolase, endoglucanase and β -glucosidase activities (Enari, 1983).

Cellobiohydrolases (EC 3.2.1.91) act on insoluble crystalline cellulosic substrates, liberating cellobiose as the main product from the cellulose chain ends (Teeri, 1997). *T. reesei* secretes two types of cellobiohydrolases, Cel7A (former CBHI) and Cel6A (former CBHII) that attack the cellulosic chains either from the reducing or non-reducing end, respectively. Characteristic for the action of cellobiohydrolases is that the DP of cellulose is affected only slightly. Kleman-Leyer et al. (1996) studied the effect of *T. reesei* Cel6A on bacterial crystalline cellulose (BMCC). Although 40 % of the substrate was solubilised, the average molecular weight of the residual BMCC was decreased only by a small extent. The action of *T. reesei* Cel7A molecules was monitored on algal crystalline cellulose fibrils using high-speed atomic force microscopy, and the data revealed unidirectional processive action of *Tr*Cel7A on top of the cellulosic fibrils (Igarashi et al., 2011). When the enzymes encounter obstacles their movement halts or slows down. Unidirectional movement of fast and slow enzymes was shown to generate situations resembling traffic jams (Igarashi et al., 2011).

Endoglucanases (EC 3.2.1.4) introduce cleavages within cellulosic chains. They decrease the DP of cellulose and generate new sites of attack for cellobiohydrolase-type enzymes (Kleman-Leyer et al., 1996). Endoglucanases are suggested to act preferentially on disordered areas of cellulose fibrils, because their action on amorphous cellulosic substrates (e.g. phosphoric acid swollen cellulose) is more pronounced compared to their action on crystalline substrates (Enari, 1983; Kleman-Leyer et al., 1996). The role of β -glucosidases (EC 3.2.1.21) is to degrade the arising cello-oligomers such as cellobiose and cellotetraose to glucose.

The different enzymes exhibit synergistic action in cellulose degradation. Simultaneous action of multiple enzyme components results in a much higher degradation rate than the sum of the degradation rates of the individual enzyme components. Synergistic action between endoglucanases and cellobiohydrolases, such as *Tr*Cel7A, (CBHI) and *Tr*Cel5A (EGII) (Medve et al., 1998) and two different types of cellobiohydrolases, such as *Tr*Cel7A (CBHI) and *Tr*Cel6A (CBHII) (Fägerstam & Pettersson, 1980; Medve et al., 1994), has been reported.

Recently, a novel oxidative enzyme class, lytic polysaccharide mono-oxygenases (LPOMs), was found to contribute to cellulose degradation. The involvement of an oxidative mechanism in cellulase degradation has been suggested already in the 1970s when oxygen was found to enhance enzymatic cellulose degradation with fungal enzymes (Eriksson, 1975). The oxidative mechanism of LPOM was first demonstrated with a chitin-oxidising enzyme, which introduces chain breaks, generates oxidised chain ends and thus enhances the action of other chitin-degrading enzymes (Vaaje-Kolstad et al., 2010). Fungal enzymes, previously classified into GH family 61, were demonstrated to have similar oxidative activity on crystalline cellulose (Harris et al., 2010; Quinlan et al., 2011). LPOMs are widespread in fungal genomes and recently a new class for auxiliary activities was created in the Carbohydrate Active enzymes (CAZy) database to accommodate the LPOMs (Levasseur et al., 2013).

1.2.1.2 Modular structures of cellulases

The structural architecture of cellulases is highly versatile especially in bacteria, where multiple domains with known or unknown functions are combined in one complex molecule (complexed cellulase systems) (Medie et al., 2012). In fungal, free-enzyme systems the enzymes may be composed of a single catalytic module or of multiple domains. A typical modular organisation of a fungal cellulase consists of a catalytic module attached to a carbohydrate binding module (CBM) through a highly glycosylated and flexible peptide linker (Gilkes et al., 1991). The major cellulases of *T. reesei*, Cel7A, Cel7B, Cel6A and Cel5A, are modular whereas only one endoglucanase of *T. reesei*, Cel12A, is composed of a single catalytic module (Karlsson et al., 2002). Fungal cellulases that lack a linker and a CBM can be found for example from *Melanocarpus albomyces* (Haakana et al., 2004) and *Talaromyces emersonii* (Tuohy et al., 2002).

Catalytic modules of cellulases are classified into families in the Carbohydrate Active Enzymes database (CAZy) (<http://www.cazy.org/>) according to their amino acid sequence similarity (Cantarel, 2009; Henrissat, 1991). Currently, endoglucanase, cellobiohydrolase and β -glucosidase activities are found from 17, 4 and 6 distinctive families, respectively. The mode of enzyme action is reflected in the topology of the enzyme's active site. Cellobiohydrolases possess a tunnel-shaped active site into to which the glycan chain end may penetrate. For example, the crystalline structure of *T. reesei* Cel7A in complex with cello-oligomers revealed the catalytic site to be located in a 50 Å long tunnel (Divne et al., 1998). The cleft-like active site of endoglucanases enables catalysis in the middle of a glycan chain and a typical pocket-type active site is found in β -glucosidases that act on small soluble substrates (Davies & Henrissat, 1995).

CBMs enhance the hydrolysis of crystalline cellulose by increasing enzyme proximity to the substrate (Reinikainen et al., 1992; van Tilbeurgh et al., 1986). CBMs are also proposed to target enzyme binding to specific features in the substrate (Carrard et al., 2000), and some CBMs are suggested to have a more active role in disrupting the crystalline structure of cellulose. For example, family 2a CBM from *Cellulomonas fimi* has been suggested to disrupt the crystalline structure, leading to an improved hydrolysis with endoglucanase (CenA) (Din et al., 1994). Based on high-speed AFM video data, Igarashi et al. (2009) concluded that the CBM of *TrCel7A* only increases the concentration of enzyme molecules on the cellulosic surfaces and does not contribute to the processive action of the enzyme. Recently, the positive effect of CBM on cellulose hydrolysis was shown to depend on dry matter content: in high dry matter (20 wt-%) enzymes lacking the CBM were found to perform as well as the corresponding intact enzymes (Várnai et al., 2013).

Currently, carbohydrate-binding modules are classified into 64 families in the CAZy database according to their amino acid sequence. The CBMs of fungal cellulases belong exclusively to the family 1, which is characterised by small size (ca. 40 amino-acid residues) and a common cysteine knot fold (Boraston et al., 2004). Family-1 CBMs have a planar face with conserved aromatic amino-acid residues that interact with crystalline surfaces of cellulose or chitin (Boraston et al.,

2004; Kraulis et al., 1989). Bacterial CBMs that bind crystalline cellulose or chitin fall into the families 2a, 3, 5 or 10 (Boraston et al., 2004). Bacterial CBMs are larger compared to the family-1 CBMs. For example, family 3 CBMs consist of ca. 150 amino-acid residues that fold into a nine-stranded β -sandwich with a jelly roll topology (Tormo et al., 1996). Despite the great differences in size, all CBMs that bind crystalline surfaces share a similar planar binding face, with which they interact with the polymeric surface (Boraston et al., 2004).

1.2.2 Enzymes acting on hemicellulose and lignin

Hemicellulases are a broad group of enzymes that degrade the complex structures of different hemicellulosic polymers composed of sugars, sugar acids and esterified acids. Synergistic action of multiple hemicellulolytic enzymes is required for complete degradation of hemicellulose. Hemicellulases may be classified into depolymerising enzymes that act on the hemicellulose backbone and into debranching enzymes (also referred to as accessory enzymes) that act on the polymer branches.

Backbone-depolymerising hemicellulases are hydrolases with different specificities for the various hemicellulose structures. They include xylanases, mannanases, β -glucanases and xyloglucanases (Decker et al., 2008). Most xylanases with endo-1,4- β xylanase activity (EC 3.2.1.8), capable of hydrolysing xylan backbone, fall into the GH families 10 and 11 in the CAZy database (Kolenová et al., 2006), although interesting enzymes with distinctive substrate specificities are also found from other GH families, such as GH30 (St John et al., 2010) and GH8 (Pollet et al., 2010). Xylanases from families 10 and 11 differ in their preference for cleavage sites in the xylan backbone: Family 10 enzymes are capable of cleaving the xylan backbone closer to the substituents compared to the family 11 enzymes (Biely et al., 1997). Mannanases (EC 3.2.1.78) are able to catalyse the cleavage of β -D-1,4 mannopyranosyl linkages within the main chain of glucomannans and galactoglucomannans. Similarly to xylanases, different endomannanases are selective for the site of attack in the polymeric backbone (Tenkanen et al., 1997). Analogously to cellulases, the depolymerising hemicellulases may also be considered as endo- or exo-acting depending on the site of attack in the hemicellulose backbone, although many enzymes have been shown to act in both modes. Certain hemicellulases (e.g. β -glucosidase, β -xylosidase and β -mannosidase) degrade only the small oligomeric fragments arising from degradation of the polymeric molecules (Decker et al., 2008). In general, depolymerising hemicellulases may be highly site- and conformation-specific or they may possess a broader spectrum potential of substrates.

Debranching hemicellulases, also referred to as accessory enzymes, cleave the side-groups bound to the polymeric backbone. The side-groups may be glycosidic or esterified acids. Enzymes involved in removing the glycosidic side-groups include α -glucuronidases, α -arabinofuranosidases and α -D-galactosidases, whereas the esterified acids may be cleaved with acetyl esterases or feruloyl esterases (Decker et al., 2008).

In nature, white-rot basidiomycete fungi are the most efficient organisms in lignin decomposition (Kirk & Farrell, 1987). To our current knowledge, enzymatic mineralisation of lignin to CO₂ occurs exclusively through oxidative enzymes that catalyse unspecific reactions through highly reactive free radicals (Hammel & Cullen, 2008). Different types of oxidative enzymes are considered important for lignin degradation, including laccases, lignin peroxidases, manganese peroxidases and versatile peroxidases (Hammel & Cullen, 2008). Traditionally lignin and polysaccharide degradation is thought to occur by separate mechanisms. However, this division may be too strict, since some enzymes are reported to act both on lignin and on carbohydrates (Henriksson et al., 2000; Lévassieur et al., 2013).

1.3 Lignocellulose pretreatment

Enzymatic hydrolysis of untreated lignocellulosic feedstocks is highly restricted, and pretreatment is therefore a prerequisite for efficient enzymatic hydrolysis of the cell wall carbohydrates (Mosier et al., 2005). Pretreatment introduces physical and/or chemical changes to the biomass structure, which leads to an improved action of enzymes on the cell wall polysaccharides. An ideal pretreatment would avoid polysaccharide degradation and subsequent formation of harmful inhibitory compounds, minimise energy and capital investments and avoid the need for biomass particle size reduction prior to the treatment (Mosier et al., 2005).

In pretreatments, improved enzymatic digestibility is attained by increasing cellulose accessibility or by altering the crystalline structure of cellulose. Accessibility may be increased by decreasing the biomass particle size (Silva et al., 2012) or by disrupting or removing the hemicellulose-lignin network surrounding the cellulose fibrils (Donaldson et al., 1988; Stenberg et al., 1998). In addition, decrease in cellulose crystallinity (Silva et al., 2012) and change in the crystalline form have also been shown to increase enzymatic degradation. For example, when the crystalline form, cellulose Ia was transformed into cellulose III by a supercritical ammonia treatment, enzymatic hydrolysis of the cellulosic material with *TrCel7A* was significantly enhanced (Igarashi et al., 2007; Igarashi et al., 2011).

Various pretreatment technologies have been and are still being developed, since none of the existing methods has proved to be superior to the others. Furthermore, different pretreatments are optimal for different feedstocks due to the structural diversity in lignocellulosic feedstocks.

Pretreatments are divided into methods that employ physical, chemical or both means to increase enzymatic digestibility in increasing cellulose accessibility. Table 2 lists the effects of some widely studied pretreatment technologies on biomass structure. In general, methods employing water or acids tend to degrade and solubilise hemicellulose and leave cellulose and lignin insoluble, whereas methods employing bases (e.g. ammonia) modify and dissolve the lignin and affect the crystallinity of cellulose.

Table 2. Effects of different pretreatments on lignocellulose structure. Dark green = major effect, light green = moderate effect, white = no effect, ND = not determined. Table modified from (Mosier et al., 2005).

Pretreatment	Principle	Increases accessible surface area	Decrystallises cellulose	Removes hemicellulose	Removes lignin	Alters lignin structure
Uncatalysed steam explosion	Treatment with high-pressure steam and termination with rapid decompression	Dark green	*	Dark green	White	Light green
Liquid hot water ^b	Treatment with water at high pressure and temperature	Dark green	ND	Dark green	**	Light green
Dilute acid	Treatment in dilute acid at high temperature	Dark green	White	Dark green	White	Dark green
AFEX (ammonia fibre explosion)	Treatment with aqueous ammonia at high temperature and pressure	Dark green	Dark green	Light green	Dark green	Dark green

^aSteam explosion pretreatment is shown to increase cellulose crystallinity (Atalla, 1991).

^bFlow-through process configuration of liquid hot water pretreatment results in moderate lignin removal (Mosier et al., 2005)

1.3.1 Steam pretreatments

Steam pretreatment is used as a general term in this thesis to describe all pretreatment technologies exploiting biomass treatment with high-pressure steam. Steam treatments are efficient for lignocellulose fractionation, resulting in degradation and solubilisation of hemicellulose, delocalisation of lignin and an accessible cellulosic fraction (Excoffier et al., 1991). In different process configurations, the treatment may be terminated with a rapid decompression (steam explosion), it may be coupled to mechanical comminution or catalysed with the addition of acid, typically H_2SO_4 . Historically, steam treatments have been of interest for the pulp and paper industry and fibreboard sector. More recently the technology has also gained interest as a pretreatment method prior to enzymatic hydrolysis (Focher et al., 1991). Limited use of chemicals, relatively low energy demand and the possibility to recover most of the cellulose- and hemicellulose-derived sugars for fermentation make steam pretreatments attractive for total hydrolysis processes (Chandra et al., 2007). Steam pretreatments are applicable for all types of lignocellulosic feedstocks, although pretreatment of softwood requires harsher conditions and an acid catalyst.

1.3.1.1 Effects of steam pretreatments on the structure of lignocellulose

Hydrothermal and steam explosion pretreatments of wheat straw are shown to separate fibres from the tissue structure (Kristensen et al., 2008). Separate fibres appear undisrupted in microscopic images, but closer examination of the fibre surfaces shows that the network of cellulose fibrils is coated with aggregated material, presumably lignin (Hansen et al., 2011; Kristensen et al., 2008). Similar aggregates have also been found from steam pretreated softwood (Donaldson et al., 1988). In general, steam pretreatment of wheat straw does not completely collapse the microfibril-network and thus cell wall structural elements are detectable after the pretreatment. Therefore, disruption of the lignin-hemicellulose network is probably an important factor in the improved hydrolysability of steam pretreated feedstocks. The effect of steam pretreatment on lignocellulose structure in nanometer-scale is schematically presented in Fig. 5.

Steam pretreatments have a major effect on the chemical structure of lignin and on the spatial distribution of lignin in the feedstock. In the treatment, most of the lignin remains insoluble, although some water soluble aromatic degradation products are known to be formed (Bobleter, 1994). Depolymerisation and repolymerisation reactions of lignin compete during steam pretreatments (Li et al., 2007). Increase in pretreatment severity favours lignin repolymerisation through condensation reactions, leading to an increased molecular weight of lignin (Li et al., 2007). In addition, increase in pretreatment severity enhances depolymerisation of β -O-4 ether bonds and thus increases the amount of phenolic hydroxyls in hardwood lignin (Robert et al., 1991). In hardwood lignin, the quantity of methoxyl groups (Chua & Wayman, 1979) and the S/G ratio (Martin-Sampedro et al., 2011) have

been shown to decrease after steam pretreatment. These changes occur probably due to preferential removal of syringyl units in the process (Shimizu et al., 1998). Steam pretreatment is also suggested to cleave lignin-carbohydrate complexes (Martin-Sampedro et al., 2011).

Steam treatments alter the cellulosic fraction by decreasing the DP (Excoffier et al., 1991) and by increasing the crystallinity of cellulose (Atalla, 1991). In general, treating cellulose at high temperatures is thought to result in tighter organisation (aggregation) of cellulose, leading to a more recalcitrant material towards degradation (Atalla et al., 2008). However, the availability of cellulosic surfaces increases (Wiman et al., 2012), leading to improved enzymatic digestion of cellulose.

Steam pretreatments fractionate the lignocellulosic feedstocks by degrading the non-cellulosic polysaccharides into water soluble oligomers and monomers. The soluble compounds may further react and form furfural, 5-hydroxymethyl furfural (HMF) and acetic acid (Larsen et al., 2008; Stenberg et al., 1998). Careful optimisation of the pretreatment conditions is necessary, because the arising compounds are inhibitory to the fermenting organisms in further biochemical processes (Palmqvist et al., 1996).

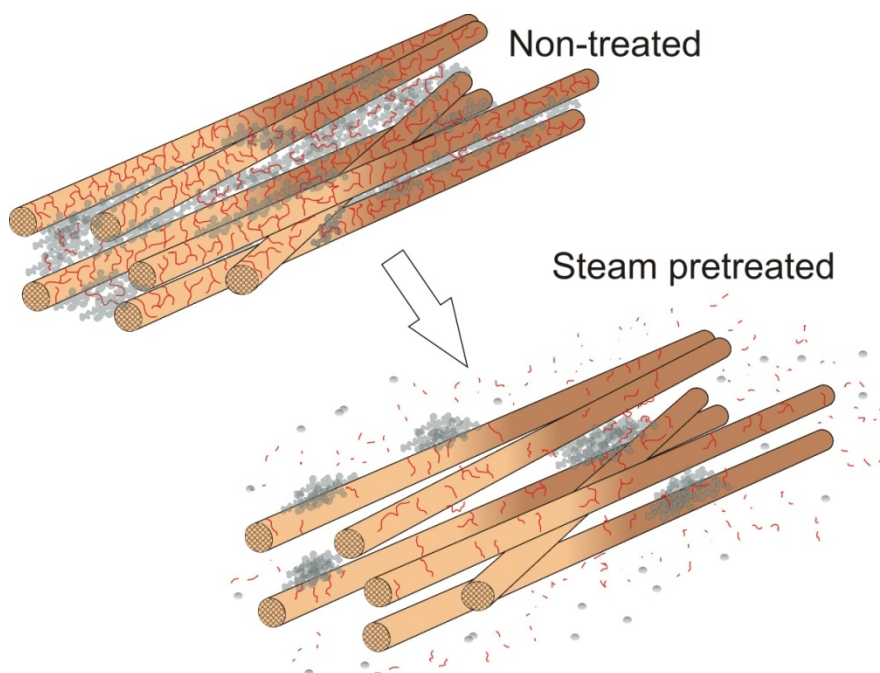


Figure 5. Schematic picture of the effects of steam pretreatment on the nanometer-scale structure of lignocellulose. Cellulose macrofibrils (long cylinders), lignin (grey), hemicellulose (orange). Steam pretreatment decreases the DP and increases the crystallinity of cellulose, although visible changes in the macrofibril network have not been detected by AFM (Hansen et al., 2011; Kristensen et al., 2008).

1.4 Inhibitory effects of lignin during enzymatic hydrolysis of lignocellulose

Lignin restricts the enzymatic hydrolysis of pretreated lignocellulosic feedstocks. After delignification, even softwood is easily hydrolysed to sugars with low cellulase dosages (Kumar et al., 2012; Mooney et al., 1998; Várnai et al., 2010). Three distinctive mechanisms are suggested to contribute to the lignin-derived inhibition: lignin shields carbohydrate surfaces from enzymatic attack (Mooney et al., 1998), lignin adsorbs enzymes (Palonen et al., 2004), and lignin-derived soluble compounds act as enzyme inhibitors (Ximenes et al., 2011). The different inhibitory mechanisms are visualised in Fig. 6, in which cellobiohydrolase-type enzymes are acting on a lignin-coated cellulose fibril.

