ISOLATION AND CHARACTERISATION OF LIGNIN FROM STEAM PRETREATED SPRUCE AND ITS UTILISATION TO STUDY CELLULASE ADSORPTION ON LIGNIN

Jenni Rahikainen

Master's thesis

University of Helsinki

Faculty of Agriculture and Forestry

2009

HELSINGIN YLIOPISTO — HELSINGFORS UNIVERSITET — UNIVERSITY OF HELSINKI

Tiedekunta/Osasto — Fakultet/Sektion — Faculty	Laitos — Institution — Department
Faculty of Agriculture and Forestry	

Tekijä — Författare — Author
Jenni Rahikainen

Työn nimi — Arbetets titel — Title
Isolation and characterisation of lignin from steam pretreated spruce and its utilisation to study cellulase adsorption on lignin

Oppiaine —Läroämne — Subject
Biotechnology

Työn laji — Arbetets art — Level	Aika — Datum — Month and year	Sivumäärä — Sidoantal — Number of pages
M. Sc. thesis	June 2009	90 pages + 3 pages of appendices

Tiivistelmä — Referat — Abstract
Environmental concerns and limited availability of fossil hydrocarbons have boosted the research of renewable feedstocks and their processing into fuels and chemicals. Currently, vast majority of transportation fuels and bulk chemicals are refined from crude oil, but renewable lignocellulosic plant biomass has long been recognised as potential feedstock for liquid fuel and chemical production. Several alternative processes exist for biomass refining; lignocellulose-to-ethanol process being among the most studied processes. First, lignocellulose is pretreated in order to deconstruct the recalcitrant structures of plant cell walls and expose cellulosic fibrils. Subsequently, biotechnical process utilises cellulolytic enzymes of fungal origin to depolymerise cellulose down to glucose monomers and oligomers. Monomeric sugars serve as a source for platform chemicals in further conversions.

Lignocellulose consists mainly of cellulose, hemicellulose and lignin. It is generally accepted that lignin has an inhibitory effect during enzymatic hydrolysis of cellulose and part of this effect is caused by irreversible cellulase adsorption on lignin. Fungal cellulase system consists of several enzyme components that contribute to the effective degradation of insoluble cellulosic substrate. Cellulases are traditionally divided to three groups according to enzymatic activity: exoglucanases, endoglucanases and β-glucosidases. Different enzyme components are shown to have different affinity to lignin which enables screening or engineering of weak lignin-binding enzymes. However, too little is still known about enzyme-lignin interactions and competitive nature of enzyme binding on lignin.

In this study, lignin-rich residues were isolated from steam pretreated spruce (SPS) using three different methods: enzymatic hydrolysis, acid hydrolysis and alkali extraction. Lignin residues were characterized and used in adsorption studies with commercial cellulase preparations from *Trichoderma reesei* (Celluclast 1.5L) and *Aspergillus niger* (Novozym 188). Enzyme activity measurements and protein analytics were employed to reveal competitive adsorption of cellulases and catalytic activity of solid-bound enzymes.

Results showed that *T. reesei* enzymes had high affinity on lignocellulosic SPS and all SPS-derived lignins, but enzyme activity measurements revealed considerably divergent competitive adsorption patterns. Among all the isolated lignins, lignin-rich residue obtained by enzymatic hydrolysis of SPS and subsequent protease purification was evaluated as most suited adsorption substrate for further adsorption studies and screening purposes.

β-glucosidases from *T. reesei* and *A. niger* were shown to have highly distinctive adsorption behaviour on the lignin-rich substrates: *A. niger β*-glucosidase lacked affinity to lignin, whereas *T. reesei* β-glucosidase adsorbed to all lignin-rich particles.

Lignin-bound *Trichoderma reesei* endoglucanases and CBH I exoglucanase were shown to retained high activity towards soluble substrates used in activity measurements. On the contrary, same enzymes were unable to processively hydrolyze insoluble crystalline cellulose.

Avainsanat — Nyckelord — Keywords
cellulase, lignin, inhibition, enzyme adsorption, enzymatic hydrolysis, steam pretreated spruce

Säilytyspaikka — Förvaringsställe — Where deposited
Helda electronic database (http://helda.helsinki.fi/)

Muita tietoja — Övriga uppgifter — Further information

HELSINGIN YLIOPISTO — HELSINGFORS UNIVERSITET — UNIVERSITY OF HELSINKI

Tiedekunta/Osasto — Fakultet/Sektion — Faculty	Laitos — Institution — Department	
Maatalous-metsätieteellinen tiedekunta		
Tekijä — Författare — Author		
Jenni Rahikainen		
Työn nimi — Arbetets titel — Title		
Ligniinin eristys höyryräjäytetystä kuusesta, sen karakterisointi ja käyttö sellulaasien adsorptiotutkimuksessa		
Oppiaine —Läroämne — Subject		
Biotekniikka		
Työn laji — Arbetets art — Level	**Aika — Datum — Month and year**	**Sivumäärä — Sidoantal — Number of pages**
Pro gradu -tutkielma	kesäkuu 2009	90 sivua + 3 sivua liitteitä

Tiivistelmä — Referat — Abstract

Fossiilisten hiilivetyjen käyttö nestemäisten polttoaineiden, kemikaalien ja materiaalien tuotannossa on ympäristön ja materiaalin rajallisen määrän vuoksi kestämätön ratkaisu. Tästä johtuen uusiutuvien luonnonvarojen hyödyntämiseen tähtäävä tutkimus on lisääntynyt merkittävästi viime vuosina. Tällä hetkellä valtaosa liikenteen polttoaineista sekä kemikaaleista tuotetaan raakaöljystä, mutta tulevaisuudessa tuotteita saatetaan jalostaa kasvien tuottamasta lignoselluloosasta. Lignoselluloosan jalostukseen on olemassa useita prosessivaihtoehtoja, joista etanolin tuotanto on yksi tutkituimpia. Prosessissa lignoselluloosan rakenne hajoitetaan esikäsittelyllä, jolloin kasvin soluseinien rakenne aukeaa ja selluloosakuidut työntyvät rakenteesta esille. Bioteknisessä prosessissa selluloosapolymeeri hajotetaan entsymaattisesti sienten selluloosaa hajoittavilla entsyymeillä. Hydrolyysin tuloksena syntyneet glukoosimonomeerit sekä oligomeerit ovat reaktiivisia molekyylejä, jotka voidaan prosessoida esimerkiksi etanoliksi.

Lignoselluloosa koostuu pääosin selluloosasta, hemiselluloosasta ja ligniinistä. Ligniinin tiedetään estävän selluloosan entsymaattista hydrolyysiä, minkä on huomattu johtuvan osin sellulaasien tarttumisesta ligniiniin. Kiinteän selluloosan hajoitukseen tarvitaan erityyppisiä sellulaaseja, jotka hajoittavat synergisesti selluloosapolymeeriä. Nämä entsyymit jaotellaan aktiivisuuden perusteella endoglukanaaseihin, eksoglukanaaseihin sekä β-glukosidaaseihin. Kullakin entsyymillä on yksilöllinen taipumus sitoutua ligniiniin, mikä mahdollistaa ligniiniin heikosti sitoutuvien entsyymien seulonnan tai proteiinimuokkauksen käyttämisen hydrolyysiin soveltuvia entsyymejä kehitettäessä. Ligniini-entsyymi -vuorovaikutuksesta sekä usean sellulaasin kompetitiivisesta adsorptiosta tiedetään kuitenkin melko vähän.

Tässä tutkimuksessa höyryräjäytetystä kuusesta eristettiin ligniinipartikkeleja kolmella eri menetelmällä: entsymaattisella hydrolyysillä, happohydrolyysillä sekä emäskäsittelyllä. Partikkelien ominaisuuksia tutkittiin ja niitä käytettiin substraatteina adsorptiokokeissa. *Trichoderma reesei* (Celluclast 1.5L) sellulaasivalmistetta sekä *Aspergillus niger* (Novozym 188) β-glukosidaasivalmistetta käytettiin sellulaasien sitoutumisominaisuuksien vertailuun. Entsyymiaktiivisuusmäärityksiä sekä proteiinianalytiikkaa käytettiin eri entsyymiryhmien adsorption vertailuun sekä ligniinissä kiinni olevien entsyymien katalyyttisen aktiivisuuden tutkimiseen. Tulokset osoittivat, että *Trichoderma reesei*n entsyymit sitoutuvat huomattavan paljon höyryräjäytettyyn kuuseen sekä siitä eristettyihin ligniinifraktioihin. Entsyymiaktiivisuusmittaukset kuitenkin osoittivat eri aktiivisuuksien sitoutuvan ligniini- ja selluloosapartikkelihin eri määriä. Eri tavoin eristettyjen ligniininäytteiden välillä ei pystytty havaitsemaan merkittäviä eroja entsyymien kiinnittymisessä. Merkittävä ero havaittiin kuitenkin kahden eri alkuperää olevan entsyymivalmisteen välillä: *T. reesei* β-glukosidaasi kiinnittyi ligniinipartikkeleihin huomattavasti enemmän kuin *A. niger β*-glukosidaasi, joka ei adsorboitunut testatuille partikkeleille. Ligniiniin sitoutuneiden *T. reesei*n endoglukanaasien ja eksoglukanaasien aktiivisuus osoittautui säilyvän hyvin immobilisoinnista huolimatta, mutta kiinteän selluloosasubstraatin hydrolyysi oli vaatimatonta ligniiniin sitoutuneilla entsyymeillä. Höyryräjäytetystä kuusesta eristettyjen ligniinipartikkelien hyödyntämistä arvioitiin myös tulevien adsorptiokokeiden kannalta. Entsymaattisella hydrolyysillä eristetyn ja proteaasilla puhdistetun näytteen arvioitiin soveltuvan parhaiten jatkotutkimuksiin.

Avainsanat — Nyckelord — Keywords

sellulaasi, ligniini, inhibitio, entsyymiadsorptio, entsymaattinen hydrolyysi, höyryräjäytetty kuusi

Säilytyspaikka — Förvaringsställe — Where deposited

Helda, elektroninen tutkielmatietokanta (http://helda.helsinki.fi/)

Muita tietoja — Övriga uppgifter — Further information

PREFACE AND ACKNOWLEDGEMENTS

In 2007 I attended a seminar where newly appointed professor Liisa Viikari gave a talk about the biorefinery concept. I found the topic highly fascinating and this single presentation gave me the first push to write my bachelor's thesis about cellulase adsorption on lignocellulose components, as well as focus my remaining studies on biorefinery-related topics. It was a real fluke for me when I got the possibility to continue with the same topic in my master's thesis and acquire hands-on experience in the laboratory.

I was privileged to get excellent guidance during this master's thesis project as the steering group composed of four skilful members: professor Liisa Viikari (University of Helsinki), Ph.D. Kristiina Kruus (VTT Technical Research Centre of Finland), Ph.D. Kaisa Marjamaa (VTT) and Ph.D. Tarja Tamminen (VTT). All members having highly different scientific backgrounds, it was evident that they also had different point of view on the subject. However, I considered it very advantageous for this work and I am grateful for all the guidance I got from them.

This work was financed by two related EU-projects: DISCO (Targeted discovery of novel cellulases and hemicellulases and their reaction mechanisms for hydrolysis of lignocellulosic biomass) and HYPE (High efficiency consolidated bioprocess technology for lignocellulose ethanol). Both, University of Helsinki and VTT are members of these joint projects. Experimental work was carried out during the period of 8 months from May 2008 until January 2009 at VTT. Accordingly, VTT is thanked for providing excellent facilities and working atmosphere.

LIST OF ABBREVIATIONS

BSA	bovine serum albumin
CBH	cellobiohydrolase
CBHI	cellobiohydrolase I (Cel7A)
CBM	carbohydrate binding module
CEL	cellulose enzyme lignin (enzymatic hydrolysis residue)
CEL-P	protease purified enzymatic hydrolysis residue
CEL-U	urea purified enzymatic hydrolysis residue
DNS	dinitrosalicylic acid
FPU	filter paper unit
HEC	hydroxy ethyl cellulose
MWL	milled wood lignin
MUL	4-methylumbelliferyl-β-D-lactoside
nkat	nanokatal
PEG	poly(ethylene glycol)
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPS	steam pretreated spruce
w/v	weight/volume

TABLE OF CONTENTS

INTRODUCTION 8

LITERATURE REVIEW ON LIGNIN, CELLULASES AND LIGNIN-CELLULASE INTERACTIONS 10

1 Wood derived lignin 10

1.1 Localisation of lignin in wood biomass 10

1.2 Lignin chemistry 14

2 Fungal cellulases 19

2.1 Structure and function of fungal cellulases 19

2.2 Classification and nomenclature of glycoside hydrolases 20

2.3 Complete cellulase system of *Trichoderma reesei* 21

3 Protein interactions with surfaces 22

3.1 Introduction to protein adsorption 22

3.2 Adsorption isotherms and models 26

4 Cellulase adsorption on lignin 28

4.1 Inhibitory mechanisms of lignin 28

4.2 Methods and studies of cellulase adsorption on lignin 30

EXPERIMENTAL WORK 36

5 Materials 36

5.1 Enzyme and protein preparations 36

5.2 Adsorption substrates 37

6 Methods 42

6.1 Analytical methods to study adsorption substrates 42

6.2 Adsorption experiments 44

6.3 Analysis of the fractions obtained from adsorption experiments 47

6.4 Total hydrolysis with solid-bound cellulases 54

7 Results 55

7.1 Characterization of adsorption substrates 56

7.2 Adsorption experiments 63

7.3 Activity and hydrolysis capacity of solid-bound *Trichoderma reesei* cellulases 71

8 Discussion 74

8.1 Isolation and characterisation of adsorption substrates 74

8.2 Competitive adsorption of *Trichoderma reesei* cellulases 78

8.3 Comparison of adsorption between *Aspergillus niger* and *Trichoderma reesei* β-glucosidases 79

8.4 BSA adsorption on different adsorption substrates 80

8.5 Enzymatic activities of lignin-bound *T. reesei* endoglucanases and CBHI (Cel7A) exoglucanase 80

8.6 Hydrolysis of crystalline cellulose with solid-bound cellulases 81

9 Conclusions and future prospects 82

10 References 84

11 Appendices 91

INTRODUCTION

Western societies have undergone a major change in their way of life during the past few centuries; self-sufficient rural communities have switched to global networks that enable constant movement of people and goods. Cheap and easily available fossil fuels have powered such development and allowed standard of living to rise. On the contrary, we have become heavily dependent on these hydrocarbons. Carbon-based energy and material industries have also detrimental effects on the environment. Burning of ancient carbon storages has influenced global carbon cycles, which contributes to global warming (IPCC 2007). Accordingly, there is urgent need for technical solutions that would respond to the growing need of energy and preserve the planet for generations to come.

Plant and microbial biomass is the only renewable carbon source that has potential to replace the use of fossil hydrocarbons. Majority of plant biomass is lignocellulose that consists primarily of structural polysaccharides, cellulose and hemicellulose, and aromatic lignin polymer. Men have always exploited lignocellulose as a source of material and energy, but the recalcitrant structure of lignocellulose has hindered its use for more fine refining processes, resulting in liquid fuels and bulk chemicals. Today, several strategies can be employed to refine carbon-based fuels and chemicals from lignocellulose. As one alternative for processing, biotechnology offers enzymatic tools for fractionation of biomass components as well as upgrading biomass components.

Lignocellulose-to-ethanol pathway is among the most studied biotechnological refining concepts. Conversion of structural polysaccharides to ethanol requires deconstruction of plant cell wall structures, depolymerisation of cell wall polymers and subsequent fermentation of carbohydrates to ethanol. Quick survey in scientific database, ISI Web of Science, showed an increasing number of scientific papers published in this field. Search with key words "lignocellulosic ethanol" listed 594 results from 1981 until 2009: 124 of them published in 2008 and 42 of them published in 2009 (prior to 15.4.2009).

Lignocellulosic biomass has to be pretreated first to make the substance fine and amenable for polysaccharide degradation. Subsequently, polysaccharides need to be degraded to fermentable sugars by chemical or enzymatic means. Biotechnical process relies on enzymatic degradation of pretreated biomass, and cellulose degrading enzymes

of fungal origin are most often used to catalyse depolymerisation of cellulose (Jørgensen 2007).

Annual plant and hardwood lignocellulosic feedstocks are favourable for such processing, whereas softwood-derived feedstocks have been called as "worst case scenario substrates" for enzymatic hydrolysis because of their recalcitrant lignin (Lu et al. 2002). Consequently, softwood waste residues are unattractive for hydrolysis from an economic perspective, although these feedstocks are abundant especially in the boreal vegetation zone.

It is generally accepted that lignin content, as well as the origin of the lignocellulosic feedstock, contributes to the efficiency of enzymatic hydrolysis. Lignin is known to interfere with enzymatic hydrolysis of lignocellulosic feedstocks by several mechanisms and irreversible enzyme adsorption on lignin has shown to be a major contributor in the inhibition especially with softwood substrates (Börjesson et al. 2007a). Hydrophobic interactions are thought to drive enzyme binding on lignin surfaces, but quite little is known about competitive adsorption and exact adsorption mechanisms. Accordingly, focus of this work is to study adsorption of fungal cellulases on steam pretreated softwood lignin (SPS).

The first aim was to isolate and characterize lignin-rich residues from SPS by different methods and evaluate their suitability for utilisation in further adsorption studies. The second was to study adsorption of well-characterized *Trichoderma reesei* cellulases on the previously isolated lignin-rich residues. Enzyme activity measurements were used as the main tool to study competitive adsorption of different cellulase activity classes. The third aim was to study activities of the adsorbed enzymes and their capability to hydrolyze solid cellulosic substrate.

LITERATURE REVIEW ON LIGNIN, CELLULASES AND LIGNIN-CELLULASE INTERACTIONS

This literature review is divided into four topics. The first topic reveals the complex nature of lignin polymer and how lignin can be isolated from lignocellulosic matrix. Emphasis is put on softwood type of lignin. The second topic gives a brief summary of fungal cellulases, and well-characterized cellulase system of *Trichoderma reesei* is reviewed in more detail. The third topic has a broad and theoretical approach to protein adsorption, whereas the fourth topic reviews techniques employed in adsorption studies. In addition to research techniques, the fourth topic summarises current knowledge about cellulase-lignin interactions.

1 Wood derived lignin

1.1 Localisation of lignin in wood biomass

Wood biomass is mainly composed of carbohydrates, cellulose and hemicellulose, and lignin, which is a complex aromatic polymer. The lignin content of softwood species is approximately one third of dry weight, whereas hardwood species contain less lignin (Sjöström 1993). Lignin has several physiological purposes in wood structure. The hydrophobic nature of lignin makes cell walls impermeable to water which allows water conduction in wood stems. In addition, lignin is needed to strengthen the tissue that supports the tree, and to hinder pathogenic attacks (Boerjan et al. 2003). The basic anatomy of trees and terminology is covered next.

Growth and anatomy of softwoods

Figure 1 illustrates a cross-section of a Scots pine (*Pinus sylvestris*) trunk. Pith is formed during the first year of growth and it is centrally located in the trunk or branch. The cambial zone consists of a thin layer of living cells (vascular cambium) that divide and produce xylem cells inwards and phloem cells outwards from the layer. Cell division is more rapid towards the centre of the trunk and accordingly, more xylem tissue is produced compared to bark (Sjöström 1993). Right after cell division, cells start to differentiate and they expand to their final dimensions. Secondary cell wall formation oc-

curs and majority of cells undergo programmed cell death (Plomion 2001). Main lignification of xylem cells takes place after the formation of cellulosic structures of the cell walls: water-filled cavities between cellulose fibrils are filled with newly synthesized lignin polymer (Plomion 2001). The above-mentioned layers and cell lignification are also visible in microscopic picture of Norway spruce stem (*Picea abies*) (Figure 2).