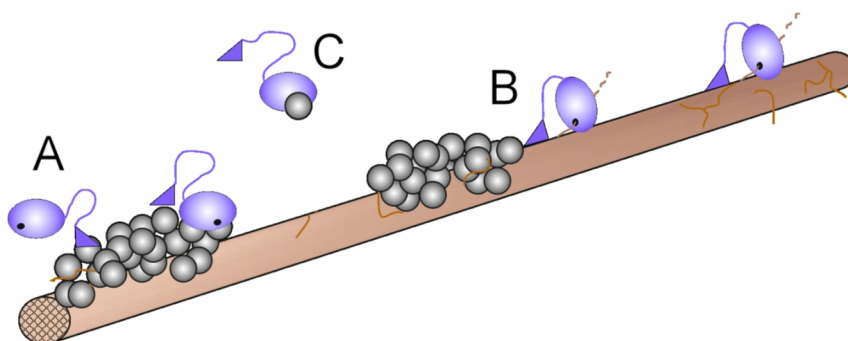


Figure 6. Inhibitory mechanisms of lignin in enzymatic depolymerisation of cell wall carbohydrates: A) enzyme adsorption onto lignin, B) restriction of enzyme accessibility to the carbohydrates and C) enzyme inhibition by soluble lignin-derived compounds.

The shielding effect as well as enzyme binding onto lignin are considered to be the most influential inhibitory mechanisms (Nakagame et al., 2011c) and recently these mechanisms were also shown to be mutually dependent (Kumar et al., 2012). When isolated lignin was added back to the hydrolysis of highly accessible delignified pulp, less inhibition due to non-productive enzyme binding occurred than when it was added to the hydrolysis of microcrystalline cellulose, a less accessible cellulosic substrate (Kumar et al., 2012).

The extent of lignin-derived inhibition is dependent on the botanical origin as well as on the pretreatment applied to the lignocellulosic feedstock, because both factors affect localisation and chemical properties of lignin. For example, localisation of lignin in the plant tissue significantly differs in herbaceous crops and in softwood. In the stems and leaves of herbaceous crops, the majority of the cell tissue (parenchyma) is composed of thin-walled cell types with low lignin content (Donaldson et al., 2001), whereas the bulk of wood tissue is composed of lignified cells (Sjöström, 1993). Furthermore, botanical differences in lignin chemistry may affect non-productive enzyme adsorption. Higher carboxylic acid content in herba-

aceous lignin has been proposed to reduce non-productive enzyme adsorption due to higher hydrophilicity of the lignin (Nakagame et al., 2011a).

In general, solvent-based or alkaline pretreatments result in lignin solubilisation, whereas acidic or steam pretreatments solubilise hemicellulose, leaving lignin insoluble in the material (Mosier et al., 2005). Nevertheless, lignin is always present to some extent in all pretreated lignocellulosic materials (Nakagame et al., 2011c). Depending on the pretreatment method, lignin is relocalised and/or solubilised from the cell walls. Both dissolution and relocalisation of lignin affect enzyme accessibility to cellulose. Pretreatments alter the chemical structure of lignin (Li et al., 2007; Pu et al., 2013), which may contribute to non-productive enzyme adsorption. Very little has been reported on the effect of pretreatment on non-productive adsorption. (Nakagame et al., 2011b) showed that softwood lignin, isolated after severe pretreatment conditions, tended to decrease hydrolysis of microcrystalline cellulose more than lignins isolated after mild pretreatment conditions.

The inhibitory effect of lignin is considered most detrimental in the hydrolysis of softwood feedstocks. Only 16 % of total carbohydrates in steam pretreated Douglas fir could be hydrolysed using low cellulase dosage (5 FPU/g cellulose) (Kumar et al., 2012), whereas similar cellulase loading solubilises approximately 80 % of total carbohydrates in acid-pretreated corn stover (Xu et al., 2008). Non-productive enzyme adsorption onto lignin is considered as a major factor preventing efficient hydrolysis of softwood feedstocks with reasonable enzyme loadings (Kumar et al., 2012). On the other hand, non-productive enzyme adsorption is considered to be insignificant in the enzymatic hydrolysis of hydrothermally pretreated corn stover and wheat straw (Barsberg et al., 2013).

1.4.1 Protein adsorption to solid surfaces

Enzyme binding onto solid surfaces is an essential phenomenon in enzymatic processing of lignocellulose, during which both cellulose-binding and non-productive binding to other cell wall polymers may occur. In general, protein adsorption to solid surfaces is a very common, sometimes desired but often unwanted phenomenon with great impact on many fields such as biomedicine and food processing (Haynes & Norde, 1994). Therefore, basic research has been carried out with model proteins and surfaces in order to understand the fundamental phenomena driving protein adsorption onto solid surfaces.

According to the thermodynamic laws, protein adsorption in constant temperature and pressure occurs only if the Gibbs energy (G) of the system decreases upon the adsorption event (Eq.1).

$$\Delta_{ads}G = \Delta_{ads}H - T\Delta_{ads}S < 0 \quad (1)$$

Δ_{ads} change upon the adsorption process

G Gibbs energy

H enthalpy

S entropy

T temperature.

Accordingly, the process may be driven by change in enthalpy or entropy. The system components that affect $\Delta_{\text{ads}}G$ include the protein, the surface, the solvent and the all soluble compounds (e.g. ions) present. In addition, intramolecular forces within the protein contribute to $\Delta_{\text{ads}}G$ since they may allow conformational changes in proteins. Therefore, knowledge on protein stability in different conditions is essential when protein-surface interactions are studied (Haynes & Norde, 1994).

1.4.1.1 Adsorption of globular proteins to solid surfaces

Globular proteins are highly surface active molecules by nature. Amino-acid residues with hydrophobic character are mostly embedded in the compact protein core, whereas charged residues are exposed to the surface. In addition to charged moieties, residues with non-polar and hydrogen-bonding character may also occur on protein surfaces.

Protein-surface interactions are usually non-covalent; the driving forces involve mostly hydrophobic and repulsive and attractive electrostatic interactions, with minor contribution from hydrogen bonding and dipolar interactions (Claesson et al., 1995; Norde, 1996). Increasing surface hydrophobicity is found to increase protein binding to the surface (Elwing et al., 1987). Hydrophobic interactions often dominate enzyme adsorption, even in conditions in which electrostatic interactions are repulsive (Norde, 1996). The formation of hydrophobic interactions is driven by entropy gain arising from the dehydration of hydrophobic surface(s).

Characteristic for protein adsorption is that conformational changes often take place upon binding. Unfolding is more likely to occur on hydrophobic surfaces (Haynes & Norde, 1994). Conformational changes contribute to the driving forces of protein adsorption (Haynes & Norde, 1995), and therefore the internal coherence of the protein structure also has an undisputed effect on the adsorption process. Structurally less stable proteins are shown to undergo more drastic conformational changes upon adsorption compared to more stable proteins (Billsten et al., 1995). Furthermore, in a study by (McGuire et al., 1995), structurally less stable mutants of T4 lysozyme were found to develop higher affinity to a silica surface.

1.4.1.2 Reversibility of adsorption

Reversibility of adsorption is often of interest when protein adsorption to solid surfaces is studied, because it provides an idea of protein affinity to the surface and defines the thermodynamic criteria that may be applied to describe the system.

If an adsorption process is reversible, the adsorbed proteins can freely exchange with proteins of the same kind in the solution and a new equilibrium is established between the free and bound fraction upon dilution or addition of fresh protein. If binding is irreversible, free exchange does not occur and the proteins mainly remain bound to the surface, although the ratio of protein and adsorbent is shifted by dilution or addition of fresh protein. Reversible adsorption is generally observed for small molecules, e.g. for ions that are able to interact with the surface

with only one type of interaction. Irreversible adsorption is often reported for proteins and polymers, probably because they are able to form multiple interactions with the surface that altogether can overcome the “dilution effect” (Haynes & Norde, 1994). An interesting exception is the reversible binding of family-1 CBM from *TrCel7A* onto cellulose (Linder & Teeri, 1996).

1.4.2 Enzyme interactions with the different components in lignocellulosic biomass

Cellulose, non-cellulosic polysaccharides and lignin are the main constituents of lignocellulosic biomass. Enzyme binding to substrate polysaccharides is a prerequisite for hydrolysis, whereas non-productive binding to the other cell wall components, such as lignin, should be avoided.

1.4.2.1 Cellulase interactions with cellulose

Cellulase binding onto crystalline cellulose has been widely studied with the main focus on CBM-cellulose interactions. At low substrate consistency (e.g. 1 % w/v), CBMs are shown to greatly increase enzyme binding to cellulosic surfaces, although the catalytic modules are also capable of low binding to cellulose (Palonen et al., 1999).

Cellulose allomorphs I_{α} and I_{β} have hydrophilic and hydrophobic faces. On the hydrophobic faces, the pyranose rings are fully exposed at the fibril surface. The hydrophobic faces are calculated to account only for 38% of surface area in crystals of 36 parallel cellulose chains (Nimlos et al., 2012). In a study by Lehtiö et al. (2003), binding of family 1 and family 3 CBMs was shown to occur preferentially on the hydrophobic planes of Valonia cellulose (I_{α}) and more recently *T. reesei* Cel7A was shown to degrade crystalline cellulose exclusively from the hydrophobic faces of a cellulose crystals (Liu et al., 2011). Nimlos et al. (2012) demonstrated with molecular simulation that the CBM from *TrCel7A* binds preferentially to the hydrophobic surfaces, but it may also be able to diffuse from hydrophilic crystal surfaces to hydrophobic surfaces.

In general, proteins are known to interact with carbohydrates through charged and aromatic amino-acid residues that may form hydrogen bonds and favourable van der Waals interactions with carbohydrates, respectively (Vyas, 1991). Binding of family 1 CBMs to crystalline cellulose have been studied the most and the amino-acid residues that are critical for CBM-cellulose interaction have been resolved using mutated CBMs. Family 1 CBMs interact with the hydrophobic face of a cellulose crystal via conserved aromatic and charged amino-acid residues that are exposed on the flat hydrophobic face of the CBM (Beckham et al., 2010; Linder et al., 1995b). The aromatic rings in these amino-acid residues are spaced so that they may stack with every other pyranose rings on a cellulosic chain (Tormo et al., 1996). Linder et al. (1995b) demonstrated with native and mutated *TrCel7A* CBMs that the aromatic amino-acid residues Y31, Y32 and supposedly Y5 are critical for

CBM-cellulose affinity, with contribution also from polar side chains, such as Gln-34 or Asn-29.

Electrostatic interactions are not considered to be of key importance for cellulase interactions with pure cellulose, although different studies have demonstrated contradicting results concerning the effect of pH on cellulase adsorption. In a study by Reinikainen et al. (1995) pH had only a modest effect on *T. reesei* Cel7A adsorption onto bacterial microcrystalline cellulose, whereas binding of the catalytic core was practically insensitive to pH.

In industrially relevant hydrolysis conditions enzyme interaction with cellulose may be less dependent on CBMs. Recently, it has been suggested that at high substrate consistency the CBM becomes less important for substrate recognition and subsequent hydrolysis (Le Costaouëc et al., 2013; Várnai et al., 2013).

1.4.2.2 Enzyme-lignin interactions

Non-productive enzyme adsorption onto the lignin-rich components in biomass is a major inhibitory mechanism preventing efficient hydrolysis of the cell wall carbohydrates especially in softwood feedstocks. The negative consequences of non-productive enzyme binding were identified already in the 1980s (Chernoglazov et al., 1988; Sutcliffe & Saddler, 1986), and interest in the topic has been increasing during recent years. The phenomenon is detrimental for process economics, since higher enzyme loadings are required to overcome the inhibitory effect and enzyme recycling is hindered after a completed reaction (Lee et al., 1995).

Three types of interactions have been suggested to mediate enzyme binding onto lignin: hydrophobic (Eriksson et al., 2002), electrostatic (Nakagame et al., 2011b) and hydrogen-bonding interactions (Berlin et al., 2006). To date, hydrophobic interactions have gained most attention and their contribution is well established compared to electrostatic or hydrogen-bonding interactions.

Hydrophobic interactions

Work carried out by Palonen et al. (2004) provides clear indications that hydrophobic interactions are involved in non-productive binding to lignin. The carbohydrate binding modules (CBMs) of *T. reesei* enzymes, Cel7A and Cel5A were shown to significantly increase enzyme binding onto lignin (Palonen et al., 2004). The fungal family-1 CBM contains a flat hydrophobic cellulose-binding face that probably interacts with lignin-rich surfaces. In a study by Börjesson et al. (2007b) *TrCel7B* (EGI) was found to bind more onto isolated lignin than *TrCel7A* (CBHI), although the catalytic modules of both enzymes bound to lignin to a similar degree. The difference in binding of the full-length enzymes was explained by the more hydrophobic character of the *TrCel7B* CBM (Börjesson et al., 2007b).

In general, lignin is more hydrophobic than cellulose, indicated by the lower water contact angle values (WCA) reported for cellulose films (ca. 20°, Eriksson et al., 2007) than for spin-coated lignin films (53–56°, Notley & Norgren, 2010). In polymer

science, 90° is defined as the lowest WCA angle for a hydrophobic surface (Förch et al., 2009). Therefore, it is inaccurate to state that lignin is a hydrophobic polymer, although this claim is widely used in the literature.

Electrostatic interactions

Less data is available concerning electrostatic interactions in lignin-binding, although both the enzymes and the isolated lignins are known to carry charged chemical groups.

In general, enzymes possess multiple amino-acid residues exposed at their surfaces that are, depending on the pH, either negatively or positively charged. Each enzyme has an individual pH (isoelectric point, pI), in which the overall charge of the molecule is zero. Above the pI, the overall charge of the enzyme is negative, whereas below pI the overall charge is positive.

Isolated lignin samples from pretreated lignocellulosics have been shown to contain carboxylic acid, phenolic and aliphatic hydroxyl groups (Berlin et al., 2006). Carboxylic acids are not included in lignin model structures (Fig. 4), but they may originate from the pretreatment or from other residual cell wall polymers (e.g. hemicellulose). Phenolic hydroxyls in lignin model compounds are shown to deprotonate in the pH range 6.2–11.3 depending on the adjacent substitution pattern (Ragnar et al., 2000). Therefore at pH 5, at which enzymatic hydrolysis is usually carried out, carboxylic acid moieties are the only groups that are likely to occur in a deprotonated (charged) state. In a recent study by (Nakagame et al., 2011b), cellulase binding onto lignin was studied at pH 4.8, at which the isolated lignin was found to be negatively charged. The negative charge of isolated lignin increases with increasing deprotonation of the carboxylic and phenolic hydroxyl groups.

Only few studies have addressed the role of electrostatic interactions in enzyme binding to lignin. In a study by Nakagame et al. (2011b), enzyme binding onto isolated lignin was studied at pH 4.8. Those *T. reesei* enzymes that were positively charged at pH 4.8 (e.g. Cel6A and Cel5A) adsorbed more onto the negatively charged lignin compared to the enzymes that were negatively charged in the same conditions (e.g. Cel7A and Cel7B). Recently, acidic groups in lignin (sulphonic and carboxylic acids) were found to reduce non-productive *T. reesei* cellulase adsorption onto lignin at elevated pH values due to increased repulsive electrostatic forces between the enzymes and lignin (Lou et al., 2013). This finding is also shown to have practical relevance; total hydrolysis of various lignocellulosic substrates was found to improve if the hydrolysis was carried out at higher pH than what is generally employed for pure cellulosic substrates (Lan et al., 2012). Interestingly, the enzyme industry appears to have been aware of the role of electrostatic interactions in non-productive binding earlier than the scientific community, as several patents claim that non-productive binding of enzymes to lignin can be decreased without compromising cellulose hydrolysis by introducing more negatively charged amino-acid residues to the enzyme structure (Cascao-Pereira et al., 2009; Lavigne et al., 2010; Scott et al., 2010; Tomashek et al., 2011).

1.4.2.3 Strategies to prevent non-productive adsorption in enzymatic hydrolysis of lignocellulose

Non-productive enzyme binding can be prevented by additives, such as proteins or surface active chemicals that bind to the lignin-rich surfaces and block the binding sites from the enzymes. Pre-incubation of steam pretreated Douglas fir with BSA protein prior to the enzymatic hydrolysis considerably decreased the need for cellulase enzyme (Kumar et al., 2012). The positive effect of BSA addition was also observed in a study by (Yang & Wyman, 2006) employing pretreated agricultural residues. Anionic and non-ionic surfactants (Eriksson et al., 2002; Tu et al., 2007) as well as lignosulphonates (Wang et al., 2013) have also been shown to improve the enzymatic hydrolysis of lignocellulose by a similar mechanism.

The use of “blocking agents” would introduce an additional cost to the process and therefore it has been suggested that low lignin-binding enzymes could be engineered (Berlin et al., 2005), or that lignin could be modified to reduce the unwanted enzyme-lignin interactions (Nakagame et al., 2011c). The biosynthetic pathway of lignin could be modified in plants (Nakagame et al., 2011a) or the specific chemical modifications could be introduced in the pretreatment. Introduction of carboxylic acid (Nakagame et al., 2011a) or sulphonic acid (Kumar et al., 2012) groups to lignin has been suggested to reduce non-productive enzyme adsorption. In a study by Pielhop et al. (2012), hydrolysis of pretreated spruce was improved when additives were used in pretreatment to reduce condensation reactions in lignin. A possible mechanism for the improved hydrolysis was reduced non-productive enzyme adsorption. Despite the research carried out on this topic, more information about the effect of lignin chemistry on non-productive binding is still required.

1.4.2.4 Surface sensitive methods to study enzyme interactions with different lignocellulose components

Real-time detection of enzyme adsorption and enzymatic hydrolysis of cellulose was long restricted by technological constraints. Recently, surface sensitive techniques such as quartz crystal microbalance with dissipation monitoring (QCM-D) (Ahola et al., 2008; Hoeger et al., 2012), ellipsometry (Eriksson et al., 2005), neutron reflectometry (Cheng et al., 2011) and surface plasmon resonance (SPR) (Ma et al., 2008) have been applied to monitor hydrolysis of cellulose thin films as well as enzyme binding onto cellulose or lignin. Undisputedly these techniques have complemented the researchers' toolbox; however, a major downside is that the methods require the use of polymer thin films that represent only a model of native polymer surface.

QCM is probably the most widely employed surface sensitive technique in hydrolysis and enzyme binding studies. A QCM device is a highly sensitive balance. In the QCM technique, a polymer coated quartz sensor oscillates in an electronic field. Loss of mass, such as polymer film degradation, or addition of mass, such as enzyme adsorption onto the film, induces a shift in the oscillation frequency. The

shift in oscillation frequency may be detected real-time and converted to mass units by modelling. In addition, a QCM-D device is capable of energy dissipation monitoring that gives information about the viscoelastic properties of the polymer films. QCM-D has been used to monitor enzymatic degradation of pure cellulosic films (Ahola et al., 2008), cellulose-lignin bicomponent films (Hoeger et al., 2012) and films made of lignocellulosic nanofibrils (Kumagai et al., 2013).

1.4.2.5 Enzymes used in studies addressing non-productive binding

Table 3 lists some key publications concerning lignin-derived inhibition of lignocellulose hydrolysis, with a focus on the role of non-productive enzyme binding onto lignin. *T. reesei* cellulases are most widely used to study cellulase-lignin interactions and lignin-derived inhibition. Most of the studies have been carried out using commercial *T. reesei* cellulase mixtures (Table 3) and only a few studies have exploited monocomponent cellulases from *T. reesei* (Börjesson et al., 2007b; Eriksson et al., 2002; Palonen et al., 2004) or cellulases/hemicellulases from other organisms (Berlin et al., 2005; Berlin et al., 2006; Krogh et al., 2009; Tu et al., 2009; Zhang et al., 2013). Therefore, our current knowledge on the topic is based on a very limited group of enzymes. In addition, the wide use of multicomponent enzyme mixtures instead of monocomponent enzymes has not allowed us to develop in-depth understanding of the molecular-level mechanisms driving enzyme binding onto lignin. Furthermore, research into the role of lignin chemistry on non-productive binding has been limited. For these reasons, work employing monocomponent cellulases and well characterised lignin preparations would provide more precise information on the interactions involved in non-productive enzyme adsorption onto lignin.

Table 3. Key publications concerning the inhibitory effect of lignin and especially the role of non-productive enzyme adsorption.