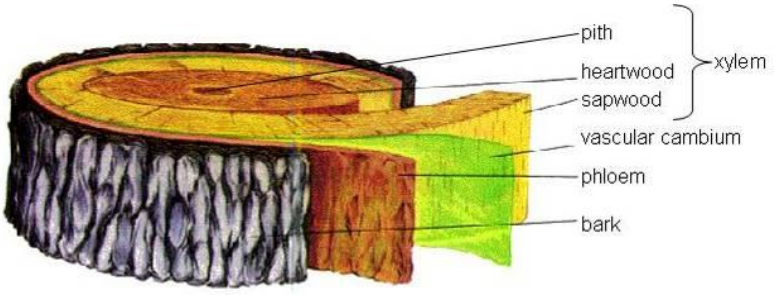

Fig. 1. Cross-section of a pine trunk (picture modified from the web page of Umeå plant science center, http://www.upsc.se/ecophysiology/anders-eriksson-project-page.html)

Cell division in vascular cambium of conifer trees is most rapid during the growth season that lasts from May up to early September in the Northern hemisphere. Light-coloured and porous earlywood is formed during the growth season and dark-coloured latewood appears when the rate of growth decreases. Latewood pine cells are thicker and radially more flat than earlywood cells which makes latewood more dense (Donaldson 1992). The inner part of the tree, designated as heartwood, is usually darker in colour than the outer sapwood, which is still active in water transport.

Seasonal changes trigger latewood and earlywood development, but strain forces are also capable of changing tissue development. Reaction wood is formed to zones that are under compression or tension. Softwood species develop compression wood to areas that are under compression, whereas hardwood species develop tension wood to the tensive ranges. Compression and tension wood differ in composition remarkably. Compression wood in softwood contains more lignin than normally grown wood tissue and lignin is also structurally different. Tissue structures in compression wood are heavier, harder and denser than in normal wood (Fengel & Wegener 1984).

Xylem of softwood species consists of axial system of cells (tracheids) and radial system of cells (ray cells). Figrue 2 is a microscopic picture of spruce stem where both the axial and radial cell systems are visible. The axial system is composed mainly of long, water conducting, tracheid cells with thickened and lignified secondary cell walls. Tra-

cheids are connected through pits and the majority of xylem tissue is composed of tracheids. The radial system of cells in softwoods is composed of living parenchymatous cells and some genera have ray tracheids as well. Cross-section is formed when tracheids and ray cells cross. (Cutler et al. 2008)

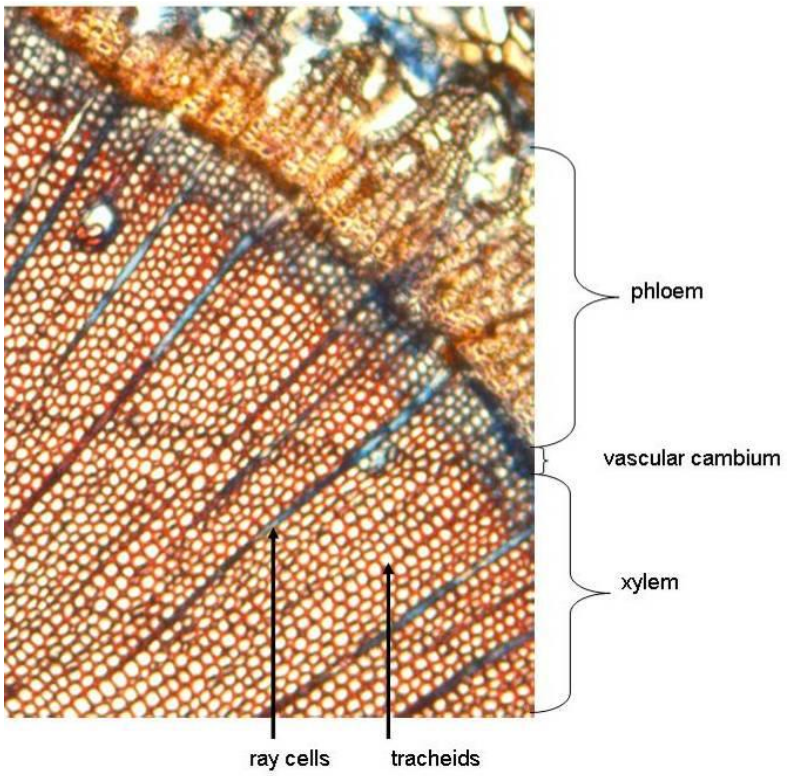

Fig. 2. Microscopic picture (100x final magnification) of Norway spruce stem (cross section). The sample has been stained with Safranin/Alcian Blue. Lignified tracheid cell walls appear red, whereas non-lignified cell walls colour blue. Picture from Kaisa Marjamaa with permission.

Cell structure and localisation of lignin in cell walls

Cell walls of xylem cells are composed of several layers which differ from each other in structure and chemical composition. Layers are visualised in figure 3. The middle lamella (ML) is highly lignified. It connects adjacent cell walls and bundles up tracheids. The primary cell wall (P) is the closest layer to middle lamella and 20 to 25% of total lignin in wood is located in middle lamellae and primary cell walls altogether. The primary cell wall is very thin, 0.1-0.2 µm, and consequently, middle lamella and primary cell wall are often together called as compound middle lamella (Sjöström 1993). Secondary cell wall is composed of three layers: outer (S_1), middle (S_2) and inner (S_3) layer. These layers differ from each other in thickness and orientation of cellulose microfibrils within the layer. Middle layer (S_2) is the thickest layer of all cell wall layers, and in

softwood tracheids its thickness varies between 1 µm (earlywood) and 5 µm (latewood) (Sjöström 1993). The innermost layer is called warty layer (W), which is a thin and amorphous structure found in all softwood cell walls.

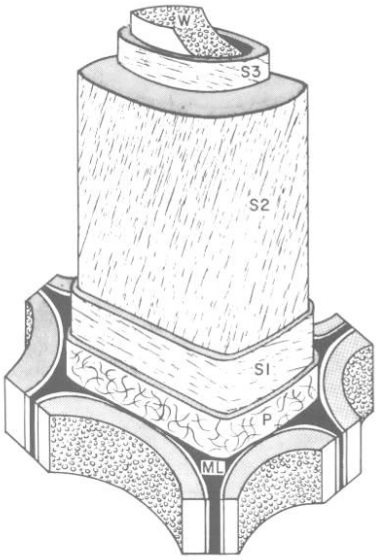

Fig. 3. Construction of a softwood tracheid cell wall. Designated structures are middle lamella (ML), primary cell wall (P), layers of secondary cell wall (S1, S2 and S3) and warty layer (W). Figure is adopted from Côté (1967).

The majority of the total lignin in wood is located in secondary cell walls, even though the matrix is not as highly lignified as in compound middle lamella. A study with black spruce (*Picea mariana*) gave the approximate values of lignin distribution between middle lamella (ML) and secondary cell wall (S) in earlywood and in latewood (Fergus et al. 1969). Results from the study are summarised in table 1. Some cell walls of ray cells are also known to be highly lignified, but the proportion of ray cells in xylem is small (5 to 10%) compared to tracheids (90 to 95%) (Sjöström 1993).

Table 1. Distribution of lignin in black spruce tracheids. Data adopted from Fergus et al. (1969).

Wood	Part of the cell wall	% of total lignin in the cell wall
Earlywood	Middle lamella	28
	Secondary cell wall	72
Latewood	Middle lamella	18
	Secondary cell wall	82

1.2 Lignin chemistry

Lignin is a hydrophobic biopolymer with low solubility to common solvents in its native form. The chemical structure is not uniform, thus it lacks precise chemical composition and repetitive polymer pattern. The chemical structure of lignin varies remarkably between plant species. Structural variation has also been found among wood tissues and even cell wall layers of one tree/plant species (Fengel & Wegener 1984; Sjöström 1993).

Lignin precursors and polymerisation

Lignins are composed of phenylpropane units that polymerize to form a netlike structure. Three different phenylpropane molecules are known to function as the major precursors for lignin polymers: *p*-coumaryl alcohol (pCA), coniferyl alcohol (CA) and sinapyl alcohol (SA). Chemical structures of the precursors are presented in figure 4. These molecules give rise to *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units after polymerisation which occurs by radical coupling. Trace amounts of other phenolic precursors can also be incorporated to lignin structures (Ralph et al. 2004).

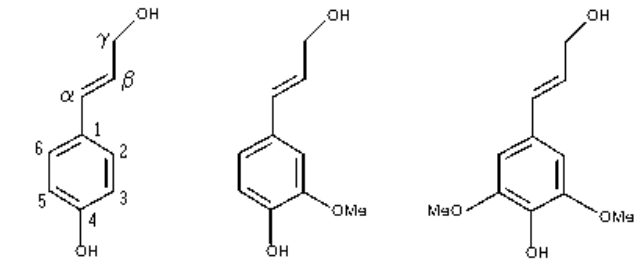

Fig. 4. Main precursors of lignin polymers

Lignin classification is traditionally done according to the precursors of the polymer. Guaiacyl lignin (G) is typical of softwood species and it is formed mostly of coniferyl alcohol precursors. The G:S:H ratio of Norway spruce (*Picea abies*) has been reported to be 94:1:5 (Erickson et al. 1973). Guaiacyl-syringyl (GS) lignin is a copolymer of coniferyl and sinapyl alcohol units and it is found in hardwood species. The ratio of the two precursors varies to a great extent. The H units are minor components of both softwood and hardwood species, whereas compression wood of softwood species are rich in H and G units. The ratios of different units affect branching of the polymer and accordingly, properties of the polymer (Sjöström 1993).

In the polymerisation process of lignin, precursors are turned into resonance-stabilised phenoxy radicals by enzymatic oxidation. Figure 5 presents the different resonance forms of coniferyl alcohol. Delocalisation of the singlet electron allows covalent bonds to be formed on three different sites of the molecule. Similar radicalisation occurs to lignin polymer when new precursors are added. However, the exact reaction mechanisms of polymer radicalisation remain controversial (Sasaki et al. 2004).

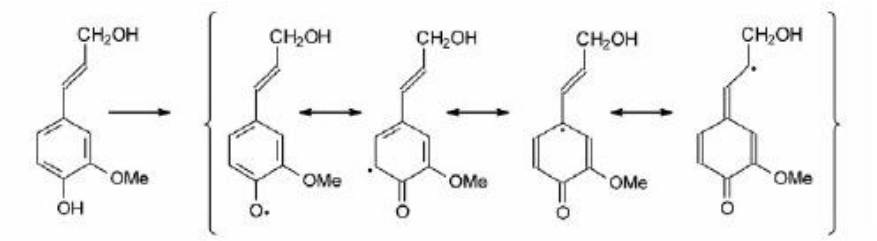

Fig. 5. Resonance forms of the coniferyl alcohol radical. Picture adopted from Koutaniemi (2007).

The structural formulas for lignin polymers have been constructed using data from structural studies of lignin. First models were published already in 1970's and a more recent formula for spruce lignin is presented in figure 6.

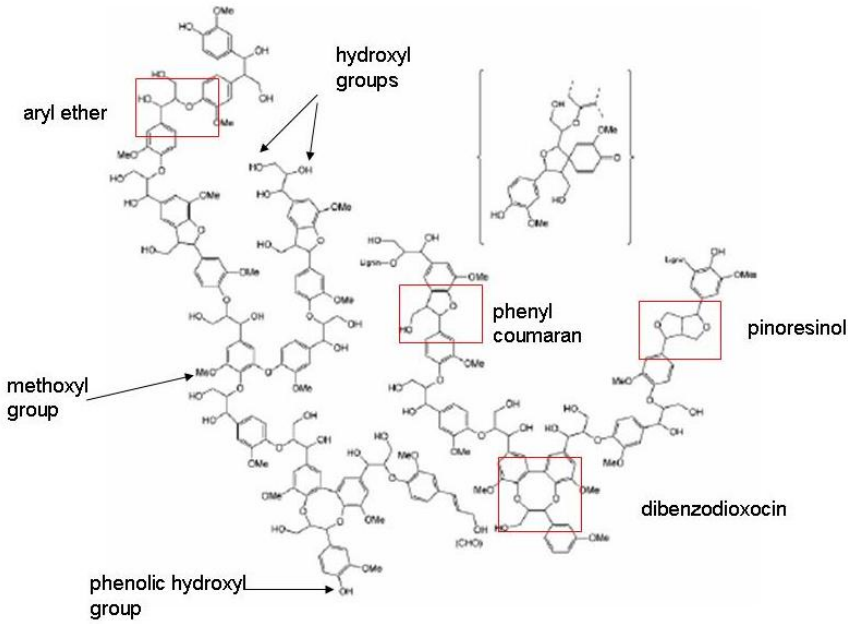

Fig. 6. Model structure of softwood lignin polymer. Figure is modified from Brunow et al. (1998). Most common structures and functional groups are pointed out.

Covalent linkages, functional groups and lignin-carbohydrate complexes

Lignin precursors are linked together with ether linkages (C-O-C) and with carbon-carbon (C-C) linkages. Carbon-carbon linkages are considered as condensed linkages, whereas ether linkages are non-condensed. Ether-type of linkages are most common and approximately two thirds of bonds between precursors are of ether-type. They are also weaker and susceptible for cleavage when lignin is depolymerised with chemicals (Sjöström 1993).

Phenylpropane units can be interconnected in several ways, and the most common linkages are presented in figure 7. Table 2 summarises the proportions of different linkages in spruce (*Picea abies*) milled wood lignin (MWL). The extraction method of MWL was first described in Björkman (1956).

Table 2. Proportions of most common covalent linkages in Norway spruce (*Picea abies*). Data adopted from Adler (1977).

Linkage type	% of all linkages in spruce MWL
β-O-4	50
α-O-4	2-8
β-5	9-12
5-5	10-11
4-O-5	4
β-1	7
β- *β*	2

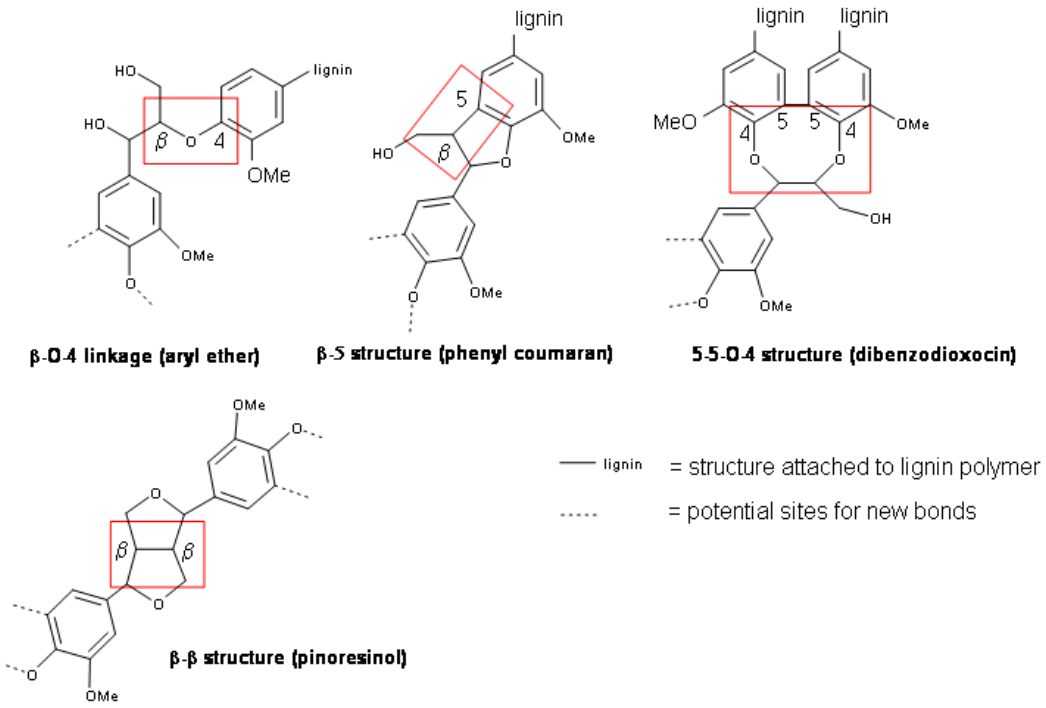

Fig. 7. The most common lignin structures. Figure slightly modified from Lewis & Davin (1999).

The same functional groups that are present in the lignin precursors are also present in the lignin polymer. Furthermore, the isolation process of lignin can introduce new functional groups to the structure (Fengel & Wegener 1984). Functional groups affect lignin reactivity and accordingly, they have been extensively studied. Native lignin contains methoxyl groups, phenolic hydroxyl groups, benzyl alcohol groups and carbonyl groups. Methoxyl groups are clearly the most abundant functional groups in wood lignins. There is considerable variation in the distribution of functional groups among different wood species. Table 3 compiles data of the functional groups in hardwood and softwoods.

Table 3. Functional groups in softwood and hardwood lignins (number per 100 phenylpropane units). Table adopted from Sjöström (1993).

Functional group	Softwood lignin	Hardwood lignin
Methoxyl	92-97	139-158
Phenolic hydroxyl	15-30	10-15
Benzyl alcohol	30-40	40-50
Carbonyl	10-15	

According to present knowledge, lignin molecules are covalently linked to the surrounding carbohydrates. Lawoko et al. (2006) isolated lignin-carbohydrate complexes (LCC) from spruce (*Picea abies*) and characterized the chemical composition of lignin-associated carbohydrates. They concluded that spruce lignin is covalently linked to all major cell wall polysaccharides: arabinoglucuronoxylan, galactoglucomannan, glucomannan, pectin and cellulose. Furthermore, the same study concluded that most probably, carbohydrate-free lignin does not exist in spruce. Carbohydrates are thought to be linked to lignin through α-ether, α-ester (Freudenberg & Grion 1959; Freudenberg & Harkin 1960) and phenyl glucosidic linkages (Kosikova et al. 1972).

Methods of lignin isolation

As previously described, lignin is tightly bound to the carbohydrate matrix and for this reason, lignin isolation is difficult. Nonetheless, lignin has to be isolated in order to study its structure with current methods. It is well-known fact that all extraction methods alter the molecular structure of lignin to some extent and interestingly, our current knowledge of native lignin structure is based on lignins extracted with milled wood lignin (MWL) method. MWL isolation is thought to preserve native lignin structures.

The macroscopic structure of wood has to be disassembled prior to lignin extraction. This is usually done by extensive milling, but other means of pretreatment can also be used. In the experimental part of this study, macroscopic wood structure was disrupted with steam explosion. Pretreatments are known to alter chemical structures of lignin. In MWL extraction, milling causes bond cleavages and depolymerisation of lignin. Prolonged milling time increases lignin yield but it also increases the number of phenolic groups (Brunow et al. 1998). Shevchenko et al. (1999) studied how SO_2-catalyzed steam explosion changed structure of the softwood lignin and these changes were reported to be dramatic in comparison with MWL lignins. Steam explosion caused lignin to become more condensed and less reactive. Hydroxyl groups of reactive α-carbons were oxidized to carbonyl groups or the α-carbon had formed carbon-carbon bonds to adjacent structures.

Two distinctive strategies can be employed for lignin isolation from fine wood material. Carbohydrate matrix of wood can be hydrolyzed with acid or enzyme catalyst. Resulting hydrolysis residues represent total lignin in wood, but the material is usually highly

contaminated with polysaccharides. Enzymatic hydrolysis retains the lignin structures, whereas strong acids cause further changes, mainly by condensation reactions (Fengel & Wegener 1984). The other strategy is lignin extraction. Native lignin is soluble only to some extent in commonly used solvents (Sjöström 1993), but it can be treated with chemicals to make it more soluble. Accordingly, new functional groups are introduced to lignin and the polymer becomes more soluble in the used solvent. Dissolved material can be separated easily from the solid carbohydrate matrix, but considerable amounts of lignin remain insoluble. For example, yields in MWL extraction remain in 25% of total Klason lignin (Brunow et al. 1998). When compared with hydrolysis residues, extracted lignins are less contaminated with carbohydrates.

2 Fungal cellulases

Cellulases are a heterologous group of enzymes able to hydrolyze the 1,4-β-glucosidic linkages of cellulose. Cellulases are part of the glycoside hydrolase superfamily. Effective degradation of insoluble crystalline cellulose requires a complete cellulase system, which is composed of several types of enzymes. White-rot and soft-rot fungi, including the genera *Phanerochaete, Trichoderma, Schizophyllum* and *Penicillium*, are the well-known organisms with an ability to secrete a complete cellulase system (Wood 1992). Several bacterial species are able to produce cellulases, but these systems often lack the ability to degrade crystalline cellulose. Bacterial systems consist of cell-bound enzymes, cellulosomes. *Cellulomonas fimi* is the most studied cellulase producing bacterial species.

2.1 Structure and function of fungal cellulases

Cellulases can be divided into three major classes according to their main catalytic activity. Enzymes function synergistically when crystalline cellulose is degraded into soluble sugars. Exoglucanases, also known as cellobiohydrolases (CBH), degrade cellulose sequentially from the free ends of glucose chains and liberate cellobiose as the main end product. *Trichoderma reesei* exoglucanases Cel7A and Cel6A have been shown to degrade cellulose polymer preferentially from the non-reducing and reducing end, respectively (Barr et al. 1996). Endoglucanases (EG) are capable of cutting glucose chains internally and β-glucosidases (BG) are specialized in the degradation of small soluble cello-oligosaccharides, such as cellobiose. Nevertheless, strict classification of

enzymes according to endo- or exoglucanase activity is not possible. It has been shown with *Trichoderma reesei* cellulases that enzymes classified as exoglucanases are also able to function in cleavage of internal polymer linkages (Ståhlberg et al. 1993).

Multidomain structure is a typical characteristic of fungal cellulases, as well as many other glycoside hydrolases that function on solid polysaccharide surfaces. The catalytic core domain (CD) is linked to a carbohydrate binding module (CBM) from its N- or C-terminus by glycosylated linker peptide. The CBM domain intensifies the affinity of the enzyme towards insoluble polysaccharide surfaces and enables effective catalytic activity on the solid-liquid interface. In addition, the CBM is responsible for the polysaccharide recognition. Different cellulose binding CBMs are able to recognise amorphous or crystalline regions of the chemically invariant polysaccharide (Boraston et al. 2004). Endo- and exoglucanases have often this modular structure, but there are also enzymes that lack the CBM. In addition, β-glucosidases degrade soluble substrates and thus, they lack CBMs.

2.2 Classification and nomenclature of glycoside hydrolases

A single enzyme may be active on multiple substrates and for this reason, new classification system and enzyme nomenclature system was developed for carbohydrate active enzymes and their carbohydrate binding domains. This classification comprises glycoside hydrolases, glycosyl transferases, polysaccharide lyases, carbohydrate esterases and carbohydrate binding modules. This new classification and nomenclature system reveals structural features of the enzymes, evolutionary relations to other enzymes and mechanistic information about their function (Henrissat 1991). Glycoside hydrolase superfamily enzymes (including all cellulases) are now divided into 114 sub-families according to their amino acid sequence. Enzyme names are in line with the classification system and renewed nomenclature is used throughout this thesis. The classification is available at Carbohydrate Active Enzymes (CAZy) online database (http://www.cazy.org) (Cantarel et al. 2009), which is updated with new enzymes and enzyme families.

Carbohydrate binding modules (CBMs) are also classified in CAZy database. At present, CBMs from all carbohydrate active enzymes are divided into 53 families according to their amino acid sequence. Fungal cellulose-binding CBMs are members of family 1

and they bind to solid crystalline cellulose or chitin. These CBMs are of similar size and they show high sequence and structural homology.

2.3 Complete cellulase system of Trichoderma reesei

Trichoderma reesei (anamorph of *Hypocrea jecorina*) (Kuhls et al. 1996) cellulases are most extensively studied among the cellulose degrading enzymes. For this reason and because of availability, they were used in this thesis to study cellulase binding on lignin. *T. reesei* cellulase system is known to be efficient in cellulose degradation and the fungus is extraordinarily efficient producer of extracellular enzymes. Surprisingly, it was found that the genome of *T. reesei* contains relatively few glycoside hydrolase encoding genes compared with other carbohydrate degrading fungi (Martinez et al. 2008).

Table 4 lists properties of all so far characterized *T. reesei* cellulases. Altogether, two exoglucanases, five endoglucanases and two β-glucosidases comprise the cellulolytic machinery. Characterization of several isoforms of Cel7A and Cel6A explain the variation of molecular weight and isoelectric point.

Table 4. Characteristics of *Trichoderma reesei* cellulases. Abbreviation na=data not available.

New name[3]	Old name[3]	Molecular weight (kDa)[3]	Isoelectric point (pI)[3]	CBM family[3]	% of total secreted proteins[2]
Cel7A	CBH I	59-68	3.5-4.2	1	50-60
Cel6A	CBH II	50-58	5.1-6.3	1	15-18
Cel7B	EG I	50-55	4.6	1	12-15
Cel5A	EG II	48	5.5	1	9-11
Cel12A	EG III	25	7.4	-	0-3
Cel61A	EG IV	56[1]	na	1	na
Cel45A	EG V	23	2.8-3.0	1	0-3
Cel3A	BGL I	71	8.7	-	na
Cel1A	BGL II	114	4.8	-	na

1. Saloheimo et al. (1997)
2. Tolan (2002)
3. Reinikainen (1994) and Palonen (2004)

3 Protein interactions with surfaces

3.1 Introduction to protein adsorption

Proteins are large molecules that are surface active by nature: they may possess charged amino acids, as well as hydrophobic residues on their surfaces. Characteristic for proteins is their affinity almost on any surface. The behaviour of the proteins on solid-liquid interface is a growingly important field due to the vast amount of applications in biomedicine and protein purification. In addition to commercial applications, protein adsorption is essential in several biochemical reactions. (Brash & Horbett 1995)

Interactions between proteins and surfaces: proteins

Protein-surface interactions are mostly non-covalent, including hydrogen bonds, electrostatic forces and hydrophobic interactions. A typical characteristic of protein adsorption is that proteins can undergo conformational changes when adsorbed. This may affect the physical and chemical characteristics of proteins as well as their catalytic activity. The conformation of adsorbed protein can vary from native structure to extensively unfolded conformations, which depend greatly on the surface and the protein in ques-

tion (Brash & Horbett 1995). Proteins can be grouped to "hard" proteins, such as lysozyme, that have strong internal coherence and "soft" proteins, such as bovine serum albumin (BSA), that are more flexible to take new conformations. Norde & Anusiem (1992) summarises that "hard" proteins adsorb to hydrophobic surfaces, whereas adsorption on hydrophilic surface occurs only if the proteins are electrostatically attracted. On the contrary, "soft" proteins adsorb even on hydrophilic and electrostatically repelling surfaces. Adsorption of "soft" proteins is dominated by structural rearrangements in the protein conformation (Norde & Anusiem 1992). The dominating phenomena in protein adsorption are suggested to be structural rearrangements in the protein molecule: dehydration of the sorbent surface, redistribution of charged groups and protein surface polarity instead of electrostatic interactions (Brash & Horbett 1995). Certain model proteins were found to adsorb on hydrophobic surface even if the protein and the sorbent repel each other electrostatically (Arai & Norde 1990).

In addition to conformational changes, proteins seem to be able to diffuse on surfaces to which they are adsorbed even though they are not able to desorb from the surface. This phenomenon has been shown to occur with BSA on acrylic polymers (Rabe & Tilton 1993) as well as with cellulases on cellulose surface (Jervis et al. 1997).

Theoretical background to assess protein-surface interactions

As noted previously, protein stuctures are likely to change during adsorption on solid surfaces. This phenomenon has also a strong theoretical basis. Protein adsorption is a system that strives to an equilibrium state. This means that proteins are distributed across the solid surface and the surrounding liquid medium as long as minimum value of total Gibbs energy (G) is achieved. If the system is departed from the equilibrium by changing the temperature, pressure or substance concentrations, there will be a spontaneous transition (also called process) towards the new equilibrium. This process can be reversible or irreversible by nature. Norde & Haynes (1995) define the difference of these two terms accordingly: "By definition, a process is reversible if, during the whole trajectory of the process, the departure from equilibrium is infinitesimally small, so that in the reverse process the variables characterizing the state of the system return through the same values but in reverse order." Figure 8 illustrates the difference between reversible and irreversible processes when the bulk concentration of protein c_p is changed in the system by adding protein (→) or by dilution (←). The difference in ascending

and descending branches is called hysteresis and such behaviour is common when protein adsorption is being studied. Hysteresis means that there are two local minima in Gibb's energy (e.g. equilibria states) for a given c_p and this indicates that a physical change has occurred in the system between the adsorption and desorption of the protein. Evidently, the structure of the protein is most likely to change during the course of adsorption. Protein adsorption on surfaces, especially on hydrophobic surfaces, is usually irreversible in nature.

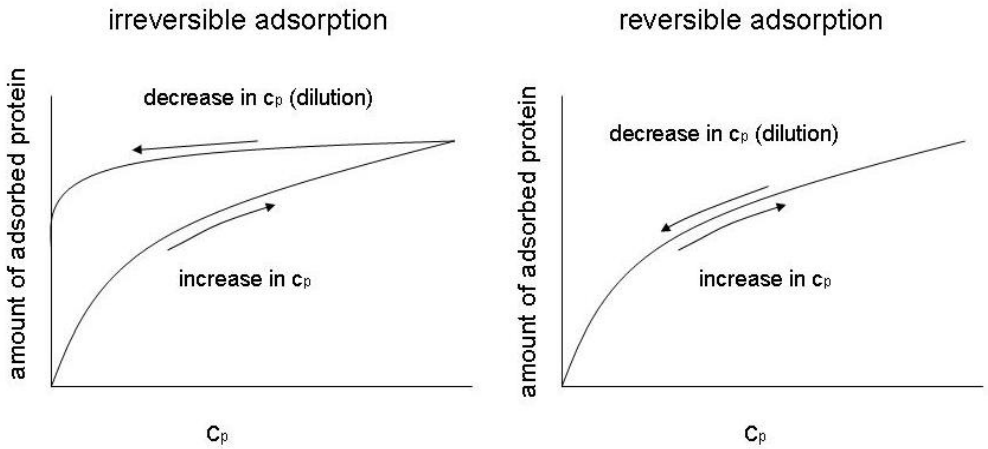

Fig. 8. Ascending and descending adsorption isotherms for irreversible and reversible process when bulk concentration of protein c_p is changed. Figure adopted from Norde & Haynes (1995).

Dynamic equilibrium and competitive binding

A dynamic equilibrium requires that the rate of interchange between bound and soluble proteins is the same. The rate of exchange varies greatly and in some cases the exchange is not detectable within a reasonable time-scale. *T. reesei* cellobiohydrolases Cel7A and Cel6A have been shown to have similar binding affinities to crystalline cellulose, but their ability to desorb is widely different. Binding of the Cel7A was shown to be truly reversible (Linder & Teeri 1996) and the enzyme had a considerable exchange rate, whereas binding of the Cel6A had more irreversible nature and exchange between free and bound enzymes was not detected (Carrard & Linder 1999).

If different proteins are present in the liquid media, they compete of the binding-sites and certain proteins have stronger affinity to the sorbent than others. If binding was reversible, which is the case with detergent molecules, the species would exchange until an equilibrium mixed layer of molecules would be achieved on the surface (Brash & Horbett 1995). However, competition is more complicated with proteins, since the na-

ture of binding is more irreversible and significant exchange does not necessarily occur. Thus, a bound protein may or may not be displaceable by a later arriving protein molecule. The probability of displacement is thought to be dependent on the elapsed time the protein is in contact with the surface. Bound proteins are thought to undergo relaxation on the surface over time which strengthens the protein-surface interaction (Brash & Horbett 1995). Figure 9 presents the simplest chronology of irreversible protein binding including three steps. The first two steps are thought to occur reversibly, thus the third relaxation step is responsible for irreversibility (Norde & Haynes 1995).

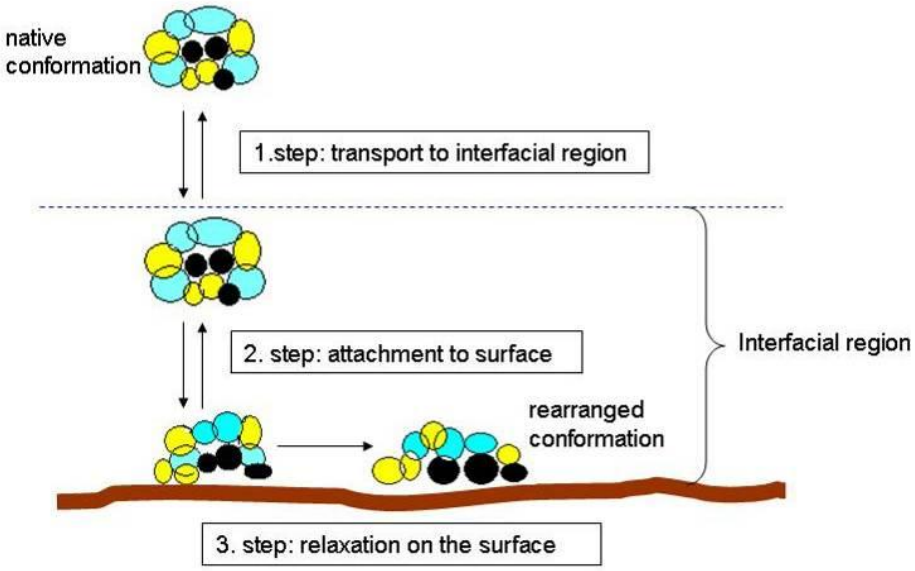

Fig. 9. Irreversible protein binding. Modified from Norde & Haynes (1995).

Hydrophobic and electrostatic interactions between proteins and surfaces

The broadest generalisation on sorbent surfaces is that hydrophobic surfaces attract proteins. Accordingly, the more hydrophobic the surface is the more it attracts proteins. This generalisation is based on studies made with hydrophobic gradient surfaces (Brash & Horbett 1995).

Electrostatic interactions are also important in protein binding. Proteins may contain several negatively or positively charged groups on their surfaces, which sum up to form the net charge of the protein. Charge of a protein is pH dependent and each protein has a pH value when there is no net charge. This value is known as isoelectric point (p*I*). Below p*I* proteins carry a positive net charge, whereas above it the charge is negative. Opposite net charges of the protein and the surface attract proteins to bind. This principle

has been widely employed in protein purification with ion exchange chromatography. Sometimes surface might adsorb a protein with a like charge. Small multivalent counterions are thought to enable such binding by forming a bridge between the protein and surface (Brash & Horbett 1995). As previously noted, the net charge of a protein comprises of several local charges and accordingly, it is interesting to know whether local charges or the net charge is dominant in protein adsorption. Several research groups have reported that the adsorption maximum of the studied protein was reached at the isoelectric point of the protein, which indicates the dominant role of the net charge to the adsorption behavior (Brash & Horbett 1995).

Surfaces containing negatively charged sulfonate groups are shown to have high affinity to various proteins and the affinity seems to be indiscriminate with respect to protein type (Brash & Horbett 1995). For this reason, technical lignins containing lignosulfonates should be avoided if protein adsorption on native structures of lignin is studied.

3.2 Adsorption isotherms and models

The binding behaviour is usually illustrated with adsorption isotherms that reveal how strongly the sorbent attracts the molecules and what is the maximum amount of adsorbed molecules on the surface in certain conditions. For isotherm construction, distribution of protein to the liquid and solid phase is measured on a broad range of protein concentrations. Proteins and sorbent are incubated for sufficient time at a fixed temperature in order to achieve the equilibrium distribution. The amount of adsorbed protein is determined by subtracting the amount of free protein left in the solution from the original amount of protein added. Sensitive protein assays need to be used in order to measure low protein concentrations.

Figure 10 illustrates the adsorption isotherm of *T. reesei* major exoglucanase Cel7A with bacterial microcrystalline cellulose (BMCC). The picture is derived from a study that showed the reversible binding behaviour of *T. reesei* Cel7A exoglucanase to BMCC when the equilibrium was disturbed by dilution. The amount of free enzyme was diluted to one tenth of the original concentration and the establishment of a new equlibrium was followed by concentration measurements. Concentrations were reverted back to the isotherm which indicates reversible binding behaviour (Linder & Teeri 1996).

The initial slope of the isotherm represents protein affinity to the surface and is widely used to compare affinities between different enzymes. Initially, isotherms reach the plateau-value, which indicates the maximum amount protein that can be adsorbed on the surface in certain conditions.

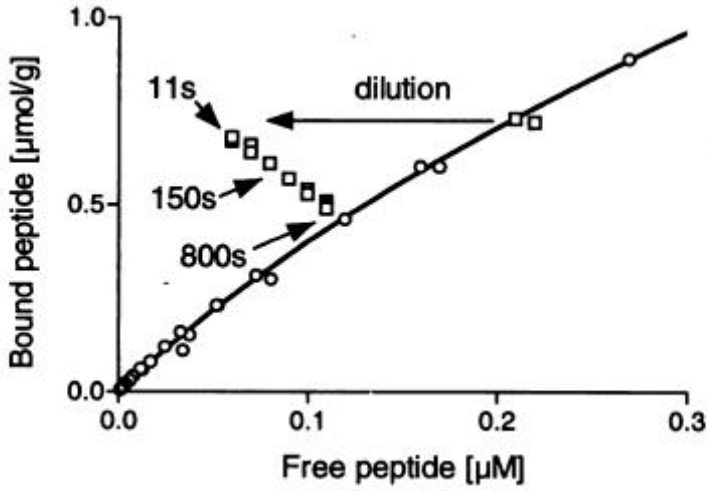

Fig. 10. Binding isotherm of *T. reesei* major exoglucanase Cel7A to bacterial microcrystalline cellulose (BMCC). Dilution shows the reversible nature of binding. Figure adopted from Linder & Teeri (1996).

Several adsorption models exist and the applicability of certain model to the experimental data has to be calculated. Models enable mathematical analysis of experimental data and derivation of constants that describe binding (e.g. affinity constant K). The Langmuir equation, originally developed by Irving Langmuir, has been widely used to model adsorption data. It was first applied to describe the dependence of surface coverage of adsorbed gas on the pressure of the gas above the surface at a fixed temperature. For this reason, one binding site Langmuir model is oversimplified description of protein adsorption and it has several well-known drawbacks. Nevertheless, it usually fits to the experimental data with satisfactory accuracy. A major disadvantage of Langmuir model is that it postulates reversible adsorption, and protein adsorption is usually irreversible. For this reason, Norde & Haynes (1995) criticize determination of binding constant K and the thermodynamic constants ($\Delta_{ads}G$, $\Delta_{ads}H$, $\Delta_{ads}S$) from irreversible processes that fit to Langmuir model, but do not obey reversible thermodynamics. Some other models used in cellulase binding studies are more sophisticated derivatives of the Langmuir model (Reinikainen 1994).