Key findings	Enzymes	Lignin/lignocellulosic material used	Reference
The presence of CBM in cellulase structure increases enzyme binding to isolated lignins	Monocomponent cellulases Cel7A (CBHI) and Cel5A (EGII) from <i>T. reesei</i> and their corresponding catalytic domains	Isolated lignins from steam pretreated spruce (SO ₂ catalysed). Lignins obtained using three different isolation procedures.	(Palonen et al., 2004)
The use of BSA protein improves enzymatic hydrolysis of lignocellulosic feedstocks by preventing non-productive enzyme adsorption	Celluclast, a commercial <i>T. reesei</i> cellulase mixture	Dilute acid and AFEX pretreated corn stover, steam exploded Douglas fir (SO ₂ catalysed)	(Yang & Wyman, 2006)
Low lignin-binding enzymes exist and <i>T. reesei</i> enzymes are not among the most lignin-tolerant cellulases	Enzyme mixtures from <i>T. reesei</i> and <i>Penicillium sp.</i> , monocomponent endoglucanases from <i>Penicillium sp.</i> and <i>Humicola sp.</i>	Organosolv –pretreated Douglas fir or mixed softwood, isolated lignin from organosolv-pretreated Yellow poplar and Douglas fir	(Berlin et al., 2005)
Isolated lignins from different feedstocks affect cellulase binding and inhibition to a different extent	Spezyme CP, a commercial <i>T. reesei</i> cellulase mixture	Isolated lignin preparations from corn stover, poplar and lodgepole pine obtained using two pretreatment types (steam and organosolv pretreatments) and two lignin isolation procedures	(Nakagame et al., 2010)
Lignin-binding of <i>T. reesei</i> cellulase mixture increases with increasing temperature and decreases with increasing ionic strength	Commercial <i>T. reesei</i> cellulase mixtures and a cellulase mixture from <i>Penicillium sp.</i>	Isolated lignins from organosolv and steam pretreated lodgepole pine	(Tu et al., 2009)

A surface active compound poly(ethylene glycol) binds to lignin and prevents non-productive enzyme adsorption in the hydrolysis. CBM-lignin interaction is mediated through a hydrophobic effect.	Celluclast, commercial <i>T. reesei</i> cellulase mixture and monocomponent <i>T. reesei</i> cellulases Cel7A (CBHI) Cel7B (EGI) and their corresponding core-domains	Steam pretreated spruce and isolated lignin from spruce sawdust	(Börjesson et al., 2007b)
Cellulose accessibility affects the extent of non-productive enzyme binding onto lignin	Celluclast, a commercial cellulase mixture from <i>T. reesei</i>	Steam pretreated Douglas fir and isolated lignin from the lignocellulosic feedstock	(Kumar et al., 2012)
Lignin modification through hydroxypropylation of phenolic hydroxyl in lignin to decrease non-productive enzyme adsorption	Commercial cellulase mixture, organism unknown	Isolated lignins from organosolv pretreated pine, steam pretreated poplar and barley straw	(Sewalt et al., 1997)
Functional groups (carboxylic acids) were suggested to alleviate non-productive enzyme adsorption onto lignin	Spezyme CP, a commercial cellulase mixture from <i>T. reesei</i>	Isolated lignin preparations from corn stover, poplar and lodgepole pine obtained using two pretreatment types (steam and organosolv pretreatments) and two lignin isolation procedures	(Nakagame et al., 2011a)
Electrostatic interactions contribute to non-productive enzyme adsorption to lignin. Increasing pretreatment severity increases non-productive enzyme adsorption to lignin	Spezyme CP, a commercial cellulase mixture from <i>T. reesei</i>	Two isolated lignin preparations from steam pretreated Douglas fir	(Nakagame et al., 2011b)

2. Aims

Cellulase adsorption onto lignin inhibits enzymatic hydrolysis of lignocellulosic feedstocks. Non-productive binding decreases hydrolysis rates and yields and prevents enzyme recycling. The aim of the thesis was to elucidate the interactions involved in non-productive cellulase binding to lignin. This information is crucial when developing technical solutions to prevent non-productive enzyme adsorption.

More specifically the aims were to elucidate the effects of the following factors on non-productive binding:

1. Structural features of enzymes
2. Botanical origin of lignin
3. Biomass pretreatment
4. External factors, such as temperature and pH.

3. Materials and methods

The materials and methods are briefly summarised here. Detailed descriptions of the methods are found in the original publications I–IV.

3.1 Materials

3.1.1 Pretreated lignocellulosic biomass and microcrystalline cellulose

The pretreated lignocellulosic materials, listed in Table 4, were used either for lignin isolation or for total hydrolysis studies. All the listed pretreatments, steam pretreatment, hydrothermal pretreatment and steam explosion employ saturated steam in the biomass treatment with different process configurations and conditions.

Microcrystalline cellulose (MCC) with a 20 μm average particle size was purchased from Serva GmbH (Germany) and used in all the publications (I–IV). MCC is produced from wood pulp with a mild acid hydrolysis treatment.

3. Materials and methods






Table 4. Steam pretreated lignocellulosic materials used for lignin isolation or for hydrolysis studies.

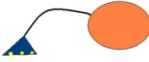


Pretreated biomass	Description of the pretreatment	Use
Steam pretreated Norway spruce	Spruce chip impregnation with gaseous SO ₂ (3 wt-%) followed by steam treatment for 5 min at 215°C. Pretreatment carried out at the Lund University, Sweden. (Stenberg et al., 1998)	Used for EnzHR lignin isolation (Publications I, IV). Used for total hydrolysis (Publication IV)
Steam pretreated Norway spruce	Material impregnation with SO ₂ , followed by steam treatment for 5–7 min at 210°C. Pretreatment carried out at Sekab E-Technology, Sweden.	Used for EnzHR lignin isolation (Publication III)
Hydrothermally pretreated wheat straw	Straw impregnation with 3 g/l acetic acid followed by steam treatment for 12 min at 190°C. Pretreatment carried out at Inbicon A/S, Denmark (Petersen et al., 2009).	Used for EnzHR lignin isolation (Publications III, IV) and for total hydrolysis (Publication IV)
Steam exploded Norway spruce	Steam treatment of air-dried spruce chips at 200°C for 10 min. Treatment terminated with a rapid decompression. Pretreatment carried out at VTT, Finland.	Used for EMAL and EnzHR lignin isolation (Publication II)
Steam exploded wheat straw	Steam treatment of air-dried wheat straw at 200°C for 10 min. Treatment terminated with a rapid decompression. Pretreatment carried out at VTT, Finland.	Used for EMAL and EnzHR lignin isolation (Publication II)

3.1.2 Enzymes

A general overview of the studied monocomponent cellulases is presented in Table 5. In addition, the commercial cellulase mixture Celluclast 1.5L, provided by Novozymes Inc. (Bagsværd, Denmark) was studied in Publication I. According to the manufacturer's product data sheet, Celluclast 1.5L contains proteins secreted by *T. reesei* as well as stabilisers and preservatives. Supplementary β -glucosidase enzyme was added to Celluclast 1.5L from Novozym 188 preparation (Novozymes Inc., Bagsværd, Denmark).

Table 5. Monocomponent cellulases, their modular structures and expression hosts.

Short name	Catalytic domain (activity)	Origin of the CBM	CBM family	Origin of the linker	Production host	Illustration of the domain structure	Used in publication
TrCel7A	<i>T. reesei</i> Cel7A (cellobiohydrolase)	<i>T. reesei</i> Cel7A	1	<i>T. reesei</i> Cel7A	<i>T. reesei</i>		II, IV
TrCel7A-core	<i>T. reesei</i> Cel7A (cellobiohydrolase)	no	no	no	<i>T. reesei</i>		II
TeCel7A-CBM1	<i>T. emersonii</i> Cel7A (cellobiohydrolase)	<i>T. reesei</i> Cel7A	1	27 amino-acid residues from <i>T. reesei</i> Cel7A	<i>S. cerevisiae</i>		IV
TeCel7A-CBM3	<i>T. emersonii</i> Cel7A (cellobiohydrolase)	<i>C. thermocellum</i> CipA	3	27 amino-acid residues from <i>T. reesei</i> Cel7A	<i>S. cerevisiae</i>		IV
MaCel45A	<i>M. albomyces</i> Cel45A (endoglucanase)	no	no	no	<i>T. reesei</i>		III

<i>Ma</i> Cel45A-CBM1	<i>M. albomyces</i> Cel45A (endoglucanase)	<i>T. reesei</i> Cel7A	1	<i>T. reesei</i> Cel7A, full length	<i>T. reesei</i>		III
<i>Ma</i> Cel45A-CBM _{Y32A}	<i>M. albomyces</i> Cel45A (endoglucanase)	<i>T. reesei</i> Cel7A with Y32A mutation	1	<i>T. reesei</i> Cel7A, full length	<i>T. reesei</i>		III
<i>Ma</i> Cel45A-CBM _{Y31W}	<i>M. albomyces</i> Cel45A (endoglucanase)	<i>T. reesei</i> Cel7A with Y31W mutation	1	<i>T. reesei</i> Cel7A, full length	<i>T. reesei</i>		III

3.2 Methods

3.2.1 Lignin isolation

Altogether three approaches were employed in lignin isolation. Lignin-rich enzymatic hydrolysis residues (EnzHR lignins), used in all four publications, were isolated from the pretreated lignocellulosics by extensive enzymatic hydrolysis of polysaccharides, followed by a protease treatment to wash off and degrade lignin-bound proteins (Publication I). A high dosage of Celluclast 1.5L supplemented with Novozym 188 preparation was used for cellulose/hemicellulose degradation. The protease treatment was carried out at pH 9.6 using pure alkaline protease (Subtilisin, Carlsberg) from *Bacillus licheniformis*. The EnzHR lignins were stored at room temperature as dry powders prior to use.

Two-step acid hydrolysis with sulphuric acid was employed in order to isolate an acid hydrolysis residue (AcidHR) from steam pretreated spruce (Publication I). This lignin-rich sample corresponds to the acid insoluble Klason-lignin residue.

Enzymatic mild acidolysis lignins (EMALs) (Guerra et al., 2006) were isolated from non-treated and steam pretreated spruce and wheat straw for the preparation of ultra-thin lignin films (Publication II). The lignocellulosic materials were ball milled into fine powders, followed by enzymatic hydrolysis of polysaccharides. The resulting insoluble residue was extracted with dioxane-water solution and the dissolved material (EMAL lignin) was re-precipitated in acidified water.

3.2.2 Analytical methods for lignocellulose and lignin characterisation

Different analytical methods, listed in Table 6, were employed to characterise biomass composition, visualise microscopic structures and analyse chemical structures of lignin.

3. Materials and methods

Table 6. Analytical methods for the characterisation of bulk lignocellulosic materials and isolated lignins.

Analysis	Short description of the method	Publication
Qualitative and quantitative polysaccharide analysis	Two-step sulphuric acid hydrolysis of structural polysaccharides followed by high-performance anion exchange chromatography (HPAEC) analysis of the solubilised monomeric sugars (Sluiter et al., 2010)	I–IV
Klason-lignin analysis	Gravimetric analysis of the residue remaining after the two-step sulphuric acid hydrolysis combined to a UV-spectroscopic analysis of acid soluble lignin (Sluiter et al., 2010)	II
Nitrogen analysis	Sample combustion and chromatographic analysis of the combustion gases to determine elemental composition (standard ASTM D-5291)	I–IV
Specific surface area analysis	Determination of specific surface area by the Brunauer–Emmett–Teller (BET) method employing N ₂ gas adsorption in different pressures (Brunauer et al., 1938)	I, IV
Microscopic analysis of biomass or lignin structures	Fluorescent microscopy of resin-embedded and sectioned lignin/lignocellulosic samples; visualisation based on lignin autofluorescence	I
Qualitative and quantitative analysis of interunit linkages and S/G ratios in lignin	Analysis of interunit linkages in lignin using a 2D NMR method, ¹ H- ¹³ C HSQC NMR (Sette et al., 2011)	II
Qualitative and quantitative analysis of hydroxyl groups in lignin	Analysis of hydroxyl groups using ³¹ P NMR after selective phosphorylation of hydroxyl groups (Granata & Argyropoulos, 1995)	II
Molecular weight distribution of lignin	Size exclusion chromatography (SEC) of dissolved lignin and detection with UV	II
Charged groups in lignin	Potentiometric titration to analyse charged groups as a function of pH (Laine et al., 1994)	III

3.2.3 Film preparation and analytical methods to study ultrathin lignin films

Ultrathin EMAL lignins films were prepared by spin-coating (Publication II). The lignin films were generated on top of clean, silica-coated QCM-sensors (BiolinScientific/Q-Sense, Sweden). The film properties were characterised using the techniques listed in Table 7.

Table 7. Analytical methods to study the surface properties of spin-coated thin lignin films prepared from the EMAL lignins (Publication II).

Analysis	Short description of the method
Film morphology and thickness by atomic force microscopy (AFM)	Nanometer-scale morphology of the lignin films was analysed by tapping mode. Thickness analysis was carried out by scratching off some of the lignin film and by analysing the depth of the groove with AFM.
Wetting properties analysed by water contact angle analysis (WCA)	A drop of water was placed on the studied surfaces. The angle between the surface and the water droplet was recorded, which is indicative of surface hydrophobicity.
Chemical composition of the film analysed by X-ray photoelectron spectroscopy (XPS)	An X-ray beam was used to excite electrons from the top 1–10 nm of the film. The kinetic energies and number of excited electrons were detected in order to determine the elemental composition quantitatively.

3.2.4 Hydrolysis experiments

The inhibitory role of lignin on the enzymatic hydrolysis of lignocellulose was studied using model systems and technical substrates. In model systems, microcrystalline cellulose was mixed with isolated EnzHR or AcidHR lignins (Publications I, II, IV). In addition, enzymatic hydrolysis of pretreated lignocellulosics was carried out in Publications II and IV. Hydrolysis reactions were followed by assaying reducing sugars (Lever, 1972) or by liquid chromatographic analysis of the solubilised monosaccharides.

3.2.5 Adsorption experiments

Conventional enzyme adsorption experiments were carried out in a suspension of EnzHR lignin or cellulose in microtubes (Publications I, IV) or in 96-well plates (Publication III). Centrifugation was used to separate solids from the liquid fraction. Enzyme distribution to the free (liquid) and bound (solid) fractions was quantified using enzyme activity assays (Publication I), protein assay (Publication I), liquid scintillation counting (Publication III) and SDS-PAGE with band quantification

3. Materials and methods

(Publication IV). When liquid scintillation counting was used, 1:20 of the enzyme was radiolabelled with ^3H -isotope (Publication III).

Quartz crystal microbalance (QCM) technique was used for on-line detection of cellulase adsorption onto ultrathin lignin model surfaces (Publication II). All experiments were carried out in a continuous flow mode, in which the lignin-coated QCM sensor is flushed with an enzyme solution throughout the experiment and enzyme binding to the sensor surface is recorded continuously. The technique is based on oscillation of a piezoelectric quartz crystal within a QCM sensor. Mass addition, such as enzyme adsorption to the sensor surface, is registered as a decrease in the oscillation frequency.

4. Results and discussion

4.1 Lignin isolation and characterisation for adsorption and inhibition studies

Lignin is an integral part of plant cell walls; its chemical structure and spatial orientation in the cell wall may both contribute to the enzyme-lignin interactions in hydrolysis processes. *In situ* study of non-productive enzyme adsorption during enzymatic hydrolysis is challenging due to the lack of research tools to differentiate non-productive adsorption from binding onto cellulose. Therefore, model systems employing isolated lignin preparations and pure cellulosic substrates have been widely exploited in research (Chernoglazov et al., 1988; Kumar et al., 2012; Palonen et al., 2004). Isolation of lignin, as it is present in the lignocellulosic material, is challenging because isolation alters the chemical structure as well as the spatial orientation of the polymer. Special attention was therefore paid in this work to evaluation of the suitability of different lignin preparations for the study.

Altogether seven different lignin preparations from spruce and wheat straw were isolated before or after steam pretreatment. Lignin-rich enzymatic hydrolysis residues (EnzHR) were isolated from steam pretreated spruce (SPS) and from hydrothermally pretreated wheat straw. Lignin-rich acid hydrolysis residue (AcidHR) was isolated from SPS and four enzymatic mild acidolysis lignin (EMAL) preparations were isolated from steam exploded and non-treated spruce and wheat straw (see Table 4 for more information).

Lignin-rich enzymatic hydrolysis residues (EnzHR lignins) were used in all four publications to represent the lignin present in process conditions. Isolation of EnzHR lignin was carried out by enzymatic hydrolysis of cell wall carbohydrates followed by a protease treatment to remove lignin-bound enzymes. Similar isolation protocols are widely documented in the literature (Berlin et al., 2006; Palonen et al., 2004). Spruce EnzHR lignin was shown to retain its spatial orientation in the cell walls upon isolation, indicated by fluorescent microscopy images taken of SPS and of spruce EnzHR lignin, isolated from SPS (Fig. 7). In a microscopic image of spruce EnzHR lignin, clear fragments with plant tissue structure were present (Fig. 7D). The advantage of using EnzHR lignins is that enzymatic isolation is not likely to introduce chemical changes to the lignin structure. However, the major down-

side of using EnzHR lignin is the amount of different contaminants (carbohydrate, protein) in the preparations that may contribute to enzyme binding and therefore to the inhibitory properties (Table 8).

The EnzHR lignins are isolated from pretreated materials and therefore the preparations are also likely to contain so-called pseudo-lignin. Pseudo-lignin is an aromatic material that increases the Klason-lignin value in lignin analytics. Pseudo-lignin is shown to form from carbohydrates during dilute acid pretreatment employing high temperatures and acidic conditions via secondary polymerisation reactions of furfural (Sannigrahi et al., 2011). Recently, pseudo-lignin was shown to bind cellulases and inhibit enzymatic hydrolysis (Hu et al., 2012; Kumar et al., 2013). Therefore, the phenomena studied with EnzHR lignin preparations do not necessarily exclusively originate from the lignin moiety of the preparation.

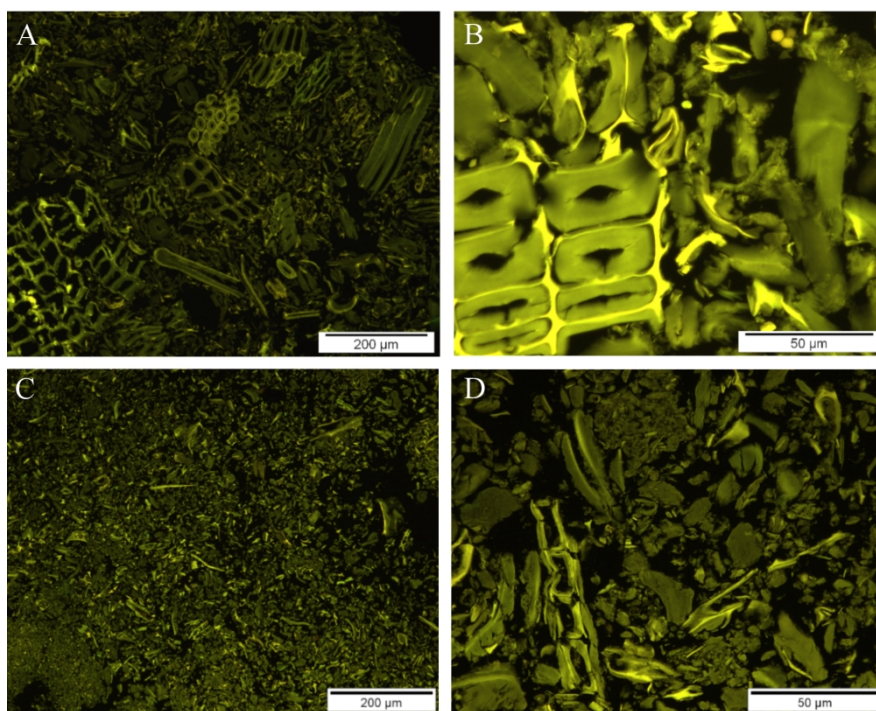


Figure 7. Fluorescent microscopy images of steam pretreated spruce (SPS) A–B and lignin-rich enzymatic hydrolysis residue (EnzHR) isolated from SPS (C–D). Lignin is visualised as yellow due to its autofluorescence. The particles in spruce EnzHR lignin retained structural features of the cell wall (Publication I).