The Langmuir model for one binding site (equation 1) has been widely used to determine the affinity constants (K_a) for cellulases.

$$[B] = \frac{[B_{max}]K_a[F]}{1 + K_a[F]} \quad (1)$$

[B] = amount of bound protein

[B_{max}] = maximum amount protein bound (saturation)

K_a = affinity constant

[F] = concentration of free protein

4 Cellulase adsorption on lignin

4.1 Inhibitory mechanisms of lignin

Lignin is considered as a major inhibitory factor in enzymatic hydrolysis of lignocellulose (Chang & Holtzapple 2000). At present, the inhibition mechanisms are being studied widely and it is believed to occur by three distinct mechanisms: 1. lignin shields cellulose fibrils from enzymatic attack, 2. enzymes adsorb on lignin surfaces and, 3. lignin-derived soluble aromatic compounds inhibit enzyme activity. The role of unproductive enzyme adsorption on lignin has been emphasized and research groups have actively studied this topic during recent years.

During the course of hydrolysis, enzyme adsorption and movement on solid cellulose is a prerequisite for effective degradation of the substrate. On the contrary, irreversible adsorption on lignin restrains the movement of enzymes and inhibits an efficient hydrolysis of cellulose. This inhibitory effect can be overcome by using high amounts of enzymes, but the high cost of the enzymes restricts this strategy. In addition to direct effects during hydrolysis, unspecific binding on lignin hinders enzyme recycling. Significant proportion of hydrolytic enzymes remains tightly bound to lignin-rich hydrolysis residues. Figure 11 presents the amount of soluble sugars (■) and proteins (●) during hydrolysis of A) microcrystalline cellulose and B) steam pretreated Douglas fir (DF) (Lu et al. 2002). After 48 hours of hydrolysis of lignocellulosic DF, 70% of cellulolytic enzymes remained bound to the hydrolysis residue. Moreover, hydrolysis had almost stopped and sugar yields remained low. On the contrary, only 15% of enzymes re-

mained bound to the microcrystalline cellulose hydrolysis residue after 48 hours. Interestingly, enzymes seem able to desorb from microcrystalline cellulose, whereas visible desorption does not occur with DF substrate.

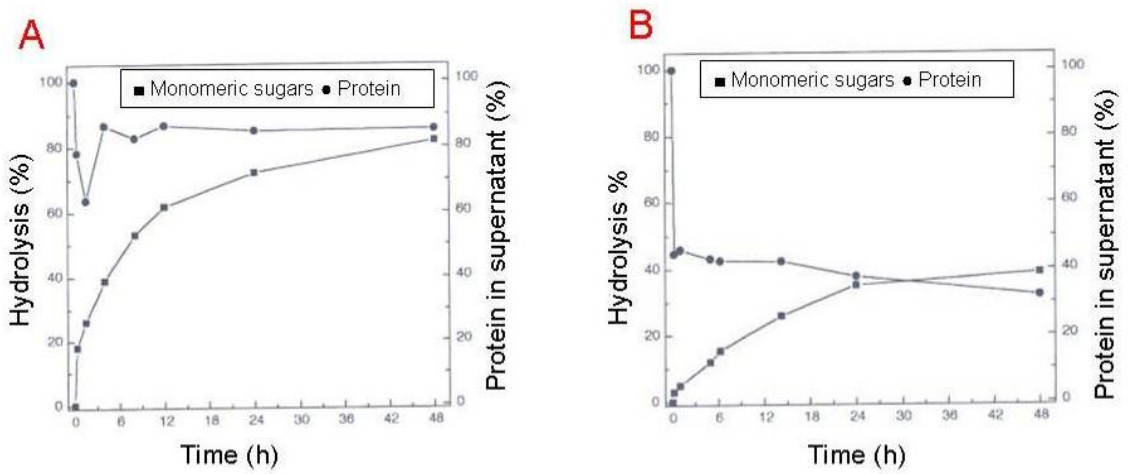

Fig. 11. Amount of monomeric sugars and protein during hydrolysis on A) microcrystalline cellulose and B) steam exploded douglas-fir (softwood). Enzyme dosage 20 filter paper units/g (FPU/g) of cellulose in 2% (w/v) substrate concentration. Adopted from Lu et al. (2002).

The extent of inhibition of this nature is shown to be dependent on several factors: the origin of hydrolytic enzymes (Berlin et al. 2006), the origin of lignocellulosic substrate (Berlin et al. 2005) and the pretreatment method affecting the amount and structure of lignin in the substrate (Tu 2007b). Accordingly, many applicable strategies already exist to avoid lignin binding in process conditions. Pretreatment methods have been developed to efficiently remove lignin prior to hydrolysis (Pan 2004). On the other hand, the chemical structure of residual lignin could be altered during pretreatment in order to weaken its affinity to hydrolytic enzymes. It was found already in the 1980's that addition of lignin-binding substances e.g. surfactant molecules improve hydrolysis efficiency (Ooshima et al. 1986). However, their use in the process introduces also extra expenditures that might turn the process uneconomical. Weak lignin binding enzymes could be screened from microorganisms or well-known enzymes could be even engineered to have lower affinity to lignin (Berlin et al. 2006). However, further development of these strategies requires detailed knowledge of enzyme-lignin interactions.

4.2 Methods and studies of cellulase adsorption on lignin

Different approaches are necessary to study cellulase adsorption on lignin and the consequences of this adsorption on the efficiency of the hydrolysis process. These approaches are grouped into three categories in figure 12.

1. Adsorption during hydrolysis of lignocellulose

2. Adsorption during hydrolysis of lignocellulose model substrate (mixture of pure cellulose and isolated lignin)

3. Adsorption on isolated lignin or lignocellulose (in the absence of hydrolytic activity)

In the first category, study conditions are closest to process conditions, but enzyme adsorption on lignin is hard to distinguish from adsorption on cellulose as well as from other inhibitory factors present in the hydrolysis conditions. Consequently, other approaches are needed to study enzyme-lignin interactions in more detail. The second category comprises studies that use isolated lignins to inhibit the hydrolysis of pure cellulosic substrates. This system is referred to as adsorption during hydrolysis of lignocellulosic model substrates. The third category represents adsorption studies without simultaneous hydrolysis of carbohydrates and these studies provide us with detailed information on enzyme-lignin interactions in experimental conditions. However, it should be taken into consideration that similar interactions are not necessarily possible in process conditions.

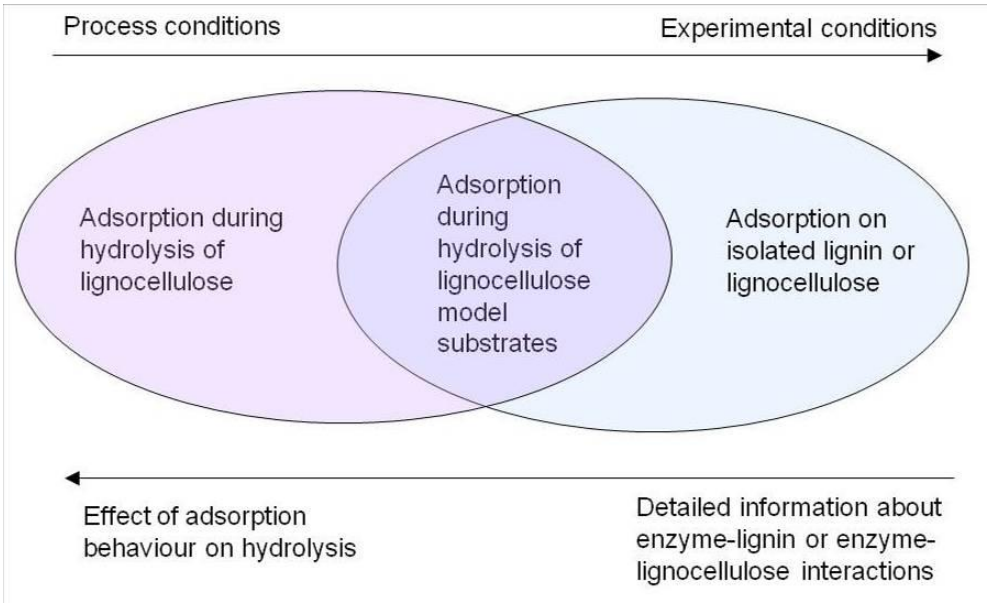

Fig. 12. Classification of strategies to study cellulase adsorption on lignin

Adsorption during hydrolysis of lignocellulose - studies and results

Surfactants (surface acting agents) have been used for decades to boost enzymatic hydrolysis of lignocellulose, but the mechanism of this boosting effect was revealed substantially later. Surfactants bind on lignin surfaces and prevent interactions of enzymes with lignin, increasing the rate of conversion and decreasing the total enzyme adsorption on lignocellulosic substrate (Eriksson et al. 2002). Consequently, the unproductive enzyme binding must have decreased. Surfactant studies are essential to study enzyme adsorption on lignin during process conditions. In addition to surfactants, some other molecules have shown similar ability to reduce enzyme binding on lignin during hydrolysis. Non-catalytic proteins, BSA (Yang & Wyman 2006) and gelatin (Xu et al. 2008), as well as a group of industrial polymers, poly(ethylene glycols) (PEGs) (Börjesson 2007b), have been used successfully. Altogether, this heterogeneous group of molecules will be referred to as lignin-binding substances in this thesis.

Surfactants are amphiphilic organic molecules. The long "tail" of the molecule is hydrophobic whereas the "head" is hydrophilic. Non-ionic surfactants, such as Tween 20, lack charged groups and they have been shown to be most efficient in boosting enzymatic conversion of lignocellulosic steam pretreated softwood (SPS) substrates (Eriksson et al. 2002). Polyethylene glycol (PEG) is a hydrophilic low molecular weight polymer that is formed in the polymerisation reaction of ethylene oxide. The hydrophobicity of PEG is shown to increase with temperature (Saeki et al. 1976) and PEG was

shown to bind on lignin more in higher temperatures (Börjesson et al. 2007a). It has been suggested that binding of surfactants and PEG on lignin is dominated by hydrophobic interactions (Eriksson et al. 2002; Börjesson et al. 2007a). The chemical structures of the non-ionic surfactant (Tween 20) and PEG are presented in figure 13.

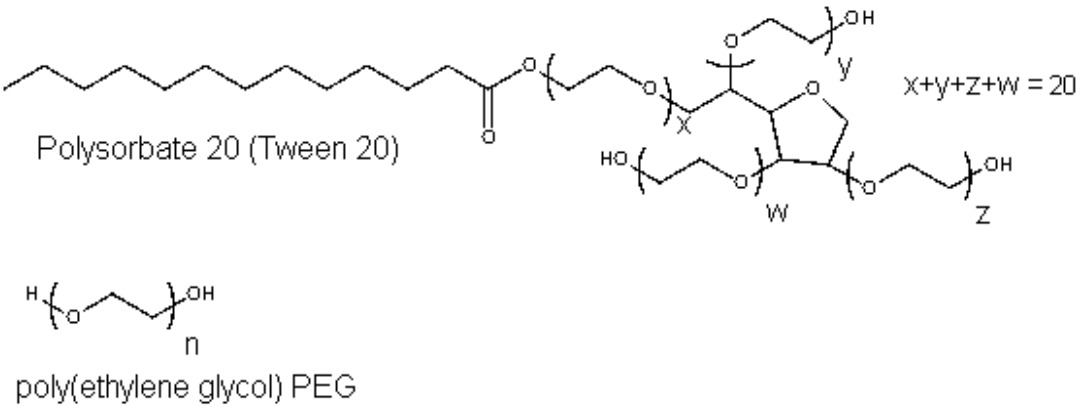

Fig. 13. Chemical structures of Tween 20 and poly(ethylene glycol) PEG.

Steam pretreated softwood (SPS) has been a widely used substrate to study enzyme adsorption on lignin: efficient hydrolysis of SPS requires high enzyme dosages and lignin preparations isolated from SPS have been shown to be good protein sorbents (Palonen et al. 2004). Strong inhibitory effect during hydrolysis has been attributed to the high lignin content of SPS (up to 50 w-%) and the boosting effect lignin-binding substances has been shown to be especially strong with SPS (Tu 2007b).

Adsorption during hydrolysis of lignocellulose model substrates - studies and results

The chemical composition of lignin varies greatly and the extent of enzyme adsorption is shown to be dependent on the chemical characteristics of lignin (Berlin et al. 2006). As previously noted, it is not possible to study all aspects of enzyme adsorption in real hydrolysis process conditions with industrial lignocellulosic substrates. Consequently, model systems have been designed in order to study factors in lignin composition that affect the adsorption capacity of the preparation.

The following model system has been widely used in various studies. Hydrolysis is carried out in a suspension of pure crystalline cellulose and isolated lignin. With this procedure, it has been possible to monitor how lignin affects the course of hydrolysis and to compare the inhibitory effect of different lignin preparations. Components of cellu-

lase mixtures are inhibited by lignin to varying extent, and comparison of cellulase preparation properties can be tested with this approach. Inhibition in this type of model system was shown to originate exclusively from enzyme adsorption on lignin (Sewalt et al. 1997). Isolated lignin preparations result in soluble compound in the hydrolysis reaction and these molecules may cause minor part of the enzyme inhibition (Sewalt et al. 1997).

Softwood-derived lignins are known to inhibit the hydrolysis of cellulose more than lignin preparations from hardwoods and herbaceous plants. This was shown, for instance, in a study by Berlin et al. (2005) when lignin preparations from softwood and hardwood were similarly isolated. Softwood preparation was found to inhibit the enzymatic hydrolysis reaction considerably more. The isolation method of lignin is also known to affect lignin structure, as well as the extent of inhibition. This was shown in a study by Berlin et al. (2006) when two lignin preparations were isolated from softwood and used to study the inhibition of hydrolysis of crystalline cellulose. One of the lignins was obtained after precipitation of dissolved lignin (DL-fraction) and the other was called enzymatic residual lignin (ERL-fraction). The ERL-fraction was obtained after extensive enzymatic hydrolysis of carbohydrates and subsequent protease purification. The DL-fraction was shown to inhibit enzyme preparations more than the ERL-fraction. Both lignin fractions were subjected to careful qualitative and quantitative structural studies (NMR methods) and their molecular weights and surface areas were shown to be similar. The higher adsorption capacity of DL was suggested to be caused by lower content of carboxyl and aliphatic hydroxyl groups that would lead to higher surface hydrophobicity of DL. As previously mentioned, hydrophobic interactions mediate the adsorption of lignin-binding substances and same forces are thought to be dominating in enzyme adsorption. Several authors have suggested that hydrophobic interactions between enzymes and lignin are mainly responsible for adsorption (Palonen et al. 2004, Börjesson et al. 2007a). However, there is still evidence of other interactions that may drive protein adsorption on lignin. Kawamoto et al. (1992) suggested that protein binding capacity of lignin depends on the phenolic hydroxyl content of lignin and some other studies gave consistent results (Sewalt et al. 1997, Pan 2008).

Lignocellulose model systems have also been used to evaluate differences between cellulase preparations. Berlin et al. (2006) tested the extent of total hydrolysis inhibition

with six cellulase preparations, capable of total hydrolysis of cellulose. Five of them were derived from *Trichoderma reesei*, whereas one preparation was obtained from *Penicillium* sp. The initial hydrolysis rate was used to measure the efficiency of hydrolysis. The extent of inhibition varied: the *Penicillium* preparation was least affected and variation occurred also among *Trichoderma* preparations. Difference in the extent of inhibition between *Penicillium* and *T. reesei* was explained by different enzyme structures. The majority of *T. reesei* enzymes possess a cellulose binding domain (table 4), whereas two main endoglucanase enzymes from *Penicillium brasilianum* (about 25% of total protein) lack CBMs (Jørgensen et al. 2003). The CBMs of *Trichoderma* cellulases are thought to be responsible for majority of enzyme adsorption on lignin (Palonen 2004). The effect of enzyme structure on lignin adsorption will be covered in the following chapter.

Adsorption on isolated lignin and lignocellulosics - studies and results

Adsorption experiments have been used to study cellulase adsorption on isolated lignins in the absence of degradable cellulosic substrates. This approach has been used to study molecular mechanisms of lignin-binding.

Traditionally, enzyme adsorption on surfaces has been measured in test tubes. Certain amount of buffer-diluted enzyme is incubated with solid particles and subsequently, solid and liquid fractions are separated. The amount of adsorbed protein is usually measured conversely from the amount of non-bound protein (protein free in the liquid fraction), although direct methods also exist (Kumar & Wyman 2008). Protein assays, based on Lowry and Bradford methods, have been widely used, although lignin-derived substances that dissolve during incubation interfere with both assays (Tan et al. 1984). In addition, sensitivities of the Lowry (Lowry et al. 1951) and Bradford (Bradford 1976) methods are often insufficient to measure low protein concentrations. New method for this field of research was ninhydrin protein assay that was used by Tu et al. (2007b). Tritium labelling of enzymes has also been used to enable accurate determination of non-adsorbed proteins (Palonen 2004). Recently, enzyme activity measurements have also been employed as a measure of cellulase adsorption (Xu et al. 2008).

Adsorption experiments are useful in studying adsorption tendencies of pure enzymes or the adsorption dynamics of enzyme mixtures. In only one publication, an adsorption

study with the complete *T. reesei* cellulase mixture was described (Xu et al. 2008). Xu et al. (2008) found that if enzymes were added to corn stover hydrolysis residue (16 mg/g solids), 90% of Cel7A and 100% of other cellulases were adsorbed on the solid fraction. Protein adsorption was quantified with protein electrophoresis and enzyme activity assays. The same approach has been used in the experimental part of this thesis.

Isotherms are useful when adsorption characteristics of purified enzymes need to be compared. Unfortunately, only few cellulase-lignin adsorption isotherms have been published. Palonen et al. (2004) constructed adsorption isotherms for two major *T.reesei* cellulases: exoglucanase Cel7A and endoglucanase Cel5A, as well as for their corresponding core domains. SPS and lignin SPS-derived lignins were used to adsorb enzymes and isotherms were formed based on concentration gradients. These isotherms show clearly that CBM-domains of the two enzymes are mainly responsible for enzyme binding on lignin substrates. The essential role of CBM in lignin-binding was confirmed also by Börjesson et al. (2007a) with *T. reesei* Cel7A and Cel7B enzymes. Comparison of adsorption capacities of different lignin preparations is a more complicated task. Lignin preparations need to have similar surface areas for the comparison of binding sites on the surfaces. In addition, the effect of pH, ionic strength and temperature on lignin-binding could be studied with isotherms, but such work has not been published to date.

Börjesson et al. (2007a) studied adsorption differences of *T. reesei* exoglucanase Cel7A and endoglucanase Cel7B. The core modules of the two enzymes adsorbed equally poorly on isolated spruce lignin, whereas the full-length enzymes were bound considerably more. Significant difference was shown: the Cel7B adsorbed about 15% more than the Cel7A and this effect was suggested to be caused by surface properties of the enzymes' CBM-domains. Both, Cel7B and Cel7A CBMs belong to the family 1 and accordingly, have highly conserved structures. However, computer modelling indicated Cel7B CBM to have more hydrophobic surface than Cel7A CBM and this was suggested to cause the difference in adsorption behaviour on lignin. Similar 12% difference in adsorption on lignin was also shown with two endoglucanases that lacked the CBM-modules (Berlin et al. 2005). Accordingly, there are major differences in adsorption behaviour of the CBM modules as well as the core modules.