Table 8. Characterisation data of the lignin-rich hydrolysis residues (EnzHR lignins) showing carbohydrate and nitrogen contaminants, charged groups at pH 5 and BET surface area (Publications III and IV).

	Glucose ^a wt-%	Xylose wt-%	Mannose wt-%	Galactose wt-%	Total polysaccharides wt-% ^b	Nitrogen wt-%	Charged groups at pH 5 (mmol/g)	BET surface area (m ² /g)
Spruce EnzHR lignin	7.6±0.0	< 0.1	0.3±0.0	< 0.1	7	0.23	0.15±0.02	3.7±0.1
Wheat straw EnzHR lignin	3.3±0.0	0.4±0.0	< 0.1	0.9±0.1	5	1.47 ^c	0.25±0.11	4.2±0.0

^a quantity of monosaccharides (glucose, xylose, mannose and galactose) presented in hydrous form

^bTotal polysaccharide content is a sum of unhydrous forms of monosaccharides

^cHigh nitrogen content in wheat straw EnzHR is due to the higher nitrogen content in hydrothermally pretreated wheat straw (see Publication IV).

The EnzHR lignins had poor solubility in the solvents used in lignin film preparation and in lignin analytics (Publication II). Therefore, for lignin-film preparation, enzymatic mild acidolysis lignins (EMALs) were isolated from steam exploded and non-treated spruce and wheat straw. The EMALs were extracted with mildly acidic dioxane-water from ball milled and enzymatically digested biomass residue (Wu & Argyropoulos, 2003). The isolated EMAL lignins showed very different spatial orientation in solution compared to the EnzHR lignins. In a confocal microscopy image, taken from a regenerated EMAL lignin sample in aqueous solution, the lignin appeared in μm -scale aggregates (Fig. 8).

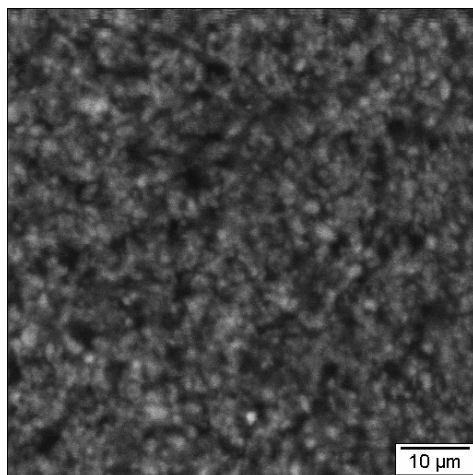


Figure 8. Confocal microscopy image of enzymatic mild acidolysis lignin (EMAL) suspension after precipitation in acidic pH and resuspension in pH 5 buffer. The lignin was extracted from steam exploded, ball milled and enzymatically hydrolysed spruce using mildly acidic dioxane-water (unpublished result).

4.2 Origin of lignin and its effects on non-productive enzyme adsorption and lignin-derived inhibition

4.2.1 Effect of EnzHR lignins isolated from steam pretreated spruce and wheat straw on the hydrolysis of microcrystalline cellulose

The inhibitory effect of lignin on *T. reesei* cellulase mixture (Celluclast) was studied at 45°C by adding EnzHR lignin preparations to the enzymatic hydrolysis of microcrystalline cellulose (MCC) (Fig. 9). Varying concentrations of EnzHR lignins (0–10 mg/ml), isolated from steam pretreated spruce or from hydrothermally pretreated wheat straw were applied to the hydrolysis of MCC. In this experimental set-up, the inhibitory effect of EnzHR lignin probably arises from non-productive enzyme adsorption onto EnzHR lignin particles. The EnzHR lignin and MCC particles

are considered to remain separate in the system and thus inhibition arising from restricted accessibility to cellulose is avoided. Some soluble lignin-derived compounds are released from the EnzHR lignin particles upon hydrolysis; however, the major cellobiohydrolase Cel7A of *T. reesei* was not inhibited by the soluble fragments (data not shown).

Spruce EnzHR lignin was found to have a strong inhibitory effect on enzymatic hydrolysis of MCC, and increase in spruce EnzHR lignin content correlated directly with stronger inhibition (Fig. 9A). Furthermore, the inhibitory effect of spruce EnzHR lignin was more pronounced after 48 hours compared to 4 hours. Interestingly, none of the tested wheat straw EnzHR lignin concentrations inhibited the MCC hydrolysis (Fig. 9B). The stronger inhibitory effect of softwood lignin compared to wheat straw lignin has been reported previously by (Barsberg et al., 2013; Kumar et al., 2012). Clearly, spruce EnzHR lignin and wheat straw EnzHR lignin had widely different surface properties and for this reason they adsorbed and inhibited the *T. reesei* cellulases to a different extent. The differences in surface properties may arise from differences in the pretreatment conditions or from the native differences in wheat straw and spruce lignin structure.

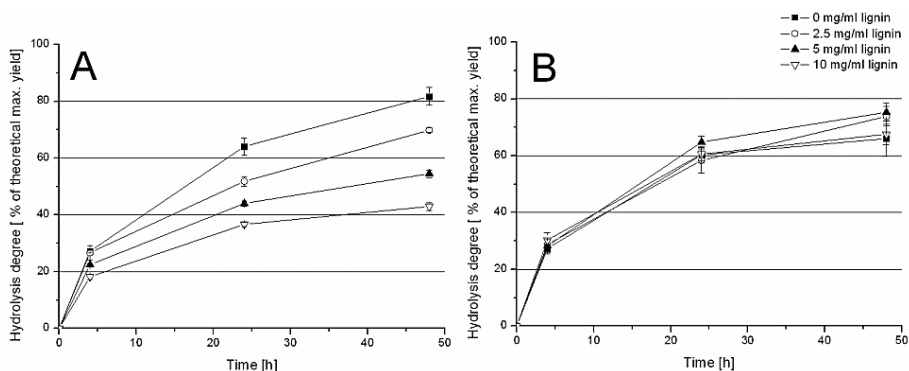


Figure 9. Effect of spruce EnzHR lignin (A, Publication I) and wheat straw EnzHR lignin (B, unpublished result) on the hydrolysis of microcrystalline cellulose at 45°C.

The spruce EnzHR lignin was shown to have an inhibitory effect on cellulolytic enzymes (Fig. 9A). On the other hand, when regenerated EMAL lignin from steam pretreated spruce (Fig. 8) was supplemented in MCC hydrolysis, it did not have any inhibitory effect on cellulolytic enzymes (Publication II). It may be concluded that the isolation procedure has a major effect on the results obtained from enzyme adsorption or inhibition studies. Similar observations have also been done by several other authors (Berlin et al., 2006; Nakagame et al., 2010).

4.2.2 Effects of pretreatment and botanical origin of lignin on non-productive cellulase adsorption

Enzyme adsorption onto lignin model surfaces was studied in order to evaluate the effect of lignin chemistry on non-productive cellulase adsorption. Botanical origin and pretreatment both give rise to differences in lignin structure and their relative contributions to non-productive binding have not been studied previously. Enzymatic mild acidolysis lignins (EMALs) were isolated from wheat straw and spruce before and after non-catalysed steam explosion (SE) pretreatment (10 min, 200°C). The four isolated lignins were used to prepare lignin model surfaces onto silica-coated QCM sensors. AFM images of the homogeneous lignin films, including roughness (R_q) and surface area values (S_{dr}), are presented in Fig. 10. The lignin films prepared from the different EMAL lignin preparations appeared to be similar in terms of surface roughness and surface area and for this reason their comparison in enzyme binding experiments was considered feasible. Interestingly, Figures 10E and 10F show that the films are actually composed of small 10–60 nm lignin aggregates.

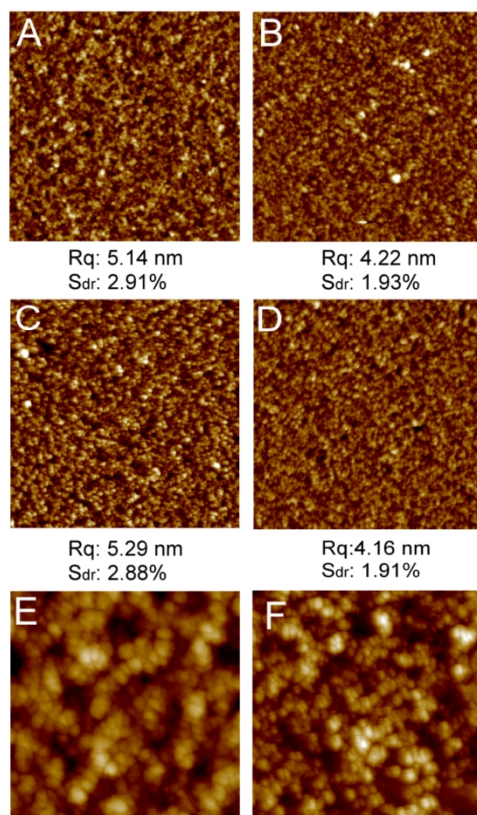


Figure 10. AFM height images of spin coated lignin films: A) Spruce EMAL (5x5 μm), B) Steam explosion pretreated spruce EMAL (5x5 μm), C) Wheat straw EMAL (5x5 μm), D) Steam explosion pretreated wheat straw EMAL (5x5 μm), E) Spruce EMAL (1x1 μm) and F) Wheat straw EMAL (1x1 μm). Surface roughness values (R_q , nm) and ratios of effective surface area and projected surface area (S_{dr} , %) are shown for A–D below each image. Z-scale for all images is 37 nm. (Unpublished result.)

The QCM technique allowed on-line follow-up of *TrCel7A* and *TrCel7A*-core adsorption onto the four different lignin model surfaces (Fig. 11). Interestingly, more *TrCel7A* and *TrCel7A*-core was adsorbed on the SE-pretreated lignin films compared to the non-treated lignin films. The differences in *TrCel7A* and *TrCel7A*-core binding will be discussed later in Section 4.5.1. Similar quantities of both enzymes were adsorbed on the non-treated spruce and wheat straw lignin films, suggesting that botanical differences in lignin chemistry, such as S/G ratio, have only a minor effect on non-productive enzyme binding. Steam explosion pretreatment was found to introduce major changes to lignin chemistry both in wheat straw and spruce substrates. The NMR and SEC analyses of lignin revealed that in the SE

treatment β -O-4 linkages were cleaved, new condensed linkages were formed, the content of phenolic hydroxyls was increased and the content of aliphatic hydroxyl groups was decreased (Publication II). Increased enzyme binding onto the SE-pretreated lignin films may originate from chemical changes in the lignin structure or from the deposition of additional material, such as pseudo-lignin, with the SE-pretreated EMAL lignin samples. Furthermore, ^1H - ^{13}C HSQC NMR could not completely reveal the chemical changes that occurred during SE-treatment, as the abundances of the native inter-unit linkages decreased without concomitant appearance of new signals. Therefore, the role of the structural changes of lignin in non-productive enzyme adsorption is difficult to evaluate and the chemical moieties responsible for increased enzyme binding may only be speculated.

Increasing steam pretreatment severity has been shown to increase non-productive enzyme adsorption onto softwood lignin (Nakagame et al., 2011b), which is in accordance with the reported results. (Sewalt et al., 1997) suggested that phenolic hydroxyl groups in lignin mediate non-productive enzyme binding and other authors have also attempted to detect the lignin structures responsible for non-productive binding (Berlin et al., 2006). However, the effect of structural features in lignin on non-productive adsorption has remained unclear likely because of the complexity of lignin chemistry.

It was previously shown in Section 4.2.1 that wheat straw EnzHR was less inhibitory to *T. reesei* enzymes than spruce EnzHR (Fig. 9). Considering the findings from the QCM studies, the higher inhibitory effect of spruce EnzHR may originate from the pretreatment rather than from the botanical origin of the lignin. As usual, spruce was pretreated in harsher conditions as compared to wheat straw (Table 4). The stronger acid catalyst and higher pretreatment temperature may generate more chemical changes in spruce lignin. These changes may result in more non-productive enzyme binding onto spruce EnzHR lignin compared to that onto wheat straw EnzHR lignin.

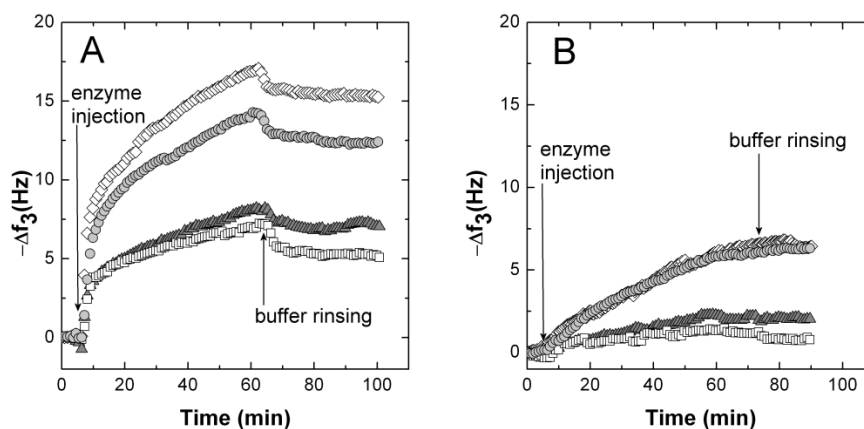


Figure 11. Enzyme binding onto the different lignin model surfaces: \blacktriangle Spruce EMAL lignin, \diamond SE-pretreated spruce EMAL lignin, \square Wheat straw EMAL lignin and \bigcirc SE-pretreated wheat straw EMAL lignin. Continuous injection of cellobiohydrolase (3.9 μ M) A) *TrCel7A* and B) *TrCel7A*-core at 40 °C was employed. Negative shift in QCM frequency, corresponding to mass addition on the lignin film, is shown as a function of time (Publication II).

4.3 Effect of temperature on non-productive cellulase adsorption

4.3.1 Effect of temperature on the activity of lignin-bound enzymes

Protein binding onto solid surface may perturb the protein folding and therefore the effect of binding on enzyme activity was studied. Protein unfolding upon binding is known to occur especially on hydrophobic surfaces and for structurally labile proteins (see Section 1.4.1). Adsorption of commercial *T. reesei* cellulase mixture (Celluclast) onto spruce EnzHR lignin was followed for 48 hours at 45 °C and at 4 °C. Cellobiohydrolase and exoglucanase activities of free and lignin-bound enzymes were measured at three time points using soluble substrates, 4-methylumbelliferyl- β -D-lactoside or hydroxyethyl cellulose, respectively (Fig. 12). At 45 °C, lignin-bound enzymes were found to gradually lose their catalytic activity towards soluble substrates (Fig. 12B and 12D). By contrast, at 4 °C the enzymes retained their full activities throughout the experiment (Fig. 12A and 12B). Loss of enzyme activity indicated that the *T. reesei* cellulases undergo unfolding on the lignin-rich surfaces at the hydrolysis temperature (45 °C) but not at 4 °C. This hypothesis was also supported by an SDS-PAGE analysis in which lignin-bound enzymes could be eluted off from lignin surfaces after binding at 4 °C but not after binding at 45 °C (Publication I). It may be concluded that temperature has an essential role in enzyme-lignin interactions: higher temperature was found to induce enzyme unfolding onto the lignin-rich surface and to increase binding of *T. reesei* cellulases onto spruce EnzHR lignin (Publication I).

4. Results and discussion

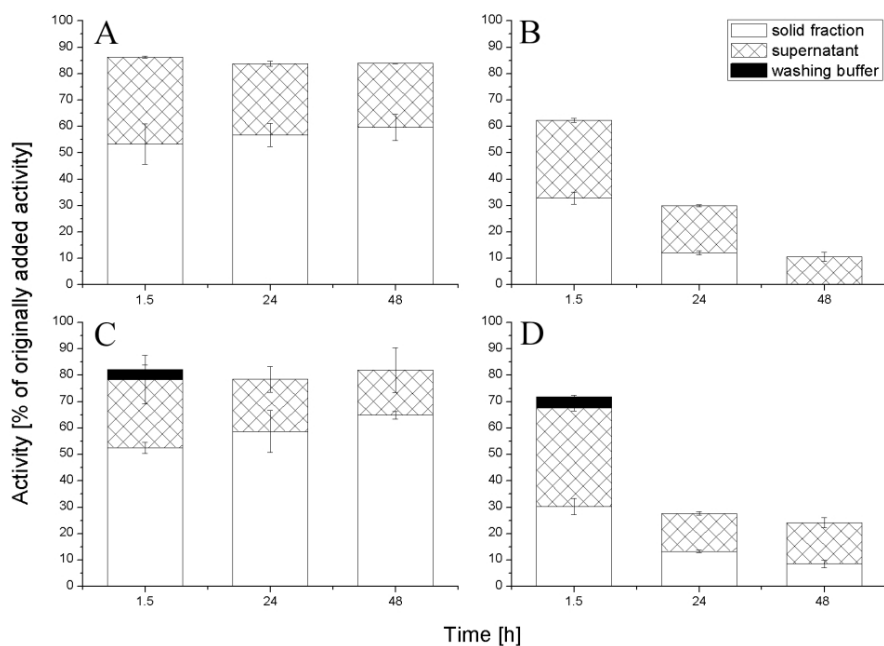


Figure 12. Effect of temperature on the catalytic activities of lignin-bound enzymes. Enzyme activities were measured after adsorption of *T. reesei* cellulase mixture (Celluclast) onto spruce EnzHR lignin. Endoglucanase activities measured after adsorption at A) 4°C or B) 45°C using hydroxyethyl cellulose. Cellobiohydrolase activities measured after adsorption at C) 4°C and at D) 45°C using 4-methylumbelliferyl-β-D-lactoside (Publication I).

4.3.2 Role of temperature in the lignin-derived inhibition of a major cellulase from *T. reesei*

Spruce or wheat straw EnzHR lignin was added to the hydrolysis of MCC either at 45°C (Fig. 13A) or at 55°C (Fig. 13B). The effect of temperature was studied using a monocomponent cellobiohydrolase, *TrCel7A*. If spruce or wheat straw EnzHR lignin was present in the hydrolysis reaction, higher temperature was found to decrease the hydrolysis yield. By contrast, if pure MCC was hydrolysed, increase in temperature was found to increase the hydrolysis yield. At 45°C, addition of wheat straw EnzHR did not result in significant inhibition (Fig. 13A), which is in accordance with the result in Fig. 9B. However, at 55°C addition of wheat straw EnzHR lignin resulted in 50 % lower hydrolysis yield compared to the control reaction without added EnzHR lignin (Fig. 13B). Again, spruce EnzHR lignin was found to be more inhibitory for *TrCel7A*. At 45°C, addition of spruce EnzHR lignin decreased the hydrolysis yield by 37 % (Fig. 13A), whereas at 55°C spruce EnzHR decreased the hydrolysis yield by 73 % (Fig 13B).

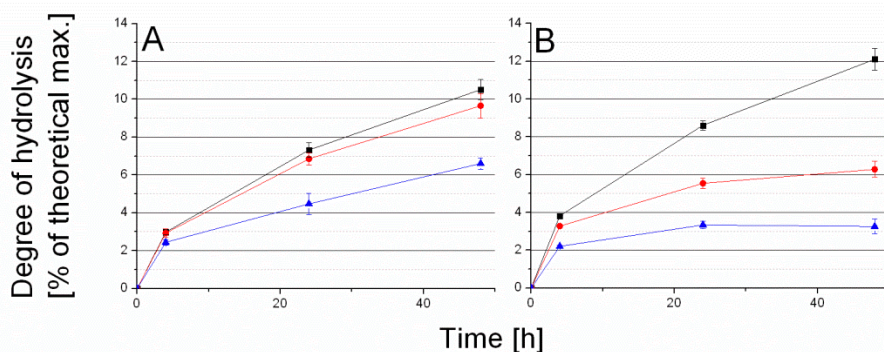


Figure 13. Hydrolysis of microcrystalline cellulose with *TrCel7A* at A) 45 °C and at B) 55 °C. Microcrystalline cellulose (10 mg/ml) (black symbols) was supplemented with 10 mg/ml wheat straw EnzHR lignin (red symbols) or with 10 mg/ml spruce EnzHR lignin (blue symbols). The same data is also presented as part of Figure 14. (Publication IV).

The effect of temperature on hydrolysis yield was also studied with technical lignocellulosic substrates. A cellulase mixture, in which *TrCel7A* represented the majority (64%) of the total protein was employed (Publication IV). Hydrolysis of hydrothermally pretreated wheat straw and steam pretreated spruce was followed at two temperatures and increase in temperature from 45 °C to 55 °C decreased the hydrolysis yields of wheat straw and spruce substrates by 25 % and 50 %, respectively. As noted in the previous section, increase in temperature from 45 °C to 55 °C was shown to increase the hydrolysis yield of pure cellulosic substrate when hydrolysis was carried out using *TrCel7A* with *TaCel3A* β -glucosidase (Fig. 13), and thus it can be concluded that the temperature stability of *TrCel7A* in solution was not limiting the hydrolysis yield at 55 °C. Possibly, the hydrolysis yields of technical substrates were lower at 55 °C due to increased non-productive binding to lignin or due to increased enzyme inactivation on lignin-rich surfaces, although other inhibitory mechanisms may also contribute in such a complex system. Previously, Börjesson et al. (2007a) and Kaar & Holtzapfle (1998) have reported that hydrolyses of technical substrates may be conducted at higher temperatures in the presence of surfactants. In general, surfactants are thought to block lignin-rich surfaces and thus prevent non-productive enzyme adsorption (see Section 1.4.2). Börjesson et al. (2007a) suggested that higher temperature increases enzyme unfolding on the lignin-rich surfaces and that for this reason the positive effect of surfactants on hydrolysis yields is also more pronounced at higher temperatures. Binding of *T. reesei* cellulases onto lignin is found to increase with increasing temperature (Tu et al., 2009). By contrast, adsorption of the major cellobiohydrolases of *T. reesei* onto cellulose is reported to decrease with increasing temperature (Medve et al., 1994). Therefore, increasing temperature may favour cellulase adsorption onto lignin in the total hydrolysis of lignocellulosic substrates.

4.3.3 Comparison of two thermostable fusion enzymes

The role of temperature in lignin-derived inhibition was also studied by using two engineered cellobiohydrolase variants. Both enzymes had a thermostable catalytic module from *Talaromyces emersonii* Cel7A but different CBMs, either from family 3 or from family 1 (see Table 5 for the enzyme structures). The enzymes, *Te*-Cel7A-CBM1 and *Te*-Cel7A-CBM3 were used in MCC hydrolysis at 45°C, 55°C and 65°C in the presence and absence of spruce and wheat straw EnzHR lignins (Fig. 14). As previously seen, the inhibitory effect of lignin was clearly temperature-dependent: the presence of lignin resulted in more inhibition at high temperature than at lower temperature. Interestingly, for the different enzymes, the inhibition was observed at different temperatures. Inhibition of *Te*-Cel7A-CBM3 was observed at 55°C (Fig. 14D), whereas clear inhibition of *Te*-Cel7A-CBM1 was observed only at the highest studied temperature, 65°C (Fig. 14H). Cellobiohydrolase *Tr*-Cel7A had the lowest thermal stability among the studied enzymes and interestingly it was also most susceptible to the presence of lignin at high temperatures (Fig. 14A). Possibly, structurally rigid protein structures may be able to resist the conformational changes at high temperatures that enable formation of strong enzyme-lignin interactions and cause loss of enzyme activity. On the other hand, *Te*-Cel7A-CBM3 and *Te*-Cel7A-CBM1 had the same thermostable core domain, but still *Te*-Cel7A-CBM3 was inhibited at lower temperature compared to *Te*-Cel7A-CBM1. At 45°C, *Te*-Cel7A-CBM3 showed more non-productive adsorption onto lignin than *Te*-Cel7A-CBM1 (Publication IV), which may explain why *Te*-Cel7A-CBM3 was inhibited more compared to *Te*-Cel7A-CBM1 at 55°C and 65°C. However, the extent of non-productive binding does not alone predict the extent of lignin-derived inhibition in hydrolysis. At 45°C, similar amounts of *Tr*-Cel7A and *Te*-Cel7A-CBM3 bound onto spruce EnzHR (Publication IV). However in the hydrolysis experiments, *Tr*-Cel7A was inhibited by spruce EnzHR lignin at 45°C (Fig. 14A), whereas *Te*-Cel7A-CBM3 was not (Fig. 13C). Probably, susceptibility of the enzymes to non-productive adsorption depends on surface properties, such as surface hydrophobicity and surface charge, as well as on structural stability. Very little research has been carried out on the role of enzyme stability for non-productive binding to lignin. (Viikari et al., 2007) reported that inhibition of cellulases on lignin-containing substrates was increased at higher temperatures. Clearly, the effect is more pronounced with *T. reesei* cellulases compared to thermostable cellulases (Viikari et al., 2007), suggesting that better lignin tolerance may be a common feature for thermostable enzymes.

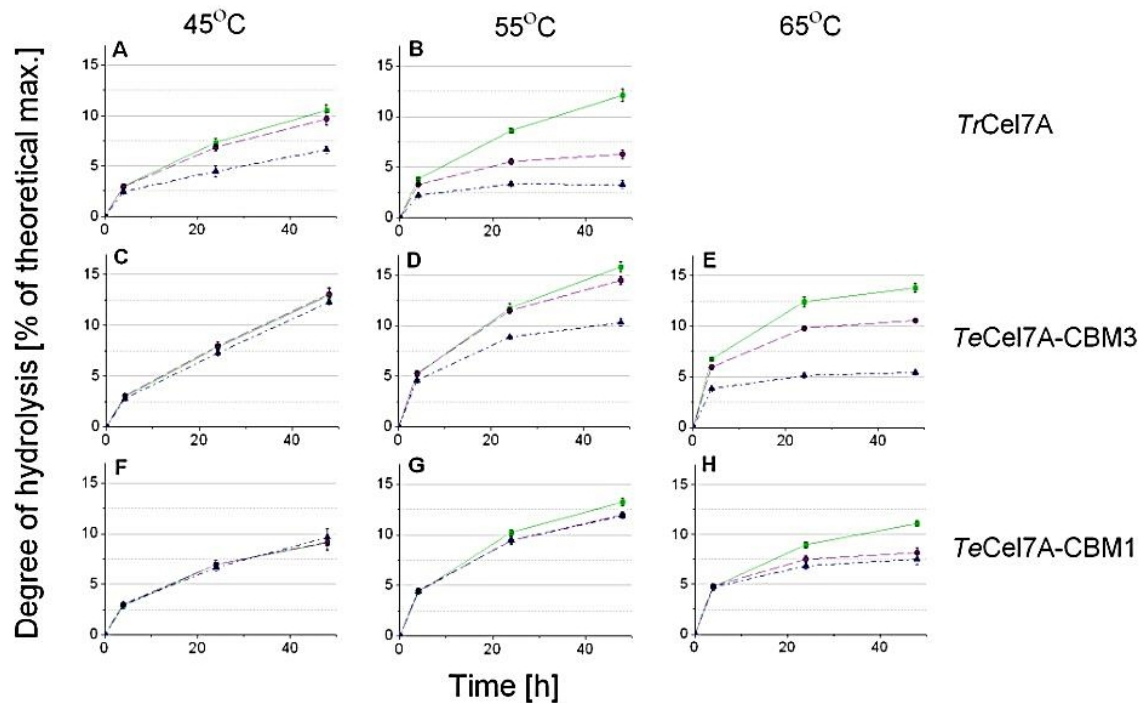


Figure 14. Effect of EnzHR lignin addition on the hydrolysis of microcrystalline cellulose. Hydrolysis with *TrCel7A* at A) 45°C and B) 55°C, with *TeCel7A-CBM3* at C) 45°C, D) 55°C and E) 65°C, and with *TeCel7A-CBM1* at F) 45°, G) 55°C and H) 65°C. Hydrolysis of MCC without added lignin (green symbols), hydrolysis of MCC supplemented with 10 mg/ml of wheat straw EnzHR (red symbols) and hydrolysis of MCC supplemented with 10 mg/ml of spruce EnzHR (blue symbols). (Publication IV).

4.4 Effect of pH on non-productive enzyme adsorption

The effect of pH on cellulase binding onto spruce and wheat straw EnzHR lignins and MCC was studied using *Melanocarpus albomyces* Cel45A (*MaCel45A*) endoglucanase and its fusion with a linker and CBM from *TrCel7A* (*MaCel45A*-CBM). Enzyme binding onto the EnzHR lignins was found to be highly pH-dependent (Fig. 15A and 15B), whereas adsorption onto MCC was similar irrespective of pH (Fig. 15C). At pH 4, ca. 65 % of *MaCel45A* and *MaCel45A*-CBM bound onto spruce EnzHR lignin, whereas at pH 7 only 20 % of *MaCel45A*-CBM and less than 10 % of *MaCel45A* was lignin-bound (Fig. 15A). The pH dependence of enzyme binding onto wheat straw EnzHR lignin was also evident (Fig. 15B), although less binding was observed onto wheat straw EnzHR lignin compared to that onto spruce EnzHR lignin. These findings indicate that electrostatic interactions are involved in enzyme adsorption to the EnzHR lignins, but not in binding to MCC. Both enzymes and EnzHR lignins show increasing negative charge between pH 4 and 7, which probably leads to increasing repulsion at high pH (Publication III).

The contribution of CBM to binding on lignin was found to be pH-dependent. At pH 7, CBM was found to increase *MaCel45A*-CBM binding onto the EnzHR lignins whereas at pH 4 the CBM did not increase binding. The effect was stronger on spruce EnzHR lignin (Fig. 15A) than on wheat straw EnzHR lignin (Fig. 15B). By contrast, CBM was found to increase binding of *MaCel45A* onto MCC at all studied pH values (Fig. 15C). At high pH, binding of the catalytic module onto lignin is probably suppressed by repulsive electrostatic interactions between the module and the lignin surface. However, hydrophobic interactions, introduced by the CBM, may still increase enzyme binding onto lignin. At pH 4, at which the enzyme and lignin charges are close to zero, electrostatic interactions may not play a major role. Instead, hydrophobic interaction may become important and allow binding of the relatively large catalytic core module. Therefore at low pH, the small CBM may not contribute to binding. Clearly, the interactions involved in enzyme binding onto lignin changed with pH, leading to a gradual transition in the adsorption mechanism. A schematic presentation of the hypothesis is shown in Fig. 16.

A few companies have applied patents for cellulases for which non-productive binding is claimed to be reduced after increasing the content of negatively charged residues in the protein structure (see Section 1.4.2). The mechanism of reduced binding may lie in increasing the repulsion between cellulases and the lignin surfaces. Unfortunately, such modifications have not yet been reported in the scientific literature.

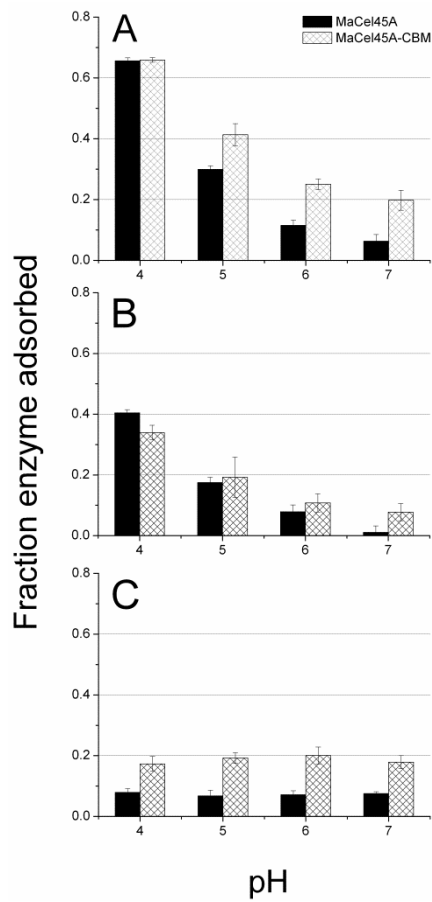


Figure 15. Effect of pH on adsorption of *MaCel45A* (black bars) and *MaCel45A-CBM* (grid bars) onto A) spruce EnzHR lignin, B) wheat straw EnzHR lignin and C) microcrystalline cellulose. The enzymes (13 μM) were incubated with the sorbent (10 mg/ml) for 24 h at 4°C (Publication III).

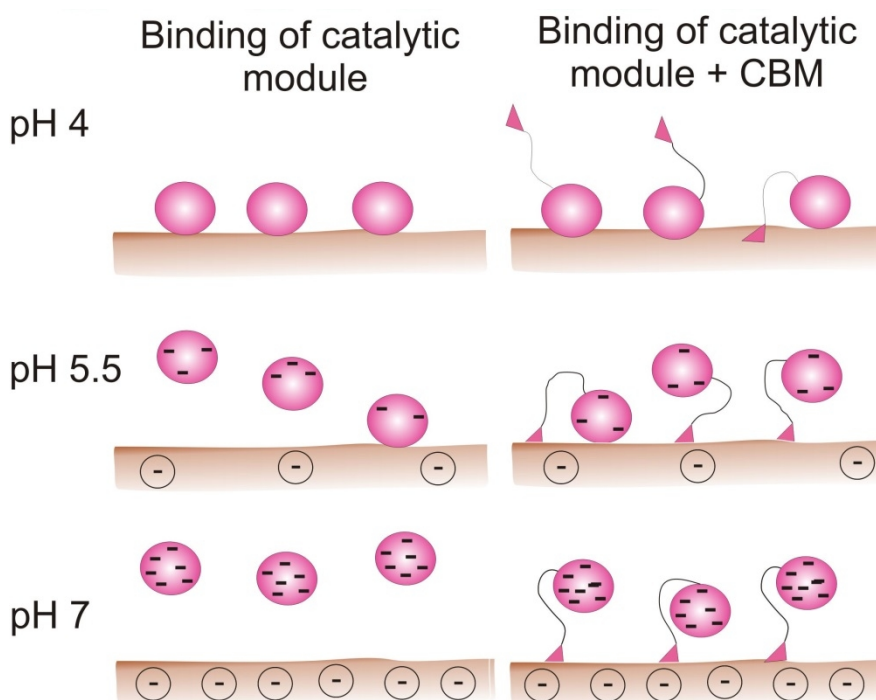


Figure 16. Effect of CBM on lignin binding at different pH conditions. Negative charge of the lignin-rich surface and enzyme surface increases with increasing pH, leading to electrostatic repulsion. At high pH, CBM is hypothesised to increase binding of a bimodular enzyme onto lignin via hydrophobic interactions.

4.5 Effects of modular structure on non-productive enzyme adsorption and inhibition

4.5.1 Role of CBM in non-productive lignin-binding

Palonen et al. (2004) demonstrated with *TrCel7A* (CBHI) and *TrCel5A* (EGII) that their family-1 CBMs increased enzyme binding onto isolated lignins. Consistently, the same was observed here in conventional binding experiments (Fig. 15) and in QCM binding experiments (Fig. 11). Hydrophobic amino-acid residues at the planar face of family-1 CBM are critical for CBM-cellulose interaction, but they are also suggested to mediate binding onto lignin (Börjesson et al., 2007b). The role of tyrosine residues 31 and 32 in the CBM-lignin interaction was explored using *MaCel45A* endoglucanase and its fusions to native and mutated CBMs from *TrCel7A* (see Table 5 for the enzyme structures). The mutations (Y32A and Y31W) were introduced to the aromatic amino-acid residues at the flat binding face of the CBM that is thought to mediate CBM binding onto cellulose. Mutation

of Tyr-32 to a small and non-aromatic alanine residue (Y32A) is shown to retain the CBM structure, but lead to reduced binding of the CBM onto crystalline cellulose (Linder et al., 1995b). Previously mutation Y5W on the flat binding face was shown to significantly increase binding of *TiCel7A* CBM onto crystalline cellulose (Linder et al., 1995a). Therefore, in this study, mutation Y31W was hypothesised to increase CBM binding onto cellulose due to the higher hydrophobicity of tryptophan compared to tyrosine.

Binding of four cellulases, *MaCel45A*, *MaCel45A*-CBM, *MaCel45A*-CBM_{Y32A} and *MaCel45A*-CBM_{Y31W}, onto spruce and wheat straw EnzHR lignins and MCC was studied by constructing adsorption isotherms (Fig. 17). Because the effect of the CBM on enzyme binding to lignin was pH-dependent (Fig. 15), adsorption isotherms were constructed around pH 6, at which the CBM was found to increase enzyme binding to lignin the most. Binding of *MaCel45A*-CBM_{Y32A} onto spruce and wheat straw EnzHR lignin and MCC was lower compared to the binding of *MaCel45A*-CBM with an intact CBM. This indicates that the tyrosine residue at position 32 participated in the binding of both lignin and cellulose. *MaCel45A*-CBM_{Y31W} bound more onto the EnzHR lignins and MCC compared to *MaCel45A*-CBM, indicating that the increased hydrophobicity at position 31 favoured binding onto the EnzHR lignins and MCC. It may be concluded that the same amino-acid residues that are critical for CBM-cellulose interaction also participated in CBM-lignin interaction. Potentially, the aromatic amino-acid residues located on the flat binding face of the CBM may interact with the aromatic rings in lignin by stacking interactions.

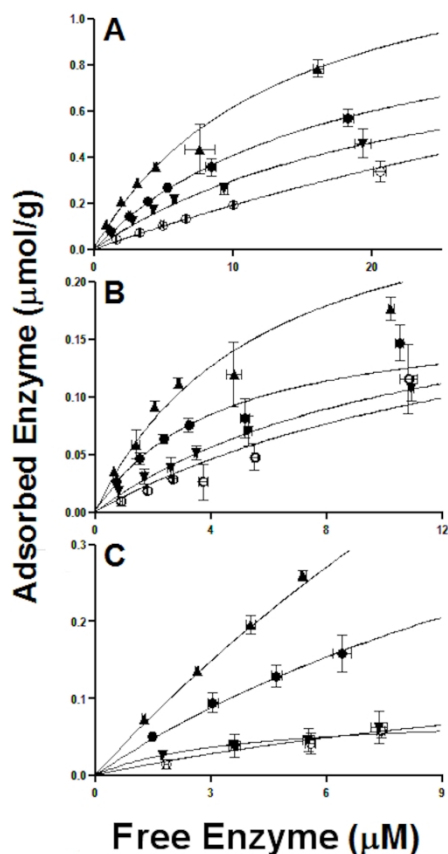


Figure 17. Adsorption isotherms of *MaCel45A* ○, *MaCel45A*-CBM ●, *MaCel45A*-CBM_{Y32A} ▼ and *MaCel45A*-CBM_{Y31W} ▲ on A) spruce EnzHR lignin, B) wheat straw EnzHR lignin and C) microcrystalline cellulose. Adsorption experiments were carried out at 4°C and at pH 5.8, 6.2 and 6 for spruce EnzHR lignin, wheat straw EnzHR lignin and MCC, respectively. One binding-site Langmuir adsorption model (solid lines) was fitted to the binding data (Publication III).

The kinetics of enzyme binding with and without CBM were evaluated when binding of *TrCel7A* and its catalytic domain (*TrCel7A*-core) to lignin model surfaces was followed on-line using QCM (Fig. 11). Binding of *TrCel7A* was clearly a two-phase process, with an initial rapid phase followed by a slower phase (Fig. 11A). Only one slow adsorption phase was observed upon adsorption of the catalytic domain (Fig. 11B), indicating that the CBM was responsible for the rapid adsorption phase observed in *TrCel7A* binding. After about 60 minutes of enzyme binding, the lignin films were rinsed with fresh buffer. Only a minor fraction of the lignin-bound *TrCel7A* and none of the lignin-bound *TrCel7A*-core could be desorbed from the films, indicating that enzyme binding to the lignin films is highly irreversible

in character. The two distinctive phases in *TrCel7A* binding suggested that the mode of adsorption changed with time. Further studies employing complementary methods would be required for a detailed understanding of the exact adsorption mechanism of *TrCel7A*.