EXPERIMENTAL WORK

5 Materials

5.1 Enzyme and protein preparations

Two commercial cellulase enzyme preparations were used during this study. Celluclast 1.5 L FG (CCN03067) was purchased from Novozymes (Bagsværd, Denmark, received 4/2003), and it is a concentrate of *Trichoderma reesei* culture broth. The following enzyme activities were measured from the preparation in 2003 at VTT:

- endoglucanase activity (hydroxyl ethyl cellulose (HEC) as substrate) 17 300 nkat/ml
- total hydrolysis activity 49 FPU/ml (FPU=filter paper units)
- β-glucosidase activity 430 nkat/ml
- xylanase activity 14 900 nkat/ml
- mannanase activity 3 500 nkat/ml
- polygalacturonase activity 82 nkat/ml
- β-glucanase activity 7 300 nkat/ml
- pectinmethylesterase activity 0 nkat/ml

Endoglucanase and FPU activities had not decreased during storage at +4°C (measured in June 2008). The protein content of the preparation was 138 g/l measured with a Lowry-based (Lowry et al. 1951) protein assay (DC-protein assay, Bio-Rad).

Novozym 188 (DCN00206) was purchased from Novozymes (Bagsværd, Denmark, received 8/2006). The enzyme preparation is a concentrated culture broth of *Aspergillus niger*. The preparation was strongly coloured and the following enzyme activities were measured from the liquor in 2008 (by the author):

- *β*-glucosidase activity 6360 nkat/ml
- endoglucanase activity (HEC) 413 nkat/ml.

Protein content was 52 mg/ml measured as previously mentioned.

Lyophilized bovine serum albumin preparation (BSA) (Sigma, USA) was also used in adsorption experiments. The preparation composed mostly of 66 kDa albumin but also some globulin impurities were present according to the product supplier.

5.2 Adsorption substrates

Microcrystalline cellulose, lignocellulosic steam pretreated spruce (SPS) and SPS-derived lignins were used in the adsorption studies. Microcrystalline cellulose was a commercial product of Serva, Germany (0.020 cm of particle size). The origin of SPS and the methods used for lignin extraction are described next in more detail.

5.2.1 Steam pretreated spruce (SPS)

The SPS was prepared in Lund, Sweden, with a previously published method (Stenberg et al. 1998). Spruce chips were treated in the following conditions: impregnation with 3% SO_2 and treatment for 5 min at 215°C. The material was washed with hot distilled water and stored frozen at -20°C in airtight plastic bags prior to use. The dry matter content after storage was 28%. Wet, as well as freeze-dried SPS were used in adsorption experiments. Figure 13 visualizes the appearance of wet SPS.



Fig. 14. Steam pretreated spruce after melting. The picture is taken by Jenni Rahikainen.

5.2.2 Alkali-lignin

Alkali-lignin was extracted with a previously published method (Palonen et al. 2004). Small proportion of total lignin in SPS dissolves readily in alkaline conditions and this dissolved fraction can be precipitated with acid.

Frozen SPS was melted at room temperature and a suspension of 10% (w/v)[1] (50 g/500 ml) was made to 0.5 M NaOH. The suspension was incubated in a +80ºC water bath, under magnetic stirring for three hours. Dissolved material was separated from solids by filtration through two 1.6 µm GF/A glass fibre filters (Whatman, UK). The filtrate was almost black in colour and its clearness was ensured visually to make sure that it contained only dissolved material. The dissolved lignin was precipitated by adjusting pH to 2.5 with 1 M HCl. During the precipitation, black colour of the solution changed to dark brown and a slurry was formed. The slurry was warmed up to +40ºC and the precipitated material was separated by centrifugation (10 min, 23 700 g, +40ºC, Sorvall RC5C with SLA-1500 rotor). The precipitate was washed three times with 100 ml of warm and pH adjusted distilled water (pH 2.5 adjusted with 1 M HCl, temperature +40ºC) and centrifuged as previously described. The wet sample was frozen at -20 °C over night and lyophilized (Christ alpha 2-4, B. Brown Biotech International). The dry sample (3.58 g) was collected to a plastic tube after freeze-drying. Subsequently, the sample was stored at room temperature prior to use.

The wet alkali-lignin sample was prepared from the lyophilized sample by dissolving 200 mg in 100 ml (0.2% w/v) of 0.1 M NaOH, and keeping the solution in magnetic stirring for 1-2 hours at room temperature. The lyophilized sample was readily dissolved and subsequently, pH was adjusted to 4.8 with 1 M HCl and the precipitate was centrifuged (15 min, 22 000 g, Sorvall RC5C with SLA-1500 rotor). The precipitate was washed twice with 100 ml of distilled water (pH 2.5 adjusted with 1 M HCl) and stored wet at +4ºC in airtight plastic tubes.

5.2.3 Cellulolytic enzyme lignin (CEL)

The principle for cellulolytic enzyme lignin isolation was adopted from Palonen et al. (2004). In this work, CEL refers to the residue that remains insoluble after extensive enzymatic hydrolysis of lignocellulosic material by cellulase.

Several batches of melted SPS were used for hydrolysis in 6% (w/v) concentration (12 g/200 ml). Hydrolysis was carried out in 50 mM sodium acetate buffer (pH 4.8) with

[1] w/v designation refers always to dry weight in biomass suspensions

total cellulase activity of 20 FPU/g cellulose (Celluclast 1.5L) and β-glucosidase activity of 450 nkat/g cellulose (Novozym 188). Based on preliminary results from carbohydrate analysis, the total amount of cellulose in SPS was about 50% of dry weight. Hydrolysis reactions were run in 1 l centrifuge bottles in an incubator (Minitron AI-70, Infors) with platform shaker. Hydrolysis conditions were +45ºC, 72 hours and 160 rpm shaking.

In order to boost the hydrolysis and minimize end-product inhibition, solids were separated by centrifugation (20 min, 5300 g, 20ºC, Sorvall RC12BP with H12000-rotor) and suspended to 200 ml of fresh buffer and enzymes every 24 hours. The supernatant was collected from each batch, and the amount of reducing sugars was measured with the dinitrosalicylic acid (DNS) -method (Bernfeld 1955) to ensure successful hydrolysis (Appendix 3).

After 72 hours of hydrolysis, solids were separated by centrifugation as previously explained and washed three times with 200 ml of pH adjusted distilled water (pH adjusted to 2.5 with HCl). Prior to the last washing step, sample was filtered through a 500 µm filtration fabric (Sefar, Switzerland) in order to remove the largest unhydrolysed particles from the sample. Batches were pooled and kept at -20ºC over night prior to freeze-drying. The freeze-dried sample was stored in a glass bottle in room temperature. The wet CEL sample was prepared using the above-mentioned method without freeze-drying. The wet CEL was stored in an airtight plastic tube at +4ºC.

5.2.4 Purified CEL samples

Cellulolytic enzymes bind to the CEL-residue during hydrolysis and it is crucial to purify the sample from solid-bound proteins prior to adsorption experiments. Enzymes are strongly bound and various methods can be employed to remove the bound enzymes. In this study, urea and protease treatments were tested to detach lignin bound enzymes from the CEL residue. Urea treatment was carried out only with lyophilized CEL but protease treatment was carried out to both, dry and wet CEL samples.

Urea treatment of CEL (CEL-U)

Urea is known as a protein denaturant as it disrupts the non-covalent bonding within the molecule. For this reason, it was tested in CEL purification. The method for urea treatment was adopted from Lawoko et al. (2006).

Lyophilized CEL (500 mg/75 ml corresponding to 0.67% w/v) was mixed to 8 M urea solution, pH 8.3. The suspension was mixed for 48 hours at +25ºC under magnetic stirring. Solids were separated by centrifugation and washed four times with 50 ml of pH adjusted distilled water (pH adjusted to 2.5 with HCl). Centrifugations were carried out accordingly: 15 min, 20ºC, 12 000 g, Sorvall RC5C with SLA-1500 rotor. The wet sample was frozen at -20ºC over night prior to lyophilization. The amount of dry sample after urea treatment was 479 mg, which was 96% of originally added dry weight (500 mg). Evidently, some lignin was dissolved from the sample during urea treatment because of elevated pH.

Protease treatment of CEL (CEL-P)

The method for protease treatment was adopted from Tamminen & Hortling (1999). Several batches of lyophilized CEL sample were suspended (500 mg/10 ml) in 0.5 M $NaHCO_3$ buffer (pH 9.6) in 5% w/v consistency. Protease solution (100 mg/ml) of subtilisin-type protease (Sigma-Aldrich, Japan) was prepared to distilled water and 100 µl of the solution was added to the reaction mixture. Accordingly, the final dosage of protease in the reaction mixture was 1 mg of enzyme per 50 mg of CEL. The treatment was carried out for 24 hours at 37ºC in magnetic stirring. Subsequently, solids were separated by centrifugation (15 min, +20ºC, 12 000 g, Sorvall RC5C with SS-34 rotor). The solid residue was washed three times with 10 ml of pH adjusted distilled water (pH 2.5, adjusted with HCl). The sample was frozen at -20ºC and lyophilized. The weight of the protease-treated solid residue after freeze-drying was 433 mg, which was 87% of originally added dry weight (500 mg). Loss of weight was presumably because of solubilisation of lignin during the treatment. Dry sample was stored in plastic tube in room temperature.

The wet CEL sample was subjected to the same purification treatment. The dry matter content of CEL was determined gravimetrically, so that a correct amount of material could be added to the reaction. In addition to protease treatment, the wet CEL sample was subjected to a reference treatment similar with the above-mentioned method, but

without the addition of protease. This reference sample was prepared in order to verify the effect of protease addition apart from treatment conditions in the purification process. Both samples were stored wet in airtight plastic tubes at +4ºC.

5.2.5 Acid hydrolysis residue

Acid hydrolysis was carried out using VTT's method for carbohydrate analysis from biomass samples. The method was scaled up in order to obtain enough of hydrolysis residue for adsorption experiments. VTT's procedure is modified from the published method of National Renewable Energy Laboratory (USA) Laboratory Analytical Procedure (LAP-003) for determination of acid-insoluble lignin in biomass.

Wet SPS was lyophilized over night and ground in a mortar. The sample was further pulverized with Fritsch pulverisette 14 –mill (cutter 0.5). Dry and pulverized SPS was suspended to 70% (w/w) H_2SO_4 to get a 10% (w/v) suspension. Treatment was carried out in several batches. The suspension was incubated for 1.5 hours in a +30ºC water bath and mixed frequently. Subsequently, the sample was transferred to a 500 ml volumetric flask with 280 ml of distilled water. The flask was sealed with aluminium foil and placed to an autoclave (Finn-Aqua 666) in order to heat up the flasks for acid catalysis. Autoclave was run with following settings: liquid program, 50 min, 120ºC, 4 pulses and 15 min cooling. The sample was cooled down and the solid hydrolysis residue was recovered by filtration through 1.6 μm glass fibre filter GF/A (Whatman, UK). Solids were washed twice with 100 ml of pH adjusted distilled water (pH adjusted to 2.5 with HCl) and centrifuged as follows: 20 min, +4ºC, 25 000 g, Sorvall RC5C with rotor SLA-1500. Batches were pooled and kept at -20 °C over night prior to lyophilization.

The above-mentioned protocol was also used to prepare wet acid hydrolysis residue. After washing steps, the residue was stored at +4ºC in an airtight plastic tube.

5.2.6 Summary of adsorption substrates

Different adsorption substrates used in adsorption experiments are summarised in table 5.

Table 5. Summary of adsorption substrates

Adsorption substrate	Dry	Wet
Microcrystalline cellulose	X	
SPS	X	X
Alkali-lignin	X	X
CEL residue	X	X
CEL urea purified residue	X	
CEL protease purified residue	X	X
CEL protease treatment control		X
Acid-lignin	X	X

6 Methods

6.1 Analytical methods to study adsorption substrates

6.1.1 Gravimetric measurement of dry matter content from biomass

Dosing of biomass samples is usually based on dry weight and accordingly, the dry matter content of wet samples was determined gravimetrically. The protocol was modified from Laboratory Analytical Procedure (LAP-001) for determination of total solids in biomass, National Renewable Energy Laboratory (USA).

A dry and clean metal vessel with a cap was preheated in a +105ºC oven (Termaks) for about 30 minutes and cooled down at room temperature in a desiccator. An analytical balance (Mettler Toledo AG204) was used to weigh the vessel and cap after cooling. The wet sample (50-500 mg) was weighed into the vessel in triplicates. Subsequently, the vessel was placed in the +105ºC oven for 12-20 hours and the cap was left partly open to facilitate water evaporation. Next, the sample was cooled down in a desiccator at room temperature, and the sample (vessel+cap+dry sample) was weighed with the analytical balance. The weight of evaporated mass was used to calculate the dry matter content of the sample.

6.1.2 Carbohydrate content analysis

A dilute acid treatment at a high temperature depolymerises the wood polysaccharides and results in soluble sugar oligo- and monomers that are weak acids. They dissociate in highly alkaline conditions and become negatively charged, which allows their detection with anion exchange chromatography.

Previously, VTT's protocol "Acid hydrolysis for analytical purposes" was used for the isolation of the acid-lignin residue (chapter 5.2.5). Here, the same protocol was employed for its original analytical purpose. The freeze-dried lignins and SPS were subjected to acid treatment in order to determine their carbohydrate content. The dry sample was mixed with 70% (w/w) H_2SO_4 in 10% (w/v) concentration (20 mg/200 µl) in triplicates. The mixture was incubated in a +30ºC water bath for 1 hour and transfered to a 10 ml volumetric flask with 5.6 ml of distilled water. Subsequently, the foil-sealed flask was put into an autoclave (Finn-Aqua 666) and the following liquid program was run: 50 min, 4 pulses, +120ºC, and 15 min cooling. After cooling, the volumetric flask was filled to the volume of 10 ml with distilled water and a 5 ml sample was taken with a syringe. In order to remove insoluble particles, the sample was filtered through a syringe filter (Pall Life Sciences, GF/0.45 µm GHP membrane) into a test tube. The filtrate was stored frozen at -20ºC prior to chromatographic analysis.

The samples were analyzed at VTT carbohydrate laboratory with anion exchange liquid chromatography. The method is published in Tenkanen & Siika-Aho (2000). Liquid chromatography was performed with Dionex ICS-3000 (dual pump model DP-1) equipment using Dionex CarboPac PA1 guard column and Dionex CarboPac PA1 analytical column. The equipment, runnig conditions, solvents and used gradient are presented in detail in appendix 1.

6.1.3 Nitrogen content analysis

Nitrogen analysis was carried out to estimate the amount of protein bound on the adsorption substrates and to compare the effects of protease and urea purification treatments of CEL. Lyophilized samples were assayed in duplicates. Total nitrogen analysis was performed at Analytische Laboratorien Gmbh, Germany (Prof. Dr. H. Malissa and G. Reuter) with a standard method ASTM D-5291 (Standard test methods for instru-

mental determination of carbon, hydrogen, and nitrogen in petroleum products and lubricants). The assay is based on combustion and subsequent ion exchange chromatography.

6.1.4 Bright-field microscopy

In addition to chemical characteristics, physical parameters of adsorption substrates, such as particle size and structure, were studied with bright-field microscopy. Lyophilized and wet adsorption substrates were examined with Olympus BX61 microscope with a digital camera. Pictures were taken of native as well as Safranin/Alcian Blue dyed samples. Objectives of x4, x10, x20, x40, x50 and x100 magnification were used and picture editing was carried out with Analysis 3.1 software.

Safranin O and Alcian Blue 8 GS dyes (Chroma-Gesellschaft Schmid GmbH & co, Germany) were used together to dye the samples prior to microscopy. Alcian Blue is known to bind on carbohydrates, whereas Safranin stains lignified particles red. Safranin solution (1% w/v) was prepared in 96.1% (v/v) ethanol and 1% (w/v) Alcian Blue solution was prepared in distilled water. Alcian Blue and Safranin solutions were mixed in 1:2 ratio (200 µl in 400 µl) and 10 mg of dry sample or 100 mg of wet sample was added to the dye mixture. The slurry was mixed properly and incubated at least for 2 minutes. Solids were separated by centrifugation (3 min, 10 600 g, Eppendorf 5417C) and thereafter, they were rinsed two times with 1 ml of distilled water and centrifuged as previously. The solid fraction was mixed properly before the sample was spread on the microscope glass slide.

6.2 Adsorption experiments

The principle of adsorption experiments was to incubate the adsorption substrates with enzyme and protein mixtures in a buffer medium. The amount of non-adsorbed protein was determined from liquid phase with activity measurements as well as protein analyses (concentration and SDS-PAGE). In addition, activities of solid-bound enzymes were determined in further experiments.

6.2.1 Adsorption conditions and sample handling

Same adsorption conditions were used in all adsorption experiments. Similar conditions have been used by Palonen et al. (2004). Adsorption experiments were carried out in a 1.5 ml total volume, in 2 ml Eppendorf tubes. The sorbent (adsorption substrate) concentration was 1% (w/v) in all experiments and water content of the wet substrates was taken into account in dosing. Freeze-dried samples were assumed to contain a negligible amount of water. A protein concentration of 0.5 mg/ml, which corresponds to 50 mg of protein/g of adsorption substrate, was used in adsorption experiments. This relatively high protein concentration was necessary to get measurable quantities of protein or enzyme activity to remain in supernatant after adsorption experiment.

The desired protein concentration was achieved by mixing protein preparation with 50 mM sodium acetate buffer (pH 4.8). Protein contents of the commercial liquid enzyme preparations (Celluclast 1.5L and Novozym 188) were determined and the preparations were diluted with the buffer to reach the desired protein concentration. The lyophilized BSA preparation was weighed with an analytical balance and dissolved in the buffer in magnetic stirring.

Each experiment was carried out using duplicate samples as well as positive and negative control samples. Positive controls contained the same amount of protein as the samples did without any adsorption substrate. Negative controls contained 1% (w/v) concentration of adsorption substrate in the buffer solution.

Prior to incubation, tubes were mixed properly to get a homogeneous suspension. Incubation was carried out for 1.5 hours, at +4ºC in 30 rpm rotary mixing (Snijders rotary-mixer 34526). Subsequently, the solid residue was separated by centrifugation incubation (10 min, 6 500 g, +4ºC, Heraeus, biofuge fresco) and the supernatant was collected to another Eppendorf tube.

In some cases, the solid residue was subjected to further fractionation. The residue was rinsed once with the buffer to remove loosely bound enzymes. The tube was filled to the total volume of 1.5 ml and solid pellet was resuspended. Centrifugation was carried out as previously described. The supernatant was collected and this fraction was designated as washing buffer of adsorption solids. The pellet was once more resuspended to the

buffer (V_{tot} = 1.5 ml) and this suspension was called as adsorption solids. Magnetic stirring was added to the Eppendorf tubes to facilitate the formation of a smooth suspension, which is easy to pipette. All fractions were stored at +4ºC in Eppendorf tubes prior to use in further analysis.

To summarise, three different fractions could be collected after an adsorption experiment: 1. adsorption supernatant, 2. adsorption solids and 3. washing buffer of adsorption solids. A scheme of fractionation is presented in figure 15.