4.5.2 Role of the catalytic domain in non-productive enzyme binding to lignin

Binding of *TrCel7A* and *TeCel7A*-CBM1 onto spruce EnzHR lignin was studied at 45°C for 48 h (Fig. 18). Enzyme distribution to free and lignin-bound fractions was quantified using SDS-PAGE and imaging tools. After 4 hours, 73 % of *TeCel7A*-CBM1 remained free in solution (Fig. 18B), whereas only 27 % of *TrCel7A* (Fig. 18A) was free. As previously discussed in Section 4.3.3., *TeCel7A*-CBM1 was considerably less inhibited by the presence of spruce EnzHR lignin in MCC hydrolysis than *TrCel7A* (Fig. 14), which may partially be explained by the low enzyme affinity to spruce EnzHR lignin. *TrCel7A* and *TeCel7A*-CBM1 share similar CBM and linker structures (see Table 5), and thus the properties of the different catalytic modules are probably responsible for the differences in enzyme binding and inhibition. Catalytic modules of *TrCel7A* and *TeCel7A* are 66 % similar in amino acid sequence and *TrCel7A* has 10°C lower thermal stability compared to *TeCel7A* (Voutilainen et al., 2008; Voutilainen et al., 2010).

In Publication I it was suggested that the strength of enzyme-lignin interactions may be detected from a simple SDS-PAGE analysis. Lignin particles carrying lignin-bound enzymes were directly applied to the SDS-PAGE gels and only the loosely bound fraction of enzyme was detached from the particles by electrical current. In Publication I, increases in time of contact and in temperature were found to increase the binding strength between the enzymes and lignin. In the SDS-PAGE analysis of *TrCel7A* and *TeCel7A*-CBM1, band intensities of bound enzymes were found to decrease over time by 39 % and 8 %, respectively (Fig. 18). Decrease in band intensities indicates that both enzymes formed stronger interaction with lignin over time, but that a higher fraction of *TrCel7A* was strongly bound to lignin compared to *TeCel7A*-CBM1. Lower thermal stability of *TrCel7A* may enable higher conformational freedom for the enzyme to develop energetically favourable interactions with surfaces. Therefore, rigid structure may shield enzymes from unfolding onto lignin surfaces. Possibly, non-productive binding and enzyme unfolding on the lignin surface is an interplay between a protein's surface properties and conformational stability.

Native catalytic domains of cellulases have been shown to differ in their binding onto isolated lignins (Berlin et al., 2005; Palonen et al., 2004) and several patent applications describe engineering of low lignin-binding cellulases by introducing negatively charged residues to the enzyme structure (Cascao-Pereira et al., 2009; Lavigne et al., 2010; Scott et al., 2010; Tomashek et al., 2011). Therefore, the use of catalytic modules with good thermal stability could be an alternative way to reduce the inhibitory effect arising from non-productive enzyme adsorption.

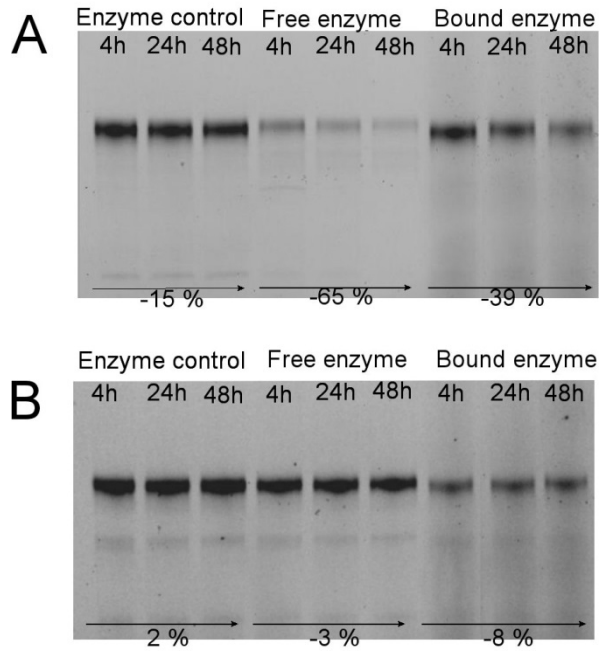


Figure 18. Enzyme binding to spruce EnzHR lignin studied by SDS-PAGE. Binding of *TrCel7A* (A) and *TeCel7A-CBM1* (B) on spruce EnzHR lignin was followed at 45°C and time three time points, 4 h, 24 h and 48 h. Enzyme distribution to free and bound fraction was followed. Time-dependent change (%) in band intensity is shown. Enzyme control samples were incubated in the absence of EnzHR lignin. (Publication IV).

5. Conclusions

The interactions involved in non-productive enzyme adsorption onto lignin have remained unclear, although the importance of the phenomenon is significant in the hydrolysis of lignocellulosic plant biomass. The aim of this thesis was to reveal the mechanisms relating to this undesired phenomenon by using isolated and well-characterised lignin preparations and monocomponent cellulases with different modular structures and temperature stabilities.

The EnzHR lignins, isolated from steam pretreated spruce and hydrothermally pretreated wheat straw, inhibited hydrolysis of microcrystalline cellulose to a different extent. Spruce EnzHR lignin was found to non-productively bind and inhibit the *T. reesei* cellulases more than wheat straw EnzHR lignin. In order to study the effect of lignin chemistry on cellulase adsorption, ultrathin lignin films were prepared from lignin isolated from steam explosion (SE) pretreated and non-treated spruce and wheat straw. More enzyme (*TrCel7A*) bound onto the SE pretreated lignin films than onto the non-treated lignin films, whereas botanical differences in lignin structure (spruce vs. wheat straw) had only a minor effect on enzyme binding. Possibly, the difference in the inhibitory effect of spruce EnzHR lignin and wheat straw EnzHR lignin arise from the pretreatment rather than from the botanical differences in lignin structure. SE pretreatment was found to depolymerise lignin by cleaving β -O-4 linkages, but simultaneous repolymerisation also occurred via formation of condensed structures. Despite serious efforts in chemical analysis, the chemical moieties responsible for the increased binding were not identified. Ideally, the unwanted reactions of lignin could be controlled during pretreatment or new technologies employing milder pretreatment conditions could also suppress unwanted chemical changes in lignin.

Inhibition arising from non-productive adsorption of cellulases was found to increase with increasing temperature. For example, for *TrCel7A*, wheat straw EnzHR lignin was not inhibitory at 45°C in the hydrolysis of microcrystalline cellulose, whereas at 55°C the inhibitory effect was evident. Different enzymes were shown to have a characteristic temperature at which the inhibition emerged. At all studied temperatures, the enzymes with a thermostable core module from *Talaromyces emersonii* Cel7A were shown to be less inhibited by the presence of lignin than *TrCel7A*. Non-productive binding onto lignin is influenced by the surface properties of an enzyme, but possibly also by the stability of the enzyme structure. Further

5. Conclusions

work is needed to generate a more complete picture of the connection between temperature, lignin-binding and enzyme stability.

Temperature was also found to have a strong effect on lignin-bound enzymes. When binding of *T. reesei* cellulases was studied at low temperature (4°C) and at hydrolysis temperature (45°C), the lignin-bound cellulases were found to lose their catalytic activity at the hydrolysis temperature but not at low temperature. In addition, at the hydrolysis temperature, the enzymes developed very strong interactions with the lignin surface. Loss of catalytic activity and increase in binding strength suggest that at the hydrolysis temperature the enzymes unfold on the lignin-rich surfaces.

Carbohydrate binding module (CBM) is known to increase non-productive binding onto lignin, although in this work it was demonstrated that CBMs do not always dominate in non-productive binding. Surface properties of the catalytic core domain were shown to be essential, therefore it is possible to engineer or screen enzymes with desired lignin-binding properties without interfering with the CBM structure. For example, repulsive electrostatic interactions were proposed to reduce non-productive enzyme binding. Negative charges were found to increase in lignin and in the enzymes with increasing pH, and lower enzyme binding onto lignin was therefore observed. By increasing the amount of charged moieties either in the enzyme structure or in lignin, non-productive adsorption could be minimised.

References

- Ahola, S., Turon, X., Österberg, M., Laine, J., Rojas, O.J., 2008. Enzymatic Hydrolysis of Native Cellulose Nanofibrils and Other Cellulose Model Films: Effect of Surface Structure. *Langmuir* 24, 11592–11599.
- Atalla, R.H., 1991. Structural transformations in celluloses, in: Focher, B., Marzetti, A., Crescenzi, V. (Eds.), *Steam Explosion Techniques: Fundamentals and Industrial Applications*. Gordon and Breach Science Publishers, Philadelphia, pp. 97–119.
- Atalla, R.H., Brady, J.W., Matthews, J.F., Ding, S.-Y., Himmel, M.E., 2008. Structures of Plant Cell Wall Celluloses, in: Himmel, M.E. (Ed.), *Biomass Recalcitrance – Deconstructing the Plant Cell Wall for Bioenergy*. Blackwell Publishing, Oxford, pp. 188–212.
- Atalla, R.H., Van der Hart, D.L., 1984. Native cellulose: A composite of two distinct crystalline forms. *Science* 223, 283–285.
- Balakshin, M.Y., Capanema, E.A., Chang, H., 2007. MWL fraction with a high concentration of lignin-carbohydrate linkages: Isolation and 2D NMR spectroscopic analysis. *Holzforschung* 61, 1–7.
- Barsberg, S., Selig, M.J., Felby, C., 2013. Impact of lignins isolated from pretreated lignocelluloses on enzymatic cellulose saccharification. *Biotechnology Letters* 35, 189–195.
- Bayer, E.A., Belaich, J.-P., Shoham, Y., Lamed, R., 2004. The cellulosomes: Multienzyme machines for degradation of plant cell wall polysaccharides. *Annual Review of Microbiology* 58, 521–554.
- Beckham, G.T., Matthews, J.F., Bomble, Y.J., Bu, L., Adney, W.S., Himmel, M.E., Nimlos, M.R., Crowley, M.F. 2010. Identification of amino acids responsible for processivity in a family 1 carbohydrate-binding module from a fungal cellulase. *Journal of Physical Chemistry B* 113, 1447–14353.
- Berlin, A., Balakshin, M., Gilkes, N., Kadla, J., Maximenko, V., Kubo, S., Saddler, J., 2006. Inhibition of cellulase, xylanase and β -glucosidase activities by softwood lignin preparations. *Journal of Biotechnology* 125, 198–209.
- Berlin, A., Gilkes, N., Kurabi, A., Bura, R., Tu, M.B., Kilburn, D., Saddler, J., 2005. Weak lignin-binding enzymes – A novel approach to improve activity of

- cellulases for hydrolysis of lignocellulosics. *Applied Biochemistry and Biotechnology* 121, 163–170.
- Biely, P., Vršanská, M., Tenkanen, M., Kluepfel, D., 1997. Endo- β -1,4-xylanase families: Differences in catalytic properties. *Journal of Biotechnology* 57, 151–166.
- Billsten, P., Wahlgren, M., Arnebrant, T., McGuire, J., Elwing, H., 1995. Structural-Changes of T4 Lysozyme upon Adsorption to Silica Nanoparticles Measured by Circular-Dichroism. *Journal of Colloid and Interface Science* 175, 77–82.
- Björkman, A., 1956. Studies on finely divided wood. Part 1. Extraction of lignin with neutral solvents. *Svensk papperstidning* 59, 477–485.
- Bobleter, O., 1994. Hydrothermal degradation of polymers derived from plants. *Progress in Polymer Science* 19, 797–841.
- Boraston, A.B., Bolam, D.N., Gilbert, H.J., Davies, G.J., 2004. Carbohydrate-binding modules: fine-tuning polysaccharide recognition. *Biochemical Journal* 382, 769–781.
- Broda, P., Birch, P., Brooks, P., Copa-Patino, J.L., Sinnott, M.L., Tempelaars, C., Wang, Q., Wyatt, A., Sims, P., 1994. *Phanerochaete chrysosporium* and its natural substrate. *FEMS Microbiology Reviews* 13, 189–195.
- Brunauer, S., Emmett, P.H., Teller, E., 1938. Adsorption of gases in multimolecular layers. *Journal of the American Chemical Society* 60, 309–319.
- Brunow, G., Lundquist, K., 2010. Functional Groups and Bonding Patterns in Lignin (Including the Lignin-Carbohydrate Complexes), in: Heitner, C., Dimmel, D.R., Schmidt, J.A. (Eds.), *Lignin and Lignans Advances in Chemistry*. CRC Press Taylor & Francis Group, Boca Raton, Florida, pp. 267–299.
- Buranov, A.U., Mazza, G., 2008. Lignin in straw of herbaceous crops. *Industrial Crops and Products* 28, 237–259.
- Börjesson, J., Engqvist, M., Sipos, B., Tjerneld, F., 2007a. Effect of poly(ethylene glycol) on enzymatic hydrolysis and adsorption of cellulase enzymes to pretreated lignocellulose. *Enzyme and Microbial Technology* 41, 186–195.
- Börjesson, J., Peterson, R., Tjerneld, F., 2007b. Enhanced enzymatic conversion of softwood lignocellulose by poly(ethylene glycol) addition. *Enzyme and Microbial Technology* 40, 754–762.

- Cantarel, B.L., Coutinho, P.M., Rancurel, C., Bernard, T., Lombard, V., Henrissat, B., 2009. The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics. *Nucleic Acids Research* 37: D233–D238.
- Carrard, G., Koivula, A., Söderlund, H., Beguin, P., 2000. Cellulose-binding domains promote hydrolysis of different sites on crystalline cellulose. *Proceedings of the National Academy of Sciences of the United States of America* 97, 10342–10347.
- Cascao-Pereira, L., Kaper, T., Kelemen, B.R., Liu, A. Compositions and methods comprising cellulase variants with reduced affinity to non-cellulosic materials. Application no. WO/2009/149202. Publication date 10/12/2009.
- Chandra, R.P., Bura, R., Mabee, W.E., Berlin, A., Pan, X., Saddler, J.N., 2007. Substrate pretreatment: The key to effective enzymatic hydrolysis of lignocellulosics? *Advances in Biochemical Engineering/Biotechnology* 108, 67–93.
- Cheng, G., Liu, Z., Murton, J.K., Jablin, M., Dubey, M., Majewski, J., Halbert, C., Browning, J., Ankner, J., Akgun, B., Wang, C., Esker, A.R., Sale, K.L., Simmons, B.A., Kent, M.S., 2011. Neutron Reflectometry and QCM-D Study of the Interaction of Cellulases with Films of Amorphous Cellulose. *Biomacromolecules* 12, 2216–2224.
- Chernoglazov, V.M., Ermolova, O.V., Klyosov, A.A., 1988. Adsorption of High-Purity Endo-1,4- β -Glucanases from *Trichoderma reesei* on Components of Lignocellulosic Materials – Cellulose, Lignin, and Xylan. *Enzyme and Microbial Technology* 10, 503–507.
- Chua, M.G.S., Wayman, M., 1979. Characterization of autohydrolysis aspen (*P. tremuloides*) lignins. Part 1. Composition and molecular weight distribution of extracted autohydrolysis lignin. *Canadian Journal of Chemistry* 57, 1141–1149.
- Claesson, P.M., Blomberg, E., Fröberg, J.C., Nylander, T., Arnebrant, T., 1995. Protein interactions at solid surfaces. *Advances in Colloid and Interface Science* 57, 161–227.
- Crestini, C., Argyropoulos, D.S., 1997. Structural Analysis of Wheat Straw Lignin by Quantitative ^{31}P and 2D NMR Spectroscopy – The Occurrence of Ester Bonds and α -O-4 Substructures. *Journal of Agricultural and Food Chemistry* 45, 1212–1219.

- Crestini, C., Melone, F., Sette, M., Saladino, R., 2011. Milled wood lignin: A linear oligomer. *Biomacromolecules* 12, 3928–3935.
- Davies, G., Henrissat, B., 1995. Structures and mechanisms of glycosyl hydrolases. *Structure* 3, 853–859.
- Decker, S.R., Siika-Aho, M., Viikari, L., 2008. Enzymatic Depolymerization of Plant Cell Wall Hemicelluloses, in: Himmel, M.E. (Ed.), *Biomass Recalcitrance: Deconstructing the Plant Cell Wall for Bioenergy*. Blackwell Publishing, Oxford, pp. 354–373.
- Dias, M.O.S., Junqueira, T.L., Cavalett, O., Cunha, M.P., Jesus, C.D.F., Rossell, C.E.V., Maciel Filho, R., Bonomi, A., 2012. Integrated versus stand-alone second generation ethanol production from sugarcane bagasse and trash. *Biore-source Technology* 103, 152–161.
- Din, N., Damude, H.G., Gilkes, N.R., Miller Jr., R.C., Warren, R.A.J., Kilburn, D.G., 1994. C1-Cx revisited: Intramolecular synergism in a cellulase. *Proceedings of the National Academy of Sciences of the United States of America*, 91, 11383–11387.
- Divne, C., Ståhlberg, J., Teeri, T.T., Jones, T.A., 1998. High-resolution crystal structures reveal how a cellulose chain is bound in the 50 ångstrom long tunnel of cellobiohydrolase I from *Trichoderma reesei*. *Journal of Molecular Biology* 275, 309–325.
- Donaldson, L., 2007. Cellulose microfibril aggregates and their size variation with cell wall type. *Wood Science and Technology* 41, 443–460.
- Donaldson, L., Hague, J., Snell, R., 2001. Lignin distribution in coppice poplar, linseed and wheat straw. *Holzforschung* 55, 379–385.
- Donaldson, L.A., Wong, K.K.Y., Mackie, K.L., 1988. Ultrastructure of Steam-Exploded Wood. *Wood Science and Technology* 22, 103–114.
- Elwing, H., Welin, S., Askendal, A., Nilsson, U., Lundström, I., 1987. A wettability gradient method for studies of macromolecular interactions at the liquid/solid interface. *Journal of Colloid and Interface Science* 119, 203–210.
- Enari, T.-M., 1983. Microbial Cellulases, in: Fogarty, W.M. (Ed.), *Microbial Enzymes and Biotechnology*. Applied Science Publishers, London and New York, pp. 183–223.

- Eriksson, J., Malmsten, M., Tiberg, F., Callisen, T.H., Damhus, T., Johansen, K.S., 2005. Enzymatic degradation of model cellulose films. *Journal of Colloid and Interface Science* 284, 99–106.
- Eriksson, K.-E., 1975. Enzyme Mechanisms Involved in the Degradation of Wood Components. Symposium on Enzymatic Hydrolysis of Cellulose. Aulanko, Finland. SITRA, Helsinki, pp. 263–280.
- Eriksson, M., Notley, S.M., Wågberg, L., 2007. Cellulose thin films: Degree of cellulose ordering and its influence on adhesion. *Biomacromolecules* 8, 912–919.
- Eriksson, T., Börjesson, J., Tjerneld, F., 2002. Mechanism of surfactant effect in enzymatic hydrolysis of lignocellulose. *Enzyme and Microbial Technology* 31, 353–364.
- Excoffier, G., Peguy, A., Rinaudo, M., Vignon, M.R., 1991. Evolution of lignocellulosic components during steam explosion – Potential applications, in: Focher, B., Marzetti, A., Crescenzi, V. (Eds.), *Steam explosion techniques: Fundamentals and industrial applications*. Gordon and Breach Science Publishers, Philadelphia, pp. 81–95.
- Fengel, D., Wegener, G. 1984. *Wood; Chemistry, Ultrastructure, Reactions*. Walter de Gruyter, Berlin.
- Focher, B., Marzetti, A., Crescenzi, V., 1991. *Steam explosion techniques: Fundamentals and industrial Applications*. Philadelphia, Gordon and Breach Science Publishers.
- Fortman, J.L., Chhabra, S., Mukhopadhyay, A., Chou, H., Lee, T.S., Steen, E., Keasling, J.D., 2008. Biofuel alternatives to ethanol: pumping the microbial well. *Trends in Biotechnology* 26, 375–381.
- Foust, T.D., Ibsen, K.N., Dayton, D.C., Hess, J.R., Kenney, K.E., 2008. The Biorefinery, in: Himmel, M.E. (Ed.), *Biomass Recalcitrance Deconstructing the Plant Cell Wall for Bioenergy*. Blackwell Publishing, Oxford, pp. 7–37.
- Fägerstam, L.G., Pettersson, L.G., 1980. The 1,4-β-D-glucan cellobiohydrolases of *T. reesei* QM 9414. A new type of cellulolytic synergism. *FEBS Letters* 119, 97–100.
- Förch, R., Schönherr, H., Jenkins, T.A., 2009. *Surface design: applications in bioscience and nanotechnology*. First ed. Wiley-VCH, Berlin.

- Gilkes, N.R., Henrissat, B., Kilburn, D.G., Miller Jr., R.C., Warren, R.A.J., 1991. Domains in microbial β -1,4-glycanases: Sequence conservation, function, and enzyme families. *Microbiological Reviews* 55, 303–315.
- Granata, A., Argyropoulos, D.S., 1995. 2-Chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane, a reagent for the accurate determination of the uncondensed and condensed phenolic moieties in lignins. *Journal of Agricultural and Food Chemistry* 43, 1538–1544.
- Guerra, A., Filpponen, I., Lucia, L.A., Saquing, C., Baumberger, S., Argyropoulos, D.S., 2006. Toward a better understanding of the lignin isolation process from wood. *Journal of Agricultural and Food Chemistry* 54, 5939–5947.
- Guerriero, G., Fugelstad, J., Bulone, V. 2010. What Do We Really Know about Cellulose Biosynthesis in Higher Plants? *Journal of Integrative Plant Biology* 52, 161–175.
- Haakana, H., Miettinen-Oinonen, A., Joutsjoki, V., Mäntylä, A., Suominen, P., Vehmaanperä, J., 2004. Cloning of cellulase genes from *Melanocarpus albomyces* and their efficient expression in *Trichoderma reesei*. *Enzyme and Microbial Technology* 34, 159–167.
- Hammel, K.E., Cullen, D., 2008. Role of fungal peroxidases in biological ligninolysis. *Current Opinion in Plant Biology*, 11, 349–355.
- Hansen, M.A.T., Kristensen, J.B., Felby, C., Jørgensen, H., 2011. Pretreatment and enzymatic hydrolysis of wheat straw (*Triticum aestivum* L.) – The impact of lignin relocation and plant tissues on enzymatic accessibility. *Bioresource Technology* 102, 2804–2811.
- Harris, P.J., Stone, B.A., 2008. Chemistry and molecular organization of plant cell walls. In: Himmel, M.E. (Eds.), *Biomass Recalcitrance – Deconstructing the Plant Cell Wall for Bioenergy*. Blackwell Publishing, Oxford, pp. 61–93.
- Harris, P.V., Welner, D., McFarland, K.C., Re, E., Navarro Poulsen, J.-C., Brown, K., Salbo, R., Ding, H., Vlasenko, E., Merino, S., Xu, F., Cherry, J., Larsen, S., Lo Leggio, L., 2010. Stimulation of lignocellulosic biomass hydrolysis by proteins of glycoside hydrolase family 61: Structure and function of a large, enigmatic family. *Biochemistry* 49, 3305–3316.
- Haynes, C.A., Norde, W., 1995. Structures and Stabilities of Adsorbed Proteins. *Journal of colloid and interface science* 169, 313–328.

- Haynes, C.A., Norde, W., 1994. Globular proteins at solid/liquid interfaces. *Colloids and Surfaces B: Biointerfaces* 2, 517–566.
- Henriksson, G., Johansson, G., Pettersson, G., 2000. A critical review of cellobiose dehydrogenases. *Journal of Biotechnology* 78, 93–113.
- Henrissat, B., 1991. A Classification of Glycosyl Hydrolases Based on Amino-Acid-Sequence Similarities. *Biochemical Journal* 280, 309–316.
- 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.
- Hoeger, I., Filpponen, I., Martin-Sampedro, R., Johansson, L.-S., Österberg, M., Laine, J., Kelley, S., Rojas, O.J., 2012. Bicomponent Lignocellulose Thin Films to Study the Role of Surface Lignin in Cellulolytic Reactions. *Biomacromolecules* 13, 3228–3240.
- Hu, F., Jung, S., Ragauskas, A., 2012. Pseudo-lignin formation and its impact on enzymatic hydrolysis. *Bioresource Technology* 117, 7–12.
- Igarashi, K., Koivula, A., Wada, M., Kimura, S., Penttilä, M., Samejima, M., 2009. High speed atomic force microscopy visualizes processive movement of *Trichoderma reesei* cellobiohydrolase I on crystalline cellulose. *Journal of Biological Chemistry* 284, 36186–36190.
- Igarashi, K., Uchihashi, T., Koivula, A., Wada, M., Kimura, S., Okamoto, T., Penttilä, M., Ando, T., Samejima, M., 2011. Traffic jams reduce hydrolytic efficiency of cellulase on cellulose surface. *Science* 333, 1279–1282.
- Igarashi, K., Wada, M., Samejima, M., 2007. Activation of crystalline cellulose to cellulose III results in efficient hydrolysis by cellobiohydrolase. *FEBS Journal* 274, 1785–1792.
- Iiyama, K., Lam Thi Bach Tuyet, Stone, B.A., 1994. Covalent cross-links in the cell wall. *Plant Physiology* 104, 315–320.
- International Energy Agency, 2012. *World Energy Outlook 2012*. OECD/IEA, Paris.
- Kaar, W.E., Holtzapple, M.T., 1998. Benefits from Tween during enzymic hydrolysis of corn stover. *Biotechnology and Bioengineering* 59, 419–427.

- 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*. *Journal of Biotechnology* 99, 63–78.
- Kemppainen, K., Ranta, L., Sipilä, E., Östman, A., Vehmaanperä, J., Puranen, T., Langfelder, K., Hannula, J., Kallioinen, A., Siika-Aho, M., Sipilä, K., von Weymarn, N., 2012. Ethanol and biogas production from waste fibre and fibre sludge – The FibreEtOH concept. *Biomass and Bioenergy* 46, 60–69.
- Kirk, T.K., Farrell, R.L., 1987. Enzymatic "combustion": the microbial degradation of lignin. *Annual Review of Microbiology* 41, 465–505.
- Kleman-Leyer, K.M., Siika-Aho, M., Teeri, T.T., Kirk, T.K., 1996. The cellulases endoglucanase I and cellobiohydrolase II of *Trichoderma reesei* act synergistically to solubilize native cotton cellulose but not to decrease its molecular size. *Applied and Environmental Microbiology* 62, 2883–2887.
- Kolenová, K., Vršanská, M., Biely, P., 2006. Mode of action of endo- β -1,4-xylanases of families 10 and 11 on acidic xylooligosaccharides. *Journal of Biotechnology* 121, 338–345.
- Kraulis, P.J., Clore, G.M., Nilges, M., Jones, T.A., Pettersson, G., Knowles, J., Gronenborn, A.M., 1989. Determination of the 3-Dimensional Solution Structure of the C-Terminal Domain of Cellobiohydrolase I from *Trichoderma reesei* – a Study using Nuclear Magnetic-Resonance and Hybrid Distance Geometry Dynamical Simulated Annealing. *Biochemistry* 28, 7241–7257.
- Kristensen, J.B., Thygesen, L.G., Felby, C., Jørgensen, H., Elder, T., 2008. Cell-wall structural changes in wheat straw pretreated for bioethanol production. *Biotechnology for Biofuels* 1, 5.
- Krogh, K.B.R.M., Kastberg, H., Jørgensen, C.I., Berlin, A., Harris, P.V., Olsson, L., 2009. Cloning of a GH5 endoglucanase from genus *Penicillium* and its binding to different lignins. *Enzyme and Microbial Technology* 44, 359–367.
- Kumagai, A., Lee, S.-E., Endo, T., 2013. Thin Film of Lignocellulosic Nanofibrils with Different Chemical Composition for QCM-D Study. *Biomacromolecules* 14, 2420–2426.
- Kumar, L., Arantes, V., Chandra, R., Saddler, J., 2012. The lignin present in steam pretreated softwood binds enzymes and limits cellulose accessibility. *Biore-source Technology* 103, 201–208.

- Kumar, R., Hu, F., Sannigrahi, P., Jung, S., Ragauskas, A.J., Wyman, C.E., 2013. Carbohydrate derived-pseudo-lignin can retard cellulose biological conversion. *Biotechnology and Bioengineering* 110, 737–753.
- Laine, J., Lövgren, L., Stenius, P., Sjöberg, S., 1994. Potentiometric titration of unbleached kraft cellulose fibre surfaces. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 88, 277–287.
- Lan, T.Q., Lou, H., Zhu, J.Y., 2012. Enzymatic Saccharification of Lignocelluloses Should be Conducted at Elevated pH 5.2–6.2. *Bioenergy Research*, in press, DOI 10.1007/s12155-012-9273-4.
- Larsen, J., Øtergaard Petersen, M., Thirup, L., Li, H.W., Iversen, F.K., 2008. The IBUS process – Lignocellulosic bioethanol close to a commercial reality. *Chemical Engineering and Technology* 31, 765–772.
- Lavigne, J., Scott, B., Whissel, M., Tomashek, J. Family 6 cellulase with decreased inactivation by lignin. Application no. WO/2010/012102. Publication date 04/02/2010.
- Le Costaouëc, T., Pakarinen, A., Várnai, A., Puranen, T., Viikari, L., 2013. The role of carbohydrate binding module (CBM) at high substrate consistency: Comparison of *Trichoderma reesei* and *Thermoascus aurantiacus* Cel7A (CBHI) and Cel5A (EGII). *Bioresource Technology* 143, 196–203.
- Lee, D., Yu, A.H.C., Saddler, J.N., 1995. Evaluation of Cellulase Recycling Strategies for the Hydrolysis of Lignocellulosic Substrates. *Biotechnology and Bioengineering* 45, 328–336.
- Lehtiö, J., Sugiyama, J., Gustavsson, M., Fransson, L., Linder, M., Teeri, T.T., 2003. The binding specificity and affinity determinants of family 1 and family 3 cellulose binding modules. *Proceedings of the National Academy of Sciences of the United States of America* 100, 484–489.
- Levasseur, A., Drula, E., Lombard, V., Coutinho, P.M., Henrissat, B., 2013. Expansion of the enzymatic repertoire of the CAZy database to integrate auxiliary redox enzymes. *Biotechnology for Biofuels* 6, 41.
- Lever, M., 1972. New Reaction for Colorimetric Determination of Carbohydrates. *Analytical Biochemistry* 47, 276–279.

- Li, J., Henriksson, G., Gellerstedt, G., 2007. Lignin depolymerization/repolymerization and its critical role for delignification of aspen wood by steam explosion. *Bioresource Technology* 98, 3061–3068.
- Linder, M., Lindeberg, G., Reinikainen, T., Teeri, T.T., Pettersson, G., 1995a. The difference in affinity between two fungal cellulose-binding domains is dominated by a single amino acid substitution. *FEBS Letters* 372, 96–98.
- Linder, M., Mattinen, M.L., Kontteli, M., Lindeberg, G., Stahlberg, J., Drakenberg, T., Reinikainen, T., Pettersson, G., Annala, A., 1995b. Identification of Functionally Important Amino Acids in the Cellulose-Binding Domain of *Trichoderma reesei* Cellobiohydrolase I. *Protein Science* 4, 1056–1064.
- Linder, M., Teeri, T.T., 1996. The cellulose-binding domain of the major cellobiohydrolase of *Trichoderma reesei* exhibits true reversibility and a high exchange rate on crystalline cellulose. *Proceedings of the National Academy of Sciences of the United States of America* 93, 12251–12255.
- Liu, Y.-S., Baker, J.O., Zeng, Y., Himmel, M.E., Haas, T., Ding, S.Y., 2011. Cellobiohydrolase Hydrolyzes Crystalline Cellulose on Hydrophobic Faces. *Journal of Biological Chemistry* 286, 11195–11201.
- Lou, H., Zhu, J.Y., Lan, T.Q., Lai, H., Qiu, X., 2013. pH-Induced Lignin Surface Modification to Reduce Nonspecific Cellulase Binding and Enhance Enzymatic Saccharification of Lignocelluloses. *ChemSusChem* 6, 919–927.
- Lynd, L.R., Weimer, P.J., van Zyl, W.H., Pretorius, I.S., 2002. Microbial cellulose utilization: Fundamentals and biotechnology. *Microbiology and Molecular Biology Reviews* 66, 506–577.
- Lynd, L.R., Wyman, C.E., Gerngross, T.U., 1999. Biocommodity engineering. *Biotechnology Progress* 15, 777–793.
- Ma, A., Hu, Q., Qu, Q., Bai, Z., Liu, W., Zhuang, G., 2008. The enzymatic hydrolysis rate of cellulose decreases with irreversible adsorption of cellobiohydrolase I. *Enzyme and Microbial Technology* 42, 543–547.
- Martin-Sampedro, R., Capanema, E.A., Hoeger, I., Villar, J.C., Rojas, O.J., 2011. Lignin changes after steam explosion and laccase-mediator treatment of eucalyptus wood chips. *Journal of Agricultural and Food Chemistry* 59, 8761–8769.

- 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.J., Grigoriev, I.V., Harris, P., Jackson, M., Kubicek, C.P., Han, C.S., Ho, I., Larrondo, L.F., deLeon, 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. & Brettin, T.S., 2008. Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. *Hypocrea jecorina*). *Nature Biotechnology* 26, 553–560.
- McGuire, J., Wahlgren, M.C., Arnebrant, T., 1995. Structural Stability Effects on the Adsorption and Dodecyltrimethylammonium Bromide-Mediated Elutability of Bacteriophage T4 Lysozyme at Silica Surfaces. *Journal of Colloid and Interface Science* 170, 182–192.
- Medie, F.M., Davies, G.J., Drancourt, M., Henrissat, B., 2012. Genome analyses highlight the different biological roles of cellulases. *Nature Reviews Microbiology* 10, 227–234.
- 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. *Biotechnology and Bioengineering* 59, 621–634.
- Medve, J., Stahlberg, J., Tjerneld, F., 1994. Adsorption and synergism of cellobiohydrolase I and II of *Trichoderma reesei* during hydrolysis of microcrystalline cellulose. *Biotechnology and Bioengineering* 44, 1064–1073.
- Meshitsuka, G., Lee, Z.Z., Nakano, J., Eda, S., 1982. Studies on the nature of lignin-carbohydrate bonding. *Journal of Wood Chemistry and Technology* 2, 251–267.
- Mooney, C.A., Mansfield, S.D., Touhy, M.G., Saddler, J.N., 1998. The effect of initial pore volume and lignin content on the enzymatic hydrolysis of softwoods. *Bioresource Technology* 64, 113–119.
- Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M., Ladisch, M., 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology* 96, 673–686.

- Nakagame, S., Chandra, R.P., Kadla, J.F., Saddler, J.N., 2011a. Enhancing the enzymatic hydrolysis of lignocellulosic biomass by increasing the carboxylic acid content of the associated lignin. *Biotechnology and Bioengineering* 108, 538–548.
- Nakagame, S., Chandra, R.P., Kadla, J.F., Saddler, J.N., 2011b. The isolation, characterization and effect of lignin isolated from steam pretreated Douglas-fir on the enzymatic hydrolysis of cellulose. *Bioresource Technology* 102, 4507–4517.
- Nakagame, S., Chandra, R.P., Saddler, J.N., 2011c. The Influence of Lignin on the Enzymatic Hydrolysis of Pretreated Biomass Substrates, in: Zhu, J.Y., Zhang, X. & Pan, X.J. (Eds.), *Sustainable Production of Fuels, Chemicals, and Fibers from Forest Biomass*, ACS Symposium Series, Vol. 1067. American Chemical Society, Washington DC, pp. 145–167.
- Nakagame, S., Chandra, R.P., Saddler, J.N., 2010. The Effect of Isolated Lignins, Obtained From a Range of Pretreated Lignocellulosic Substrates, on Enzymatic Hydrolysis. *Biotechnology and Bioengineering* 105, 871–879.
- Nimlos, M.R., Beckham, G.T., Matthews, J.F., Bu, L., Himmel, M.E., Crowley, M.F., 2012. Binding preferences, surface attachment, diffusivity, and orientation of a family 1 carbohydrate-binding module on cellulose. *Journal of Biological Chemistry* 287, 20603–20612.
- Norde, W., 1996. Driving forces for protein adsorption at solid surfaces. *Macromolecular Symposia* 103, 5–18.
- Notley, S.M., Norgren, M., 2010. Surface energy and wettability of spin-coated thin films of lignin isolated from wood. *Langmuir* 26, 5484–5490.
- Palmqvist, E., Hahn-Hägerdal, B., Galbe, M., Zacchi, G., 1996. The effect of water-soluble inhibitors from steam-pretreated willow on enzymatic hydrolysis and ethanol fermentation. *Enzyme and Microbial Technology* 19, 470–476.
- Palonen, H., Tenkanen, M., Linder, M., 1999. Dynamic Interaction of *Trichoderma reesei* Cellobiohydrolases Cel6A and Cel7A at Equilibrium and during Hydrolysis. *Applied and Environmental Microbiology* 65, 5229–5233.
- 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. *Journal of Biotechnology* 107, 65–72.

- Petersen, M.Ø, Larsen, J., Thomsen, M.H., 2009. Optimization of hydrothermal pretreatment of wheat straw for production of bioethanol at low water consumption without addition of chemicals. *Biomass and Bioenergy* 33, 834–840.
- Pielhop, T., Studer, M., von Rohr, P.R., 2012. Use of carbonium ion scavengers in the pretreatment of spruce wood. *Proceedings of the 12th European Workshop on Lignocellulosics and Pulp*. Espoo, Finland, 27.–30.8.2012. pp. 20–23.
- Pollet, A., Schoepe, J., Dornez, E., Strelkov, S.V., Delcour, J.A., Courtin, C.M., 2010. Functional analysis of glycoside hydrolase family 8 xylanases shows narrow but distinct substrate specificities and biotechnological potential. *Applied Microbiology and Biotechnology* 87, 2125–2135.
- Pu, Y., Hu, F., Huang, F., Davison, B.H., Ragauskas, A.J., 2013. Assessing the molecular structure basis for biomass recalcitrance during dilute acid and hydrothermal pretreatments. *Biotechnology for Biofuels* 6, 15.
- Quinlan, R.J., Sweeney, M.D., Lo Leggio, L., Otten, H., Poulsen, J.-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., Walton, P.H., 2011. Insights into the oxidative degradation of cellulose by a copper metalloenzyme that exploits biomass components. *Proceedings of the National Academy of Sciences of the United States of America* 108, 15079–15084.
- Ragnar, M., Lindgren, C.T., Nilvebrant, N., 2000. pKa-values of guaiacyl and syringyl phenols related to lignin. *Journal of Wood Chemistry and Technology* 20, 277–305.
- Reinikainen, T., Ruohonen, L., Nevanen, T., Laaksonen, L., Kraulis, P., Jones, T.A., Knowles, J.K.C., Teeri, T.T., 1992. Investigation of the function of mutated cellulose-binding domains of *Trichoderma reesei* cellobiohydrolase I. *Proteins: Structure, Function and Genetics* 14, 475–482.
- Reinikainen, T., Teleman, O., Teeri T.T., 1995. Effects of pH and High Ionic Strength on the Adsorption and Activity of Native and Mutated Cellobiohydrolase I from *Trichoderma reesei*. *Proteins: Structure, Function and Bioinformatics* 22, 392–403.
- Robert, D., Bardet, D., Gagnaire, D., 1991. Fractionation and structural studies by ¹³C NMR of lignins from steam exploded aspen wood, in: Focher, B., Marzetti, A., Crescenzi, V. (Eds.), *Steam explosion techniques: Funda-*

- mentals and industrial applications. Gordon and Breach Science Publishers, Philadelphia, pp. 121–124.
- Salanti, A., Zoia, L., Tolppa, E., Orlandi, M., 2012. Chromatographic detection of lignin-carbohydrate complexes in annual plants by derivatization in ionic liquid. *Biomacromolecules* 13, 445–454.
- Sannigrahi, P., Kim, D.H., Jung, S., Ragauskas, A., 2011. Pseudo-lignin and pre-treatment chemistry. *Energy and Environmental Science* 4, 1306–1310.
- Schmoll, M., Schuster, A., 2010. Biology and biotechnology of *Trichoderma*. *Applied Microbiology and Biotechnology* 87, 787–799.
- Schüleïn, M., 1997. Enzymatic properties of cellulases from *Humicola insolens*. *Journal of Biotechnology* 57, 71–81.
- Scott, B.R., StPierre, P., Lavigne, J., Masri, N., White, T.C., Tomashek, J.J. Novel lignin-resistant cellulase enzymes. Application no. WO2010096931. Publication date 02/09/2010.
- Sette, M., Wechselberger, R., Crestini, C., 2011. Elucidation of lignin structure by quantitative 2D NMR. *Chemistry – A European Journal* 17, 9529–9535.
- Sewalt, V.J.H., Glasser, W.G., Beauchemin, K.A., 1997. Lignin impact on fiber degradation .3. Reversal of inhibition of enzymatic hydrolysis by chemical modification of lignin and by additives. *Journal of Agricultural and Food Chemistry* 45, 1823–1828.
- Shimizu, K., Sudo, K., Ono, H., Ishihara, M., Fujii, T., Hishiyama, S., 1998. Integrated process for total utilization of wood components by steam-explosion pretreatment. *Biomass and Bioenergy* 14, 195–203.
- Silva, G.G.D., Couturier, M., Berrin, J., Buléon, A., Rouau, X., 2012. Effects of grinding processes on enzymatic degradation of wheat straw. *Biore-source Technology* 103, 192–200.
- Sjöström, E., 1993. *Wood chemistry: fundamentals and applications*, second ed. Academic Press, San Diego, California.
- Sluiter, J.B., Ruiz, R.O., Scarlata, C.J., Sluiter, A.D., Templeton, D.W., 2010. Compositional analysis of lignocellulosic feedstocks. 1. Review and description of methods. *Journal of Agricultural and Food Chemistry* 58, 9043–9053.