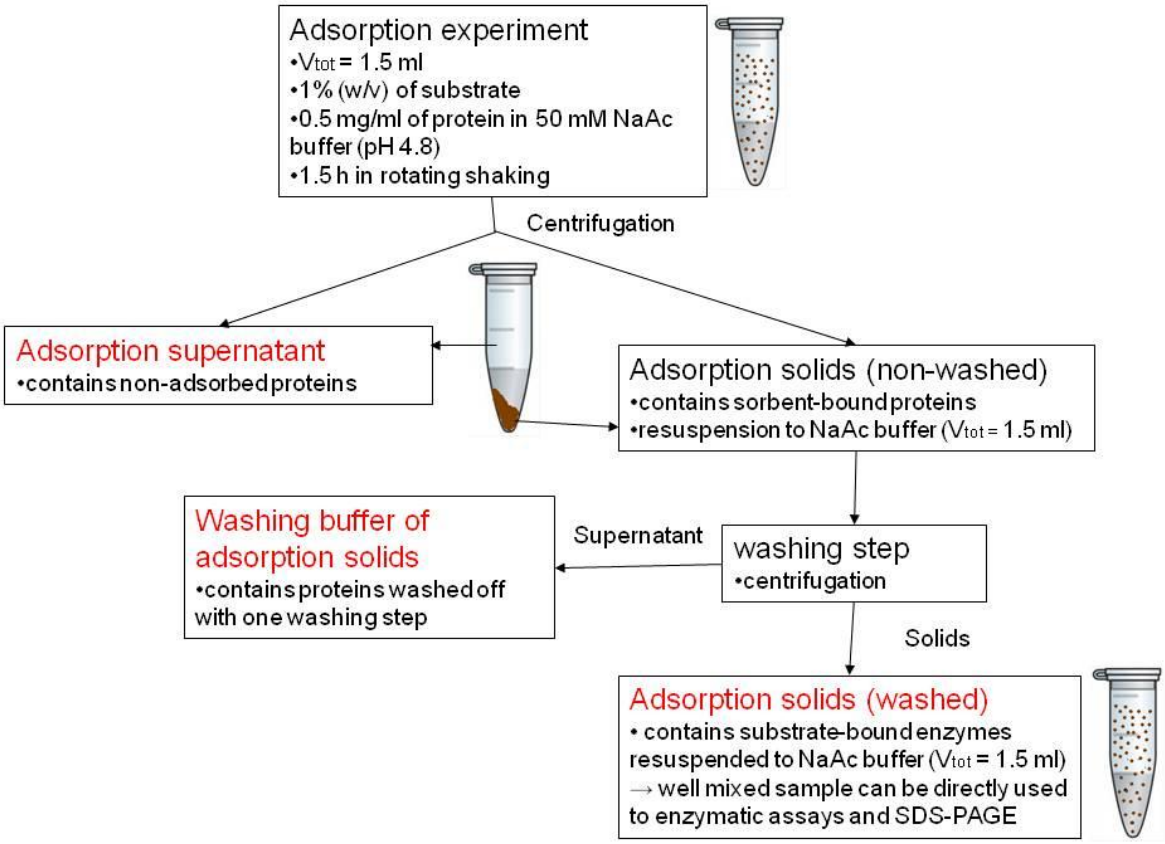

Fig. 15. Scheme of adsorption experiments and sample handling. Different fractions obtained after adsorption experiments are labelled with red font

6.3 Analysis of the fractions obtained from adsorption experiments

6.3.1 Protein analyses

Determination of protein content with Bradford protein assay

The protein contents of adsorption supernatants and washing buffers were measured with a commercial protein assay kit which is based on the Bradford method (Bradford 1976). Bradford method is a dye-binding assay in which a Coomassie Brilliant Blue G-250 dye molecule binds to a protein molecule in acidic conditions. Accordingly, the absorbance maximum of the dye shifts from 465 nm to 595 nm. Coomassie is a polyaromatic molecule that binds on basic amino acids, especially arginine (Compton & Jones 1985) as well as on other aromatic residues.

The reagent for the assay was purchased from Bio-Rad (USA) and the standard and microassay procedures in microtiter plate scale were employed. The Bio-Rad product manual reported linear ranges of 0.05-0.5 mg/ml and 8-80 µg/ml for standard and microassay procedure, respectively.

For the standard procedure, the dye reagent was diluted in 1:5 ratio with distilled water and filtered through a cellulose filter (Whatman 1, UK) prior to use. The sample was pipetted on a microtiter plate (Nunc, Denmark) in triplicates (10 µl for standard procedure and 160 µl for microassay procedure) and Bradford reagent was added (200 µl for standard procedure and 40 µl for microassay procedure) with a multichannel pipette. The microtiter plate was mixed at least for 5 minutes on a microtiter plate shaker and the colour reaction was measured at 595 nm with a microplate reader (Thermo Varioskan with SkanIt software 2.2.1).

BSA and immunoglobulin G protein preparations (Bio-Rad, USA) were used for making the standard curve for the assays. IgG and BSA do not form equally strong colour response with Coomassie dye and accordingly, the slope of the BSA standard curve is steeper than that of IgG. BSA seemed to form a stronger colour response with Coomassie than the Celluclast enzymes did, even though the protein concentration in the mixture should have been the same. Accordingly, the protein concentrations of Celluclast enzymes were compared to the IgG standard curve, whereas the BSA concentra-

tions were naturally compared to the BSA standard curve. Similarly, IgG was used as protein standard for Celluclast and Novozym enzymes in Lee et al. (1995).

SDS-PAGE and gel imaging methods

The adsorption supernatant and the adsorption solid fractions were assayed with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Saphiro et al. 1967). SDS binds to the sample proteins and denaturates their folded structure together with boiling. Simultaneously, proteins become negatively charged. If SDS-treated samples are loaded to a polyacrylamide gel and electrical currency is conducted through the gel, negatively charged proteins start to migrate towards the positively charged electrode. Small proteins migrate faster than large proteins in the gel matrix and consequently, the proteins are separated according to their size.

In this study, two gel-imaging methods were employed to visualise proteins on the PAGE gels: Coomassie staining and Criterion gel imaging system. Detailed information about the used buffers, staining solutions as well as destaining solutions is available in Appendix 2.

Sample handling and gel running

The sample handling was carried out as follows: 30 µl of the sample was mixed with 10 µl of sample buffer and the mixture was boiled for 5 minutes in a heating block (Norlab, blockheater SBH130D). Molecular size standard (10 µl), Precision Plus Protein stain free (Bio-Rad, USA), was boiled simultaneously. After boiling, the condensed moisture was spinned down with a table centrifuge.

Precast gradient gels (4 to 20% in Tris-HCl buffer (Bio-Rad, USA)) were used for SDS-PAGE. Gels contained 12 sample wells of 45 µl volume, and 40 µl and 10 µl of sample and size standard were loaded in the wells, respectively. Precast gels were compatible with the Criterion imaging system, but they could be stained with Coomassie as well. During the run, the voltage was set to be constant 200 V (LKB Bromma, 2301 Macrodrive I Power supply) and an adequate running time was about 55 minutes.

After the run was completed, gels were stained either with Coomassie staining or they were visualised with Bio-Rad Criterion imaging system. Coomassie staining was used, when there was no access to the Criterion imaging system.

Gel staining with Coomassie stain and gel imaging with Criterion system

Polyacrylamide gels were stained with Blue R (Serva, Germany) which is similar to the Coomassie Brilliant Blue R-250 stain. Staining was carried out for 15 minutes on a platform shaker. Destaining was done using two destain solutions with different ratios of ethanol and acetic acid. Gels were usually treated 2x15 minutes with the first destain solution and over night with the second solution. The gel images were scanned with a Hewlett Packard Scanjet 5590P scanner. The picture contrast and colour setting were adjusted with HP SolutionCenter software.

Gel imaging with Bio-Rad Criterion imaging system was carried out with a UV-table and Image Lab Software. According to the manual of the company, precast gels contain molecules that react with proteins when the gel is subjected to UV-light. Fluorescent reaction product can be visualised from the gel and image is recorded directly to the computer software.

6.3.2 Enzyme activity assays

Three cellulase activity assays were used to measure enzyme activities after adsorption experiments: endoglucanase, cellobiohydrolase I and β-glucosidase activity assays. All the three methods are based on the same principle: the enzyme hydrolyzes the substrate molecule down and the amount of reaction product can be measured with a spectrophotometer at a certain range of wavelength.

Econase cellulase mixture (AB Enzyme Oy, Finland, lot 100068259) was used to control the repeatability of the enzyme assays. One Econase sample was always added to the assay to ensure that the assay conditions and reagents were of standard quality. Enzyme blank samples were used if samples were coloured and if it was to be expected that the enzyme preparation or soluble lignin caused background absorbance. The absorbances of all samples were compared against blank samples that were treated as other samples, but 50 mM sodium citrate buffer (pH 5) was added instead of enzyme solutions.

Endoglucanase and cellobiohydrolase I assays were used to study if lignin-bound enzymes remained active against soluble substrates. Samples of adsorption solids contained lignin particles and hydrolytic enzymes were immobilized on their surfaces. Magenetic stirring was used with these samples to prevent particle sedimentation during the activity measurements. Solid material had to be removed from these samples by centrifugation prior to the measurement of absorbance.

Endoglucanase activity assay (HEC-assay)

The endo-β-1, 4-glucanase activity (EC 3.2.1.4) was assayed using hydroxyethyl cellulose (HEC) as a substrate (Ghose 1987). HEC is a water soluble cellulose derivative that has hydroxyl groups substituted with hydroxyethyl groups (figure 16). The degree of substitution and polymerisation varies. Endoglucanases introduce internal cuts to HEC-polymers decreasing the degree of polymerisation. Accordingly, endoglucanase activity adds the amount of reducing chain ends in the solution. DNS-reagent reacts with the reducing chain ends at high temperatures and forms a reaction product with distinctive absorption spectrum.

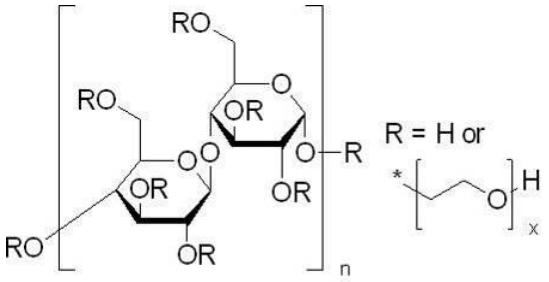

Fig. 16. Hydroxyethyl cellulose polymer. Source of the structure: Sigma-Aldrich internet product catalogue www.sigmaaldrich.com, 19.2.2009.

Solution of hydroxyethyl cellulose (1% w/v) was prepared by mixing HEC (Fluka Chemie, medium viscosity, degree of polymerisation 450, degree of substitution 0.9-1.0) in 50 mM sodium citrate buffer (pH 5). The HEC substrate solution was mixed at least for 1 hour and then the solution was let to stand for one more hour (clarification) prior to use. The solution could be stored at +4ºC up to one week. Citrate buffer served as the blank sample and the standard curve was prepared of glucose (from 2.5 to 10 µmol/ml) (BDH, UK) in the citrate buffer. The assay was carried out in 1/2 or 1/4 scale of the original method.

Test tube containing substrate solution (1.8 ml) was preheated in a +50ºC water bath. Buffer-diluted enzyme sample (200 µl) was added and the tube was mixed. The incubation time was precisely 600 seconds and the enzymatic reaction was stopped by mixing with 3.0 ml of highly alkaline DNS-reagent (Sigma, Germany; Appendix 3). Next, the tube was boiled for 5 minutes in a vigorously boiling water bath and cooled down in a cold water bath. The amount of reaction product was measured in plastic cuvettes against the blank sample at 540 nm. The standard/blank samples were treated in the same manner.

A standard curve was constructed from 2.5 to 10 µmol/ml glucose solutions that correspond to the activity range from 4.2 to 16.7 nkat/ml (nkat/ml = nmol/s x ml). Absorbances of the samples were compared to those of the standard curve and converted to units of activity. The HEC enzyme activity measurement for *Trichoderma* enzymes is known to be linear only in the range of 5-15 nkat/ml (Ghose 1987) and for this reason, correct sample dilutions are essential in the HEC-assay.

Cel7A (CBH I) activity assay (MUL-assay)

The idea of using fluorogenic and chromogenic glycosides in cellulase activity assays was first described by van Tilbeurgh et al. (1988). The method used in this study has been used in a study published by Bailey & Tähtiharju (2003) and it is described in detail in VTT-3712-2000 protocol.

In this study, cellobiohydrolase I (CBH I) was assayed using 4-methylumbelliferyl-β-D-lactoside (MUL) as substrate. Few *Trichoderma reesei* enzymes are known to be able to cleave the MUL molecule, which liberates a chromogenic substance, 4-methylumbelliferone (MU), as a reaction product (Figure 17). These enzymes include the exoglucanase Cel7A, the endoglucanase Cel7B (EG I) and the β-glucosidases (Reinikainen 1994). For this reason, enzyme activity inhibitors need to be used in the assay to solve the proportion of Cel7A activity. However, enzyme inhibition is not complete and for this reason the assay is only indicative of Cel7A activity.

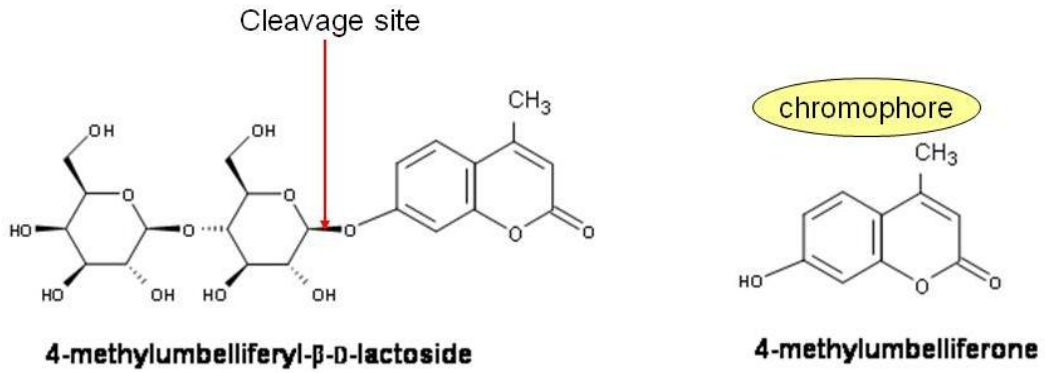

Fig. 17. 4-methylumbelliferyl-β-D-lactoside (MUL) substrate and chromogenic reaction product 4-methylumbelliferone (MU).

Substrate solution of 4-methylumbelliferyl-β-D-lactoside (1 mM) (Sigma-Aldrich, Israel) was prepared in 50 mM sodium citrate buffer (pH 5). The solution could be stored at +4ºC and used for 2-3 days. Enzyme inhibition was carried out with the following sugar solutions: 1 M glucose solution (BDH, UK) and 1 M glucose-50 mM cellobiose (Sigma-Aldrich, Slovakia) solutions were prepared to citrate buffer and stored frozen. Highly alkaline 1 M Na_2CO_3 (Sigma-Aldrich, Germany) solution was prepared to distilled water and used as stopping reagent to stop the enzymatic activity. The standard sample, 4-methylumbelliferone (MU) (Sigma-Aldrich, Switzerland), was diluted to the stopping reagent in concentrations ranging from 0.01 to 0.1 mM.

Two test tubes were needed to assay one sample. The first tube contained 50 µl of glucose solution and the other 50 µl of glucose-cellobiose solution to inhibit β-glucosidase and β-glucosidase+exoglucanase activities, respectively. In addition, 400 µl of substrate solution was added to both tubes. The tubes were mixed and preheated to +50ºC in a water bath. Subsequently, 50 µl of sample solution was added and tubes were incubated for 600 seconds. Enzymatic activity was stopped with 0.5 ml of stopping solution and test tubes were let to cool down at room temperature. Absorbances were read against blank at 370 nm in disposable plastic cuvettes.

Standard samples, enzyme blanks and blank samples were treated as follows:

- Standard samples: 400 µl substrate solution, 10 min at +50ºC, 0.5 ml of standard solution, 50 µl of 1 M glucose solution and 50 µl of Na-citrate buffer (pH 5).

- Enzyme blanks: 400 µl substrate solution, 10 min at +50ºC, 0.5 ml of stopping reagent, 50 µl of 1 M glucose solution and 50 µl of sample solution.
- Blank samples: 400 µl substrate solution, 10 min at +50ºC, 0.5 ml of stopping reagent, 50 µl of 1 M glucose solution and 50 µl of Na-citrate buffer (pH 5).

β-Glucosidase activity assay

β-Glucosidase activity (EC 3.2.1.21) was assayed using 4-nitrophenyl-β-D-glucopyranoside (PNPG) as a substrate. The used method is published in Bailey & Linko (1990) and detailed protocol of the assay is described in VTT-3720-93 protocol. The molecular structure of PNPG is not coloured whereas the hydrolysis product, 4-nitrophenol, becomes chromogenic in alkaline conditions when the molecule is converted to the corresponding salt, 4-nitrophenolate (figure 18).

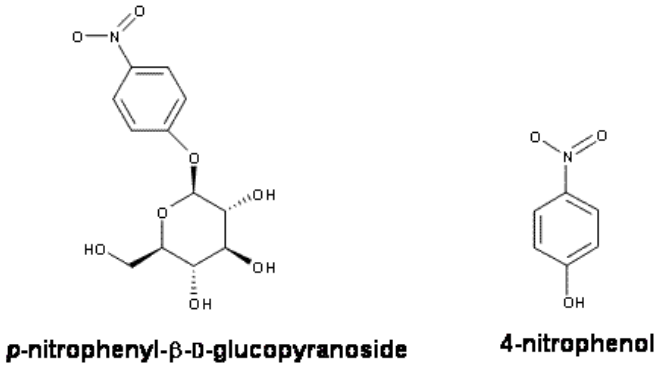

Fig. 18. β-glucosidase assay substrate (p-nitrophenyl-β-D-glucopyranoside) and the hydrolysis end product (4-nitrophenol).

Substrate solution for the PNPG assay was 1 mM 4-nitrophenyl-β-D-glucopyranoside (Calbiochem, USA) in 50 mM sodium citrate buffer (pH 5) and stopping reagent was 1 M Na_2CO_3 in distilled water. Standard curve (from 0.05 to 0.4 µmol/ml) was constructed from buffer-diluted 4-nitrophenol (Merck, Germany) and the citrate buffer served as the blank sample. The assay was usually carried out in 1/2 or 1/4 scale of the original method.

The substrate solution (1.8 ml) was preheated in a +50ºC water bath and 200 µl of buffer-diluted enzyme sample was added and mixed to the solution. Next, the test tube was incubated for 600 seconds and 1.0 ml of stopping reagent was added. Sample was cooled down at room temperature and the absorbance was read against the blank sample

at 400 nm in plastic cuvettes. The standards and the blanks were prepared similarly, except that the standard/blank sample was added after the stopping reagent.

6.4 Total hydrolysis with solid-bound cellulases

Lignin-bound Celluclast 1.5L cellulases were tested for their ability to hydrolyze insoluble microcrystalline cellulose. The solid residues of wet CEL-P and alkali-lignin, containing lignin-bound enzymes, were obtained after adsorption experiments and used in hydrolysis experiments. In addition to lignin-bound cellulases, reactions were supplemented with β-glucosidase activity.

The used protocol of microtiter-scale hydrolysis has been developed at VTT. The total reaction volume was 0.5 ml and reactions were carried out on 96-well microtiter plates (Nunc U96 PP 2 ml, Denmark). Hydrolysis substrate was microcrystalline cellulose (1% w/v) (Serva, Germany) in 50 mM Na-citrate buffer (pH 5). Reactions were run for 72 h at +45ºC and in 1000 rpm shaking in a thermomixer (Mxi4t, FinePCR).

The hydrolysis end products, reducing sugars, were measured at four time points: 4, 24, 48 and 72 hours and reactions for each time point were run parallel on separate microtiter plates. Reactions were stopped by centrifugation (5 min, 3500 rpm, +4ºC, Eppendorf centrifuge 5810R) and supernatant (250 µl) was pipetted from each well on a new microtiter plate. In order to stop all enzymatic activity, pH of the supernatant was raised with 5 µl of 10 M sodium hydroxide. Sealed microtiter plates were stored at +4ºC until all time points were ready to be assayed for reducing sugars.

Cellulase dosing was a more complicated task, but the following idea was applied in dosing of solid-bound enzymes. According to protein and enzyme activity measurements of adsorption supernatants, it was estimated that during the course of adsorption experiment about 70% of the initial amount (0.5 mg/ml) of Celluclast enzymes were bound to the solid CEL-P and about 90% of enzymes were bound to the solid alkali-lignin fraction. Based on these figures, the amount of solid-bound protein and corresponding FPU-activity of soluble enzymes was calculated. Lignin suspension was mixed carefully and 210 µl of it was added to the reaction in order to reach reasonable enzyme dosing in the reaction. If solid-bound enzymes were equally active as free enzymes, enzyme activities would have been 16.3 FPU/g cellulose and 12.7 FPU/g cellu-

lose in alkali-lignin samples and CEL-P samples, respectively. The positive controls of adsorption experiments (0.5 mg/ml of enzyme) were used as positive controls in the hydrolysis as well. For CEL-P and alkali-lignin samples they were diluted with buffer, so that there was 70% and 90% of the cellulase activity left in the solution, respectively. Celluclast enzyme preparations have quite low β-glucosidase activities for hydrolysis of cellulose. Accordingly, to ensure complete hydrolysis of cellulose all the reactions were supplemented with β-glucosidase activity (Novozym 188; 300 nkat/ g cellulose).

The hydrolysis reactions with positive controls and solid-bound enzymes were performed in duplicate and triplicate reactions, respectively. Dark colour of the supernatant indicated that some lignin was dissolved during the hydrolysis. For this reason, negative controls were used in order to see if dissolved lignin caused background in the sugar assay.

Reducing sugars were assayed from liquid fraction with the PHBAH-method (*p*-hydroxybenzoic acid hydrazide) (Lever 1972). Fresh reagent was prepared for each assay by mixing *p*-hydroxybenzoic acid hydrazide (Sigma-Aldrich, Germany) with 0.5 M NaOH (15 mg/ml). Samples were diluted with distilled water to reach the right concentration range (0.01-0.125 mg/ml). Glucose (BDH, UK) was mixed with distilled water for the standard curve (0.01-0.125 mg/ml) and distilled water was used in the blank samples.

The sample (100 µl) was mixed with 65 µl of PHBAH-reagent (Sigma, Germany) on a 96-well PCR-plate (AB-gene, UK) and the properly sealed plate was boiled in a heating block for 10 minutes. The plate was cooled down on ice and condensed water was spinned down with a microtiter plate centrifuge. Samples (100 µl) were moved to a flat-base microtiter plate (Nunc, Denmark) and absorbances were read at 405 nm (Labsystems, Multiskan MS) against the blank sample. Samples, standards as well as blank samples were treated in the same manner.

7 Results

The results are presented in three sections. Section 7.1 summarises the results of characterization of the adsorption substrates. Next section, 7.2, shows results from adsorption

experiments and the final section, 7.3 comprises results from the activity measurements and the hydrolysis experiments with lignin-bound enzymes.

7.1 Characterization of adsorption substrates

The chemical composition and particle size of adsorption substrate defines its adsorption capacity. For this reason, carbohydrate and nitrogen contents, as well as particle characteristics were determined.

Three different methods were employed to isolate lignin from steam pretreated spruce. Alkali-lignin was extracted in alkaline conditions and precipitated with acid. CEL-lignin is the residue left after extensive enzymatic hydrolysis and protease as well as urea treatment were used to clean the residue from solid-bound hydrolytic enzymes. Acid-lignin was obtained after the hydrolysis of carbohydrates with an acid catalyst.

The carbohydrate compositions of SPS and isolated lignins were measured with a method based on acid hydrolysis and subsequent analysis of dissolved monosaccharides. Results from the analysis are shown in table 6. The acid-lignin is not included in the table, as the sample was assumed to be free of carbohydrates since it was isolated by scaling up the analytical method.

Table 6. Carbohydrate contents of lignocellulosic steam pretreated spruce (SPS) and isolated lignins (CEL, CEL-P, CEL-U and Alkali-lignin).

Sample	Glucose w-%	Xylose w-%	Mannose w-%	Total polysaccharides w-%
SPS[1]	57.2	0.18	0.11	51.7
CEL[2]	5.6	<0.1	0.93	5.9
CEL-P[3]	6.1	<0.1	0.26	5.7
CEL-U[4]	5.9	<0.1	0.61	5.9
Alkali-lignin	0.2	<0.1	<0.1	0.2

1. Steam pretreated spruce
2. Enzymatic hydrolysis residue
3. Protease purified enzymatic hydrolysis residue
4. Urea purified enzymatic hydrolysis residue

The total polysaccharide content of SPS was 51.7%, which is a typical result for this kind of raw material. The pretreatment has efficiently removed hemicellulose from spruce chips and cellulose was almost exclusively the only polysaccharide left in the

steam pretreated biomass (SPS). This is also a characteristic consequence of acid catalyzed steam pretreatment. When it comes to isolated lignins, unpurified and purified CEL residues contained more carbohydrates than alkali-lignin, which is typical for hydrolysis residues. According to these results, alkali-lignin is highly pure from carbohydrate contaminants.

Interestingly, mannose content was highest in CEL-residues and purification of CEL seems to decrease the proportion of mannose in the samples. The most likely explanation for this is that mannose is originated from glycosylated cellulases. Protease and urea treatments have removed solid-bound proteins, and accordingly, the amount of mannose was also reduced in the purified samples. Mannose content of CEL-P was lower than that of CEL-U which infers that protease treatment was more efficient in removing solid-bound enzymes. The following results from nitrogen analysis confirm this assumption.

Adsorption substrates were assayed for their nitrogen content in order to compare the impact of protease and urea purifications on the amount nitrogen. The analysis was carried out in Germany with combustion and chromatographic methods.

Table 7. Nitrogen contents of SPS and isolated lignins.

Sample	Nitrogen content (w-%)
SPS[1]	0.06
CEL	1.21
CEL-P	0.29
CEL-U	1.07
Alkali-lignin	0.05
Acid-lignin	<0.05

1. nitrogen content of SPS is adopted from Palonen et al. (2004).

Lignocellulosic SPS contains only trace amounts of nitrogen (0.06%), whereas over twenty-fold higher nitrogen content was measured from CEL (1.21%) (Table 7). Evidently, the nitrogen in CEL samples originated from solid-bound cellulases. Urea treatment resulted in only a minor reduction (12%) of the nitrogen content of CEL, whereas protease treatment caused a 76% decrease. Acid- and alkali-lignins were also subjected to the analysis, but very low nitrogen contents, <0.05 w-% (under detection limit) and 0.05 w-%, respectively, were obtained.

Visualisation of adsorption substrates

In addition to the chemical structure, the adsorption capacity of a sorbent is also dependent on the available surface area. When the samples were assessed visually, all the lignins had highly similar appearances: the freeze-dryed samples had powdery consistency, whereas the wet samples resembled melted chocolate. Only the brown colour of alkali-lignin was darker than that of enzymatic and acid hydrolysis residues. Accordingly, magnification was needed to reveal the structures of the sample particles. The adsorption substrates were visualised with light microscopy and Safranin and Alcian Blue dyes were used to localize carbohydrates and lignin in the samples.

Figure 19 illustrates how native microcrystalline cellulose appears in bright-field microscopy (19A) and how pure carbohydrates are coloured with Safranin/Alcian Blue dyes (19B). The particles of microcrystalline cellulose are approximately 20 µm in diameter and the structure looks compact in comparison with SPS (figure 20), meaning also that the available surface area of a pure cellulose particle may be smaller.

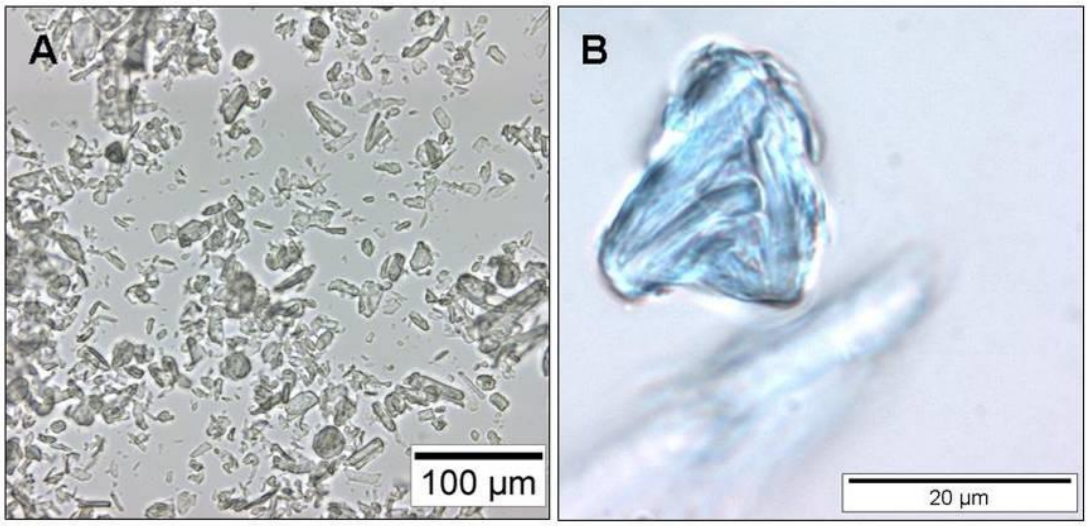

Fig. 19. Microcrystalline cellulose A) native sample with x20 magnification B) dyed crystal with x100 magnification

The microscopic pictures of SPS and isolated lignin samples are presented in figures 20 to 25. All pictures were taken of wet samples, excluding alkali-lignin. The dry and wet alkali-lignin samples showed considerable difference in particle sizes, thus pictures of the two samples are here for comparison. Drying did not alter the appearance of other lignin samples.

Figure 20 shows pictures of lignocellulosic steam pretreated spruce (SPS). Particle sizes and shapes varied to a great extent; from few to several hundreds of micrometers. The wood tissue structure has been severely damaged in the steam pretreatment, but cell wall components are still visible from the pictures. Safranin and Alcian Blue dyed the SPS sample purple, because both lignin and carbohydrates are present in a ratio of about 1:1 (Figure 20B). In general, wood tissue dyes red with Safranin if lignin is an abundant component in the structure, although considerable amounts of carbohydrates may also be present. On the contrary, blue colour indicates that the amount of lignin is low in the particle (Figure 19B).

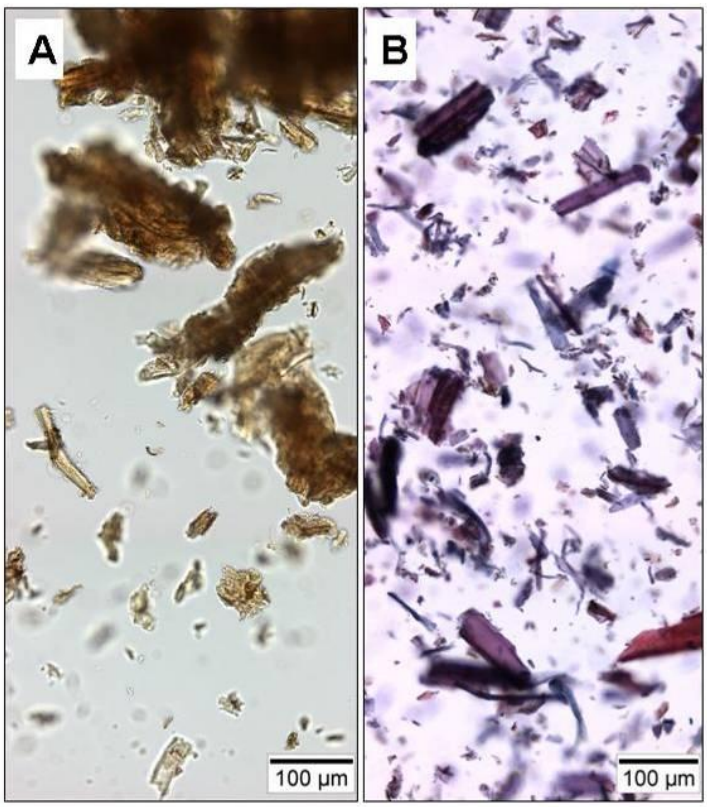

Fig. 20. Lignocellulosic SPS A) native sample with x20 magnification, B) dyed sample with x20 magnification

Figures 21 and 22 show the particles of CEL and protease purified CEL, respectively. The majority of particles appear to be considerably smaller than in SPS and the colouring is more red than purple due to carbohydrate degradation. However, some big particles are still present in both, CEL and CEL-P samples and many of them resemble the cross-field areas of spruce tissue figure (21C).

Pictures of the acid-residue are presented in figure 23. The sample consisted of surprisingly large particles that resembled wood tissue structures. Figure 23C visualises a particle that is composed of six tracheid cell walls. As previously mentioned, the acid-lignin was assumed to be completely pure from carbohydrate contaminants. Considering the various cell wall structures in the sample, it is highly unexpected that pure lignin could form such structures. Generally lignin is considered as a glue-like substance that strengthens the polysaccharide backbone of the cell wall.

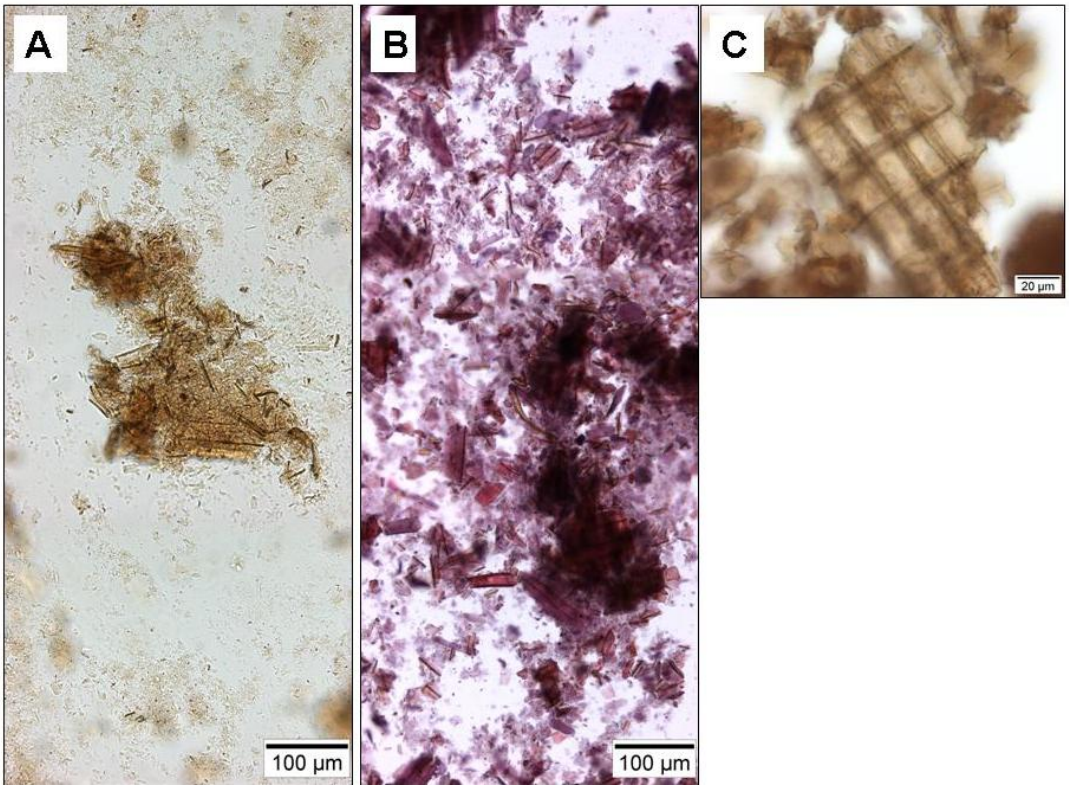

Fig. 21. CEL-lignin residue: A) native sample with x20 magnification, B) dyed sample with x20 magnification, C) native sample with x50 magnification, cross-field area of wood tissue

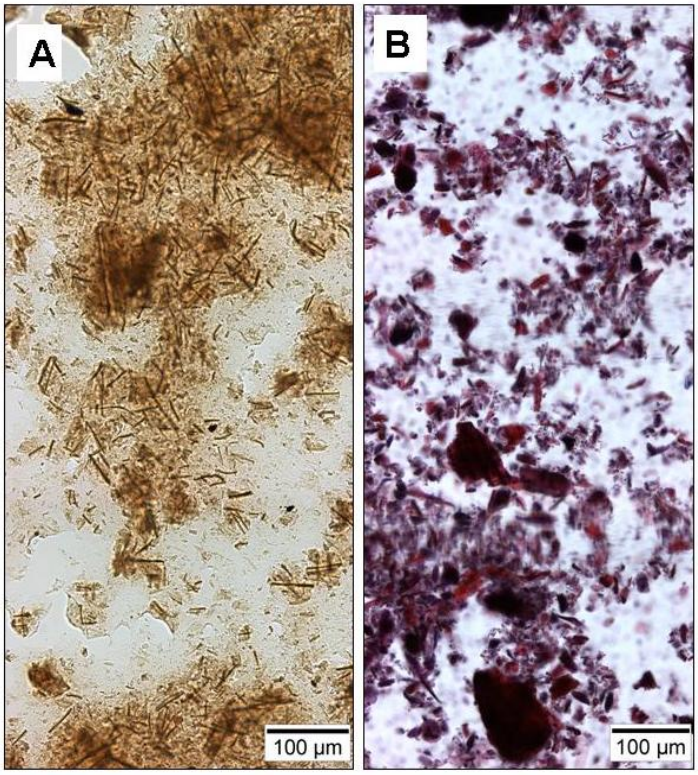

Fig. 22. CEL-P residue A) native sample with x20 magnification, B) dyed sample with x20 magnification

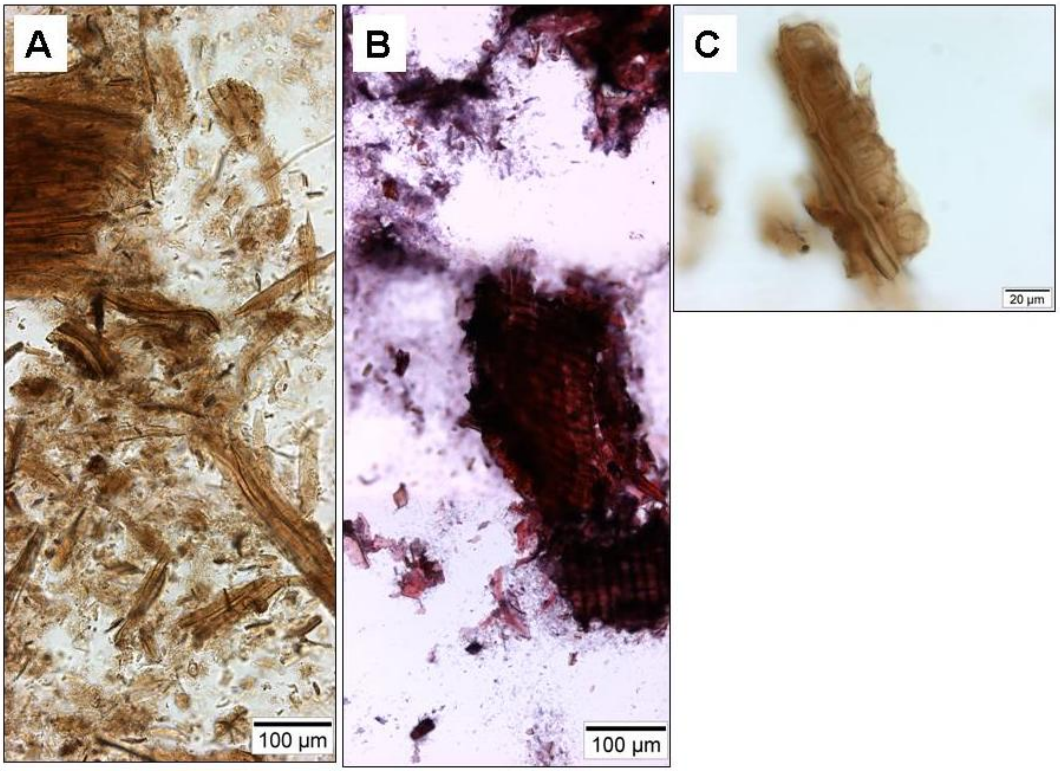

Fig. 23. Acid-residue A) native sample with x20 magnification, B) dyed sample with x20 magnification, C) native sample with x50 magnification

Microscopic pictures of the freeze-dried and wet alkali-lignin samples are shown in figures 24 and 25, respectively. Drying has caused alkali-lignin to pack into angular particles of varying shapes and sizes (figure 24A). The majority of particles was coloured deep red with Safranin and Alcian Blue dyes (figure 24B), but higher magnification reveals the presence of small blue particles in the sample (figure 24C). This indicates that in addition to lignin, some other substance may have dissolved and precipitated in the extraction process. The native wet alkali-lignin had a syrup-like appearance and separate particles were not visible in the light microscope (figure 25A). The colour of the dyed sample was dominated by purple, when viewed with lower magnifications (figure 25B), but agglomerates of red and blue particles could be visualised with higher magnifications (figure 25C). Drying was shown to have a dramatic effect on the structure of alkali-lignin: particle sizes of the dry sample were enormous when compared with those of the wet sample. Expectedly, adsorption experiments with these samples showed also considerable difference in adsorption capacity.