- Soimakallio, S., Koponen, K., 2011. How to ensure greenhouse gas emission reductions by increasing the use of biofuels? – Suitability of the European Union sustainability criteria. *Biomass and Bioenergy* 35, 3504–3513.
- Solomon, B.D. 2010. Biofuels and sustainability. *Annals of the New York Academy of Sciences* 1185, 119–134.
- Somerville, C., Youngs, H., Taylor, C., Davis, S.C., Long, S.P., 2010. Feedstocks for lignocellulosic biofuels. *Science* 329, 790–792.
- St John, F.J., González, J.M., Pozharski, E., 2010. Consolidation of glycosyl hydrolase family 30: A dual domain 4/7 hydrolase family consisting of two structurally distinct groups. *FEBS Letters* 584, 4435–4441.
- Stenberg, K., Tengborg, C., Galbe, M., Zacchi, G., 1998. Optimisation of steam pretreatment of SO₂-impregnated mixed softwoods for ethanol production. *Journal of Chemical Technology and Biotechnology* 71, 299–308.
- Sutcliffe, R., Saddler, J.N., 1986. The role of lignin in the adsorption on cellulases during enzymatic treatment of lignocellulosic material, in: Scott, C.D. (Ed.), *Biotechnology and Bioengineering Symposium*, John Wiley & Sons, New York, pp. 749–762.
- Teeri, T.T., 1997. Crystalline cellulose degradation: New insight into the function of cellobiohydrolases. *Trends in Biotechnology* 15, 160–167.
- Tenkanen, M., Makkonen, M., Perttula, M., Viikari, L., Teleman, A., 1997. Action of *Trichoderma reesei* mannanase on galactoglucomannan in pine kraft pulp. *Journal of Biotechnology* 57, 191–204.
- Thomas, C.D., Cameron, A., Green, R.E., Bakkenes, M., Beaumont, L.J., Collingham, Y.C., Erasmus, B.F.N., Ferreira De Siqueira, M., Grainger, A., Hannah, L., Hughes, L., Huntley, B., Van Jaarsveld, A.S., Midgley, G.F., Miles, L., Ortega-Huerta, M.A., Peterson, A.T., Phillips, O.L., Williams, S.E., 2004. Extinction risk from climate change. *Nature* 427, 145–148.
- Tomashek, J.J., Scott, B.R., Kolczynski, D. Carbohydrate binding modules with reduced binding to lignin. Application no. WO/2011/097713. Publication date 18/08/2011.
- Tormo, J., Lamed, R., Chirino, A.J., Morag, E., Bayer, E.A., Shoham, Y., Steitz, T.A., 1996. Crystal structure of a bacterial family-III cellulose-binding domain:

- A general mechanism for attachment to cellulose. *EMBO Journal* 15, 5739–5751.
- Tu, M., Chandra, R.P., Saddler, J.N., 2007. Recycling cellulases during the hydrolysis of steam exploded and ethanol pretreated lodgepole pine. *Biotechnology Progress* 23, 1130–1137.
- Tu, M., Pan, X., Saddler, J.N., 2009. Adsorption of Cellulase on Cellulolytic Enzyme Lignin from Lodgepole Pine. *Journal of Agricultural and Food Chemistry* 57, 7771–7778.
- Tuohy, M.G., Walsh, D.J., Murray, P.G., Claeysens, M., Cuffe, M.M., Savage, A.V., Coughlan, M.P., 2002. Kinetic parameters and mode of action of the cellobiohydrolases produced by *Talaromyces emersonii*. *Biochimica et Biophysica Acta – Protein Structure and Molecular Enzymology* 1596, 366–380.
- Vaaje-Kolstad, G., Westereng, B., Horn, S.J., Liu, Z., Zhai, H., Sørtie, M., Eijsink, V.G.H., 2010. An oxidative enzyme boosting the enzymatic conversion of recalcitrant polysaccharides. *Science* 330, 219–222.
- van Tilbeurgh, H., Tomme, P., Claeysens, M., Bhikhabhai, R., Pettersson, G., 1986. Limited Proteolysis of the Cellobiohydrolase I from *Trichoderma reesei* – Separation of Functional Domains. *FEBS Letters* 204, 223–227.
- Várnai, A., Siika-Aho, M., Viikari, L., 2013. Carbohydrate-binding modules (CBMs) revisited: reduced amount of water counterbalances the need for CBMs. *Biotechnology for Biofuels* 6, 30.
- Várnai, A., Siika-aho, M., Viikari, L., 2010. Restriction of the enzymatic hydrolysis of steam-pretreated spruce by lignin and hemicellulose. *Enzyme and Microbial Technology* 46, 185–193.
- Viikari, L., Alapuranen, M., Puranen, T., Vehmaanperä, J., Siika-Aho, M., 2007. Thermostable enzymes in lignocellulose hydrolysis. *Advances in Biochemical Engineering/Biotechnology* 108, 121–145.
- Voutilainen, S.P., Murray, P.G., Tuohy, M.G., Koivula, A., 2010. Expression of *Talaromyces emersonii* cellobiohydrolase Cel7A in *Saccharomyces cerevisiae* and rational mutagenesis to improve its thermostability and activity. *Protein Engineering, Design and Selection* 23, 69–79.
- Voutilainen, S.P., Puranen, T., Siika-Aho, M., Lappalainen, A., Alapuranen, M., Kallior, J., Hooman, S., Viikari, L., Vehmaanperä, J., Koivula, A., 2008.

- Cloning, expression, and characterization of novel thermostable family 7 cellobiohydrolases. *Biotechnology and Bioengineering* 101, 515–528.
- Vyas, N.K. 1991. Atomic features of protein-carbohydrate interactions. *Current Opinion in Structural Biology* 1, 732–740.
- Wang, Z.J., Lan, T.Q., Zhu, J.Y., 2013. Lignosulfonate and elevated pH can enhance enzymatic saccharification of lignocelluloses. *Biotechnology for Biofuels* 6, 9.
- Watanabe, H., Tokuda, G., 2001. Animal cellulases. *Cellular and Molecular Life Sciences* 58, 1167–1178.
- Weng, J., Chapple, C., 2010. The origin and evolution of lignin biosynthesis. *New Phytologist* 187, 273–285.
- Willför, S., Sundberg, A., Hemming, J., Holmbom, B., 2005a. Polysaccharides in selected industrially important softwood species. 59th Appita Annual Conference and Exhibition, incorporating the 13th ISWFPC: International Symposium on Wood, Fibre and Pulping Chemistry. Auckland, New Zealand, 16–19 May 2005. Proceedings, Appita Inc., Carlton Vic, pp. 415–422.
- Willför, S., Sundberg, A., Pranovich, A., Holmbom, B., 2005b. Polysaccharides in some industrially important hardwood species. *Wood Science and Technology* 39, 601–617.
- Willför, S., Sundberg, K., Tenkanen, M., Holmbom, B., 2008. Spruce-derived mannans – A potential raw material for hydrocolloids and novel advanced natural materials. *Carbohydrate Polymers* 72, 197–210.
- Wilson, D.B., 2011. Microbial diversity of cellulose hydrolysis. *Current Opinion in Microbiology* 14, 259–263.
- Wiman, M., Dienes, D., Hansen, M.A.T., van der Meulen, T., Zacchi, G., Lidén, G., 2012. Cellulose accessibility determines the rate of enzymatic hydrolysis of steam-pretreated spruce. *Bioresource Technology* 126, 208–215.
- Wright, M.M., Brown, R.C., 2007. Comparative economics of biorefineries based on the biochemical and thermochemical platforms. *Biofuels, Bioproducts and Biorefining* 1, 49–56.
- Wu, S., Argyropoulos, D.S., 2003. An improved method for isolating lignin in high yield and purity. *Journal of Pulp and Paper Science* 29, 235–240.

- Ximenes, E., Kim, Y., Mosier, N., Dien, B., Ladisch, M., 2011. Deactivation of cellulases by phenols. *Enzyme and Microbial Technology* 48, 54–60.
- Xu, F., Ding, H., Osborn, D., Tejirian, A., Brown, K., Albano, W., Sheehy, N., Langston, J., 2008. Partition of enzymes between the solvent and insoluble substrate during the hydrolysis of lignocellulose by cellulases. *Journal of molecular catalysis B: Enzymatic* 51, 42–48.
- Yang, B., Wyman, C.E., 2006. BSA treatment to enhance enzymatic hydrolysis of cellulose in lignin containing substrates. *Biotechnology and Bioengineering* 94, 611–617.
- Zhang, J., Moilanen, U., Tang, M., Viikari, L., 2013. The carbohydrate-binding module of xylanase from *Nonomuraea flexuosa* decreases its non-productive adsorption on lignin. *Biotechnology for Biofuels* 6, 18.

Appendices of this publication are not included in the PDF version.

Title	Cellulase-lignin interactions in the enzymatic hydrolysis of lignocellulose
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Abstract	<p>Lignin, a major non-carbohydrate polymer in lignocellulosic plant biomass, restricts the action of hydrolytic enzymes in the enzymatic hydrolysis of lignocellulosic feedstocks. Non-productive enzyme adsorption onto lignin is a major inhibitory mechanism, which results in decreased hydrolysis rates and yields and difficulties in enzyme recycling. The mechanisms of non-productive binding are poorly understood; therefore, in this thesis, enzyme-lignin interactions were studied using isolated lignins from steam pretreated and non-treated spruce and wheat straw as well as monocomponent cellulases with different modular structures and temperature stabilities.</p> <p>The origin of the isolated lignin had an undisputable effect on non-productive binding. Ultrathin lignin films, prepared from steam pretreated and non-treated lignin preparations, were employed in QCM adsorption studies in which <i>Trichoderma reesei</i> Cel7A (<i>TrCel7A</i>) was found to bind more onto lignin isolated from steam pretreated biomass than onto lignin isolated from non-treated lignocellulosic biomass. Botanical differences in lignin chemistry had only a minor effect on non-productive binding when enzyme binding to non-treated wheat straw and spruce lignin was compared.</p> <p>Increase in temperature was found to increase the inhibitory effect arising from non-productive enzyme binding to lignin. Different enzymes were shown to have a characteristic temperature at which the inhibition emerged. Thermostable enzymes were the most lignin-tolerant at high temperatures, suggesting that in addition to the surface properties of an enzyme, non-productive binding onto lignin may be influenced by stability of the enzyme structure. In addition, for lignin-bound <i>T. reesei</i> cellulases, increase in temperature resulted in loss of catalytic activity and tighter binding, suggesting that at high temperature enzyme binding to lignin was probably coupled to conformational changes in the protein folding.</p> <p>With <i>TrCel7A</i>, carbohydrate-binding module (CBM) was found to increase non-productive adsorption to lignin. The <i>Talaromyces emersonii</i> Cel7A catalytic module was linked to a CBM from <i>TrCel7A</i>, giving rise to a fusion enzyme <i>TeCel7A</i>-CBM1. Despite a similar CBM, <i>TeCel7A</i>-CBM adsorbed significantly less to lignin than <i>TrCel7A</i>, indicating that the catalytic module (<i>TeCel7A</i>) had a strong contribution to the low binding. Probably, the contribution of CBM or catalytic core module in non-productive binding varies between different enzymes, and the role of the CBM is not always dominant.</p> <p>To date, very little attention has been paid to the role of electrostatic interactions in lignin-binding. In this work, binding of <i>Melanocarpus albomyces</i> Cel45A endoglucanase onto lignin was found to be very dependent on pH, suggesting that electrostatic interactions were involved in the binding. At high pH, significantly less non-productive binding occurred, probably due to increasing electrostatic repulsion between negatively charged enzymes and lignin. Modification of the charged chemical groups in enzymes or lignin may be a viable strategy to reduce non-productive enzyme binding in the hydrolysis of lignocellulosic substrates.</p>
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Nimeke	Sellulaasi-ligniini-vuorovaikutukset lignoselluloosan entsyymattisessa hydrolyysissä
Tekijä(t)	Jenni Rahikainen
Tiivistelmä	<p>Kasvien lignoselluloosa on vaihtoehtoinen uusiutuva raaka-aine liikennepolttoaineiden sekä erilaisten kemikaalien tuotantoon. Lignoselluloosan biokemiallisella prosessoinnilla pyritään hajottamaan biomassan rakennepolysakkaridit, selluloosa ja hemiselluloosa, entsyymattisesti liukoiksi sokereiksi, joista pystytään esimerkiksi mikrobin avulla tuottamaan haluttuja yhdisteitä. Lignoselluloosa koostuu pääosin rakennepolysakkarideista (selluloosa ja hemiselluloosa) sekä ligniineistä, joka on aromaattinen polymeeri. Ligniinin läsnäolo estää rakennepolysakkarideja hajottavien entsyymien toimintaa useilla mekanismeilla, joista entsyymien epäspesifi sitoutuminen ligniiniin on eräs tärkeimmistä. Entsyymien sitoutuminen ligniiniin heikentää niiden toimintaa sekä rajoittaa entsyymien kierrätettävyyttä. Molekyyllitason mekanismit, jotka mahdollistavat entsyymien sitoutumisen ligniiniin, tunnetaan heikosti. Tämän väitöskirjan tavoitteena oli tutkia entsyymi-ligniini-vuorovaikutuksia käyttäen hyödyksi eristettyjä ligniininäytteitä sekä rakenteeltaan ja lämpöstabiilisuudeltaan erilaisia sellulaasi-entsyymejä. Ligniininäytteet eristettiin joko höyry-esikäsitelystä tai käsittelemättömästä kuusesta tai vehnän korjuutähteestä.</p> <p>Eristetyn ligniinin alkuperällä oli selvä vaikutus sellulaasien sitoutumiseen, kun sitoutumista tutkittiin eri ligniininäytteistä valmistetuilla ohuilla ligniiniakvoilla käyttäen QCM-tekniikkaa. <i>Trichoderma reesei</i> -sellobiohydrolaasi (Cel7A) sitoutui huomattavasti enemmän ligniiniin, joka oli eristetty esikäsitelystä biomassasta kuin ligniiniin, joka eristettiin käsittelemättömästä materiaalista. Vehnän ja kuusen ligniinin kemiallisella erolla oli huomattavasti heikompi vaikutus entsyymien sitoutumiseen.</p> <p>Lämpötilan nousu lisäsi selkeästi ligniinin haitallista vaikutusta entsyymien toimintaan, mutta haitan suuruus riippui tutkittavasta entsyymistä. Lämpötila, jossa ligniinistä johtuva inhibiitio huomattiin, oli kullekin tutkitulle sellulaasientsyymille yksilöllinen. Lämpöstabiilit sellobiohydrolaasientsyymit kestivät paremmin ligniiniä korkeissa lämpötiloissa verrattuna <i>T. reesei</i> Cel7A -sellobiohydrolaasiin. Näin ollen entsyymiproteiinin pinnan ominaisuudet sekä proteiinin rakenteen stabiilisuus saattavat molemmat vaikuttaa entsyymi-ligniini-vuorovaikutukseen. Lisäksi lämpötilan huomattiin vaikuttavan merkittävästi ligniiniin sitoutuneiden <i>T. reesei</i> -entsyymien toimintaan. Korkeassa lämpötilassa sitoutuneet entsyymit menettivät katalyyttistä aktiivisuuttaan sekä sitoutuivat voimakkaammin ligniinin pintaan. Näiden huomioiden perusteella voidaan olettaa, että ligniiniin sitoutuneiden entsyymien rakenne purkautuu korkeassa lämpötilassa.</p> <p><i>T. reesei</i> Cel7A -sellobiohydrolaasin hiilihydraatteihin sitoutuva moduuli (CBM) lisäsi entsyymien adsorptiota ligniiniin. Kun sama CBM liitettiin toiseen, <i>Talaromyces emersonii</i> -homeesta peräisin olevaan sellobiohydrolaasiin, fuusioentsyymi sitoutui huomattavasti vähemmän ligniiniin kuin <i>T. reesei</i> -homeen Cel7A sellobiohydrolaasi. Näin ollen katalyyttisen domeenin vaikutus entsyymi-ligniini-vuorovaikutuksessa voi olla hyvinkin merkittävä, ja aiemmasta tutkimuksesta poiketen voidaan todeta, että CBM:n merkitys entsyymi-ligniini-vuorovaikutuksessa ei ole aina määräävä.</p> <p>Tutkimus elektrostaattisten vuorovaikutusten roolista entsyymi-ligniini-vuorovaikutusten yhteydessä on ollut vähäistä. Työssä huomattiin, että <i>Melanocarpus albomyces</i> Cel45A -endoglukanaasin sitoutuminen ligniiniin on pH-riippuvaista: korkeassa pH:ssa vähemmän entsyymiä sitoutui ligniiniin. pH-Riippuvuuden perusteella elektrostaattiset vuorovaikutukset vaikuttanevat entsyymien sitoutumiseen, ja sitoutumisen väheneminen korkeassa pH:ssa saattaa johtua lisääntyvästä repulsiosta negatiivisesti varautuneiden entsyymien sekä ligniinin välillä. Negatiivisesti varautuvien kemiallisten ryhmien lisääminen entsyymi- tai ligniinirakenteeseen saattaa olla keino vähentää sitoutumista teollisissa prosesseissa.</p>
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Cellulase-lignin interactions in the enzymatic hydrolysis of lignocellulose

Today, the production of transportation fuels and chemicals is heavily dependent on fossil carbon sources, such as oil and natural gas. Their limited availability and the environmental concerns arising from their use have driven the search for renewable alternatives. Lignocellulosic plant biomass is the most abundant, but currently underutilised, renewable carbon-rich resource for fuel and chemical production. Enzymatic degradation of structural polysaccharides in lignocellulose produces soluble carbohydrates that serve as ideal precursors for the production of a vast amount of different chemical compounds. The difficulty in full exploitation of lignocellulose for fuel and chemical production lies in the complex and recalcitrant structure of the raw material. Lignocellulose is mainly composed of structural polysaccharides, cellulose and hemicellulose, but also of lignin, which is an aromatic polymer. Enzymatic degradation of cellulose and hemicellulose is restricted by several substrate- and enzyme-related factors, among which lignin is considered as one of the most problematic issues. Lignin restricts the action of hydrolytic enzymes and enzyme binding onto lignin has been identified as a major inhibitory mechanism preventing efficient hydrolysis of lignocellulosic feedstocks. In this thesis, the interactions between cellulase enzymes and lignin-rich compounds were studied in detail and the findings reported in this work have the potential to help in controlling the harmful cellulase-lignin interactions, and thus improve the biochemical processing route from lignocellulose to fuels and chemicals.

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