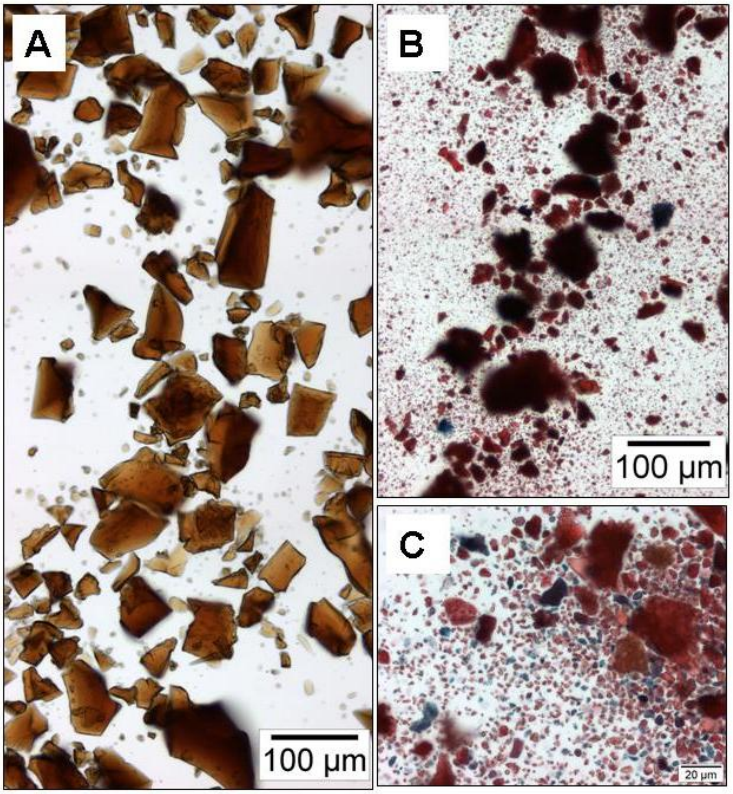

Fig. 24. Freeze-dried alkali-lignin: A) native sample with x20 magnification, B) dyed sample with x20 magnification, C) dyed sample with x50 magnification

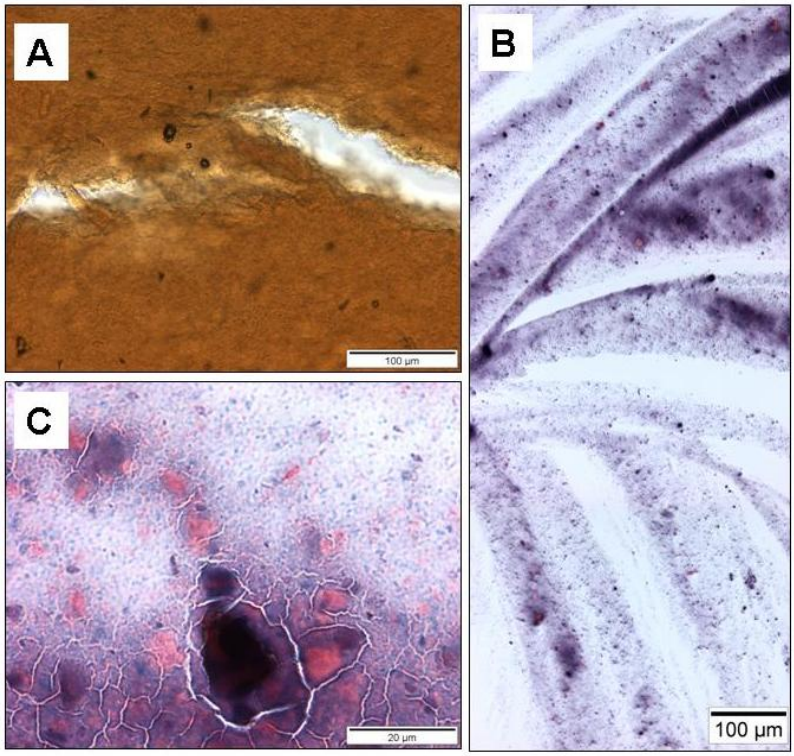

Fig. 25. Wet alkali-lignin A) native sample with x20 magnification, B) dyed sample with x20 magnification, C) dyed sample with x100 magnification. Cracks in C are caused by drying.

7.2 Adsorption experiments

The results from adsorption experiments are presented in histograms. The category axis (x-axis) lists the adsorption substrates used in the experiment and the value axis (y-axis) shows the protein content or enzyme activity that was measured from the supernatant after the adsorption experiment. The values on y-axis are given as percentages of originally added protein content or enzyme activity. Accordingly, good sorbents result in lower bars. The top ends of the bars show the arithmetic means of measured values and the error bars define the 95% confidence interval. In some cases, the value of the bar is based on one measurement and the confidence interval could not be defined.

Adsorption conditions were the same in all adsorption experiments: 50 mg of protein/g of adsorption substrate and tube incubation at +4ºC for 90 min in a rotating mixer.

7.2.1 The effect of CEL purification and drying on the adsorption capacity of the lignin samples

After the isolation procedure, two purification methods were tested for CEL-lignin and the effect of purification on the adsorption capacity of the sample was studied. In addition, the essential role of the protease in the purification was confirmed. It was also studied if freeze-drying affects the adsorption capacities of isolated lignins.

The enzymatically isolated hydrolysis residue (CEL) is contaminated with cellulolytic enzymes that remain bound to the residue after isolation and two methods, protease and urea treatments, were employed to remove the solid-bound enzymes. The effect of protein removal on the adsorption capacity was tested accordingly: CEL, protease treated CEL and urea treated CEL samples were used in an adsorption experiment with Celluclast 1.5L cellulase preparation. Adsorption was quantified with protein content measurements from the adsorption supernatant and the results are presented in figure 26. Protease purified CEL was found to be the best protein sorbent, which shows that purification is essential in order to restore the adsorption capacity of the CEL residue.

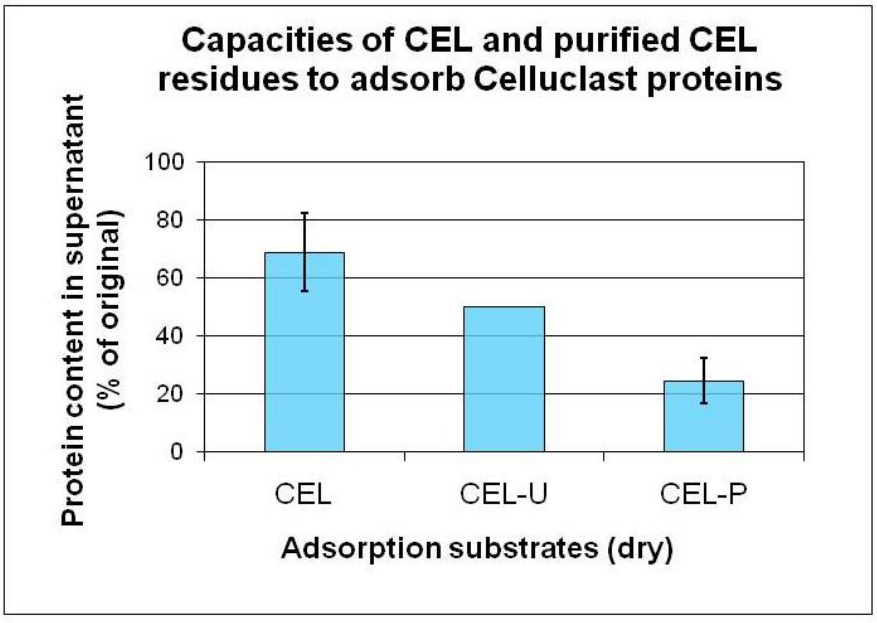

Fig. 26. Adsorption capacities of the unpurified residue (CEL), urea purified residue (CEL-U) and protease purified residue (CEL-P) to adsorb Celluclast 1.5L enzymes.

The protease purification was carried out in alkaline conditions (pH 9.6) and obviously some lignin was dissolved from the sample in the treatment conditions. Thus, the better adsorption capacity of CEL-P could be caused by changes in the sample structure and

for this reason, CEL was treated in similar conditions with and without the addition of protease and both samples were used in adsorption experiments with Celluclast 1.5L enzymes. Treatment in purification conditions without the addition of protease (CEL*) affected the adsorption capacity of the sample to some extent, but the enzymatic removal of proteins (CEL-P) was shown to be the main reason for the better adsorption capacity (figure 27). Based on this and the previous result, CEL-P was the only purified lignin sample chosen to be used in further adsorption studies.

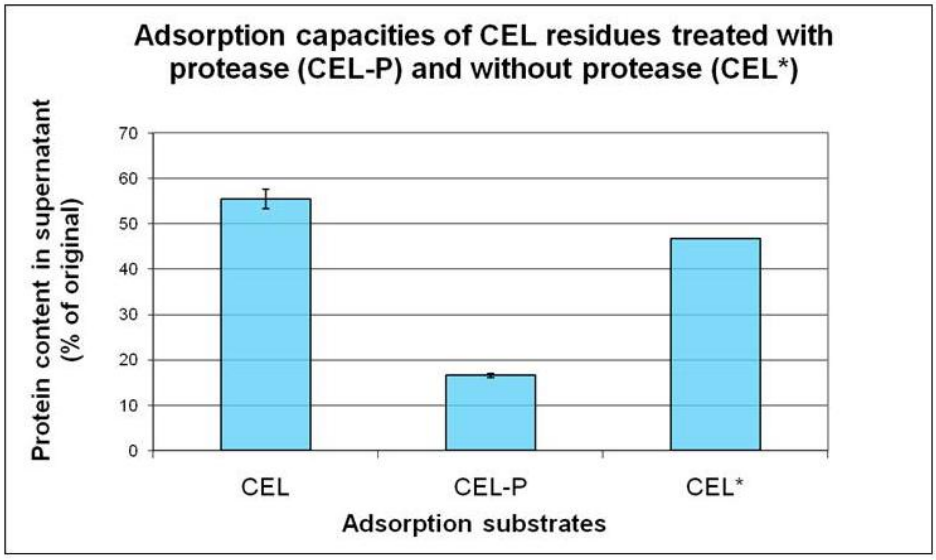

Fig. 27. Adsorption capacities of CEL, protease purified CEL (CEL-P) and CEL treated in purification conditions without the addition of protease (CEL*). The lack of error bar in CEL* sample is because the exact values were obtained of the duplicate samples.

The adsorption capacities of freeze-dried and wet lignin samples were compared to study the effect of drying on the adsorption capacity. Dry and pulverised samples would be easier to store and handle, and thus more practical for further studies. Figures 28_A and 28_B show the adsorption capacities of freeze-dried and wet adsorption substrates, respectively. As measures of adsorption, endoglucanase activity and protein contents were determined from the adsorption supernatant. Wet and dry CEL, CEL-P and acid-lignin had highly similar capacities to adsorb cellulases, whereas the behaviour of alkali-lignin differed significantly. Wet alkali-lignin adsorbed enzymes (figure 28_B) whereas dry alkali-lignin lacks the capacity to adsorb enzymes (figure 28_A). An apparent reason for these results is that drying was shown to have a tremendous effect on the available surface area of alkali-lignin (figures 24 and 25). Drying may also introduce minor changes to the structures of other adsorption substrates. Consequently, wet adsorption substrates were exclusively used in further studies.

The protein concentrations were under the quantification limit in certain adsorption supernatants. More sensitive method should have been used and for this reason, data from endoglucanase activity measurement was included to give an indicative estimate of total protein in the adsorption supernatant.

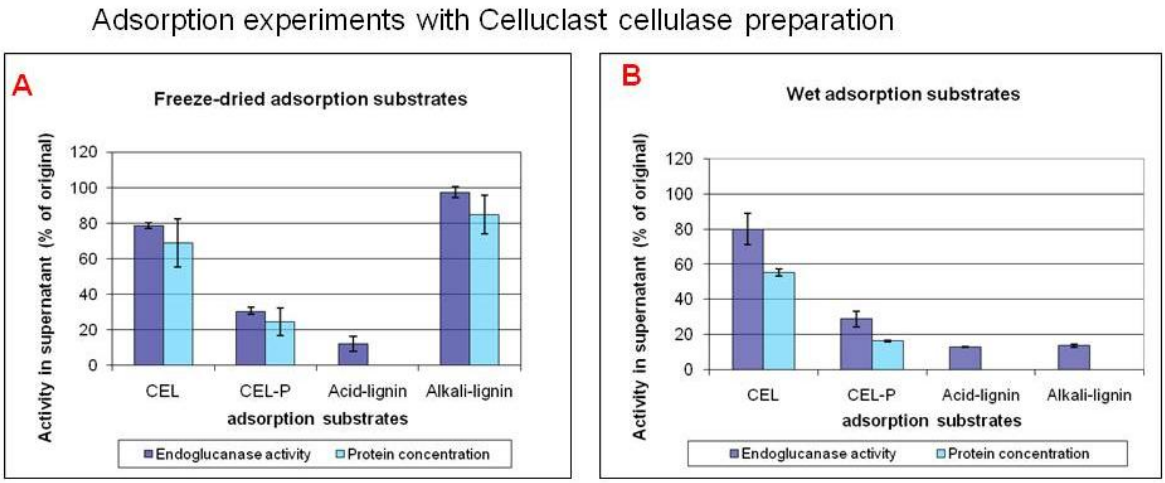

Fig. 28. The effect of drying on the adsorption capacities of adsorption substrates: A) adsorption capacities of freeze-dried adsorption substrates, B) adsorption capacities of wet adsorption substrates.

7.2.2 Adsorption of *Trichoderma reesei* cellulases (Celluclast 1.5L)

Protein content of the supernatant does not reveal details of the competitive nature of cellulase adsorption. The Celluclast preparation contains many cellulases and certain enzymes tend to have higher affinity to the sorbent than others. In this study, enzyme activity measurements were employed to find out if there are differences in the adsorption behaviour of *Trichoderma reesei* cellulases and if these differences can be studied with enzyme activity measurements.

Isolated lignin samples were chosen to be used in the wet formulation in further studies and wet lignocellulosic SPS and dry microcrystalline cellulose powder were served as cellulose-rich adsorption substrates. After the adsorption experiment, the supernatant was subjected to the three cellulase activity assays: endoglucanase, Cel7A (CBH I) and β-glucosidase assays. In addition, the total protein content was measured from the adsorption supernatant. Results are shown in figure 29. All the three lignin-rich samples (CEL-P, acid-lignin, alkali-lignin) seem to have a high capacity to adsorb all types of *Trichoderma* cellulases and similar adsorption pattern is visible with all the three lignin samples: β-glucosidase activity was reduced the most, whereas CBHI and endogluca-

nase activities were less decreased than β-glucosidase activity. However, differences between the enzyme activities were relatively small and not necessarily statistically significant.

The adsorption patterns of cellulose-rich sorbents, SPS and microcrystalline cellulose, are distinctive from that of the lignin samples. Enzyme activities in the supernatant showed considerable variation: β-glucosidase activity was least decreased and there is a clear difference in endoglucanase and CBHI activities. Protein concentration, β-glucosidase activity or both remained under the detection limit in three samples (SPS, acid-lignin and alkali-lignin), and hence these results are not given in figure 29.

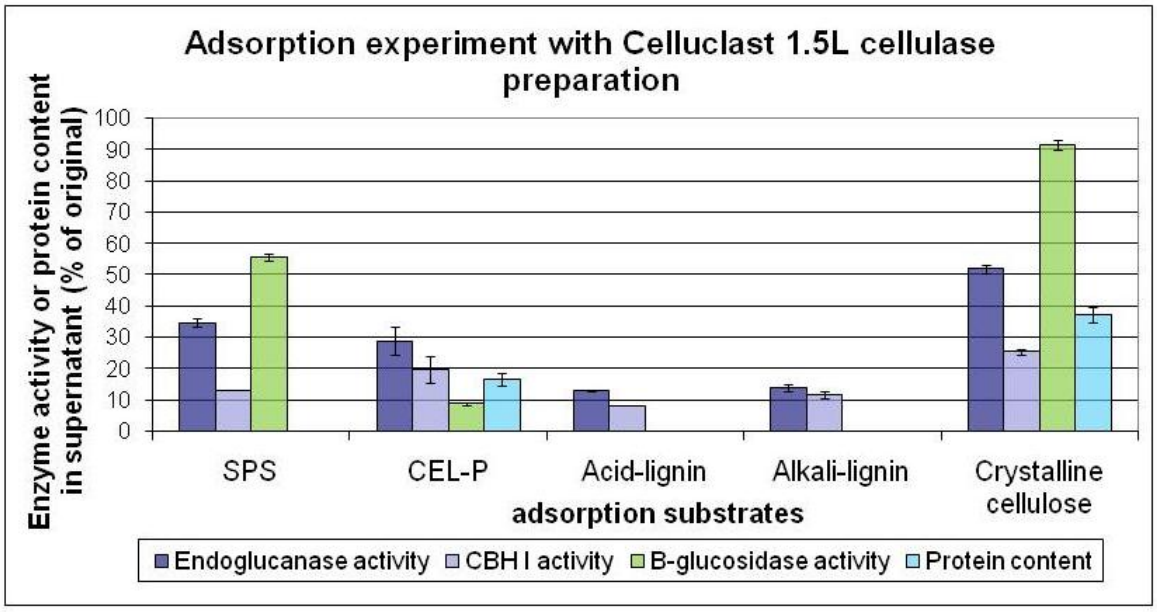

Fig. 29. Adsorption of Celluclast 1.5L enzyme preparation on different adsorption substrates. Adsorption was measured with cellulase activity measurements and protein content measurement from adsorption supernatant.

Furthermore, SDS-PAGE was used to visualise the distribution of Celluclast proteins among the solid fraction and the adsorption supernatant (figure 30). All samples, excluding microcrystalline cellulose, were used for the analysis. The picture shows clearly, that solid fractions of lignin (CEL-P, alkali-lignin and acid-lignin) have adsorbed effectively all kinds of proteins and little was left in the supernatant. Obviously, there is more variation in the distribution of proteins on the lanes that represent fractions from SPS. Accordingly, the results from protein electrophoresis are consistent with the results from enzyme activity measurements.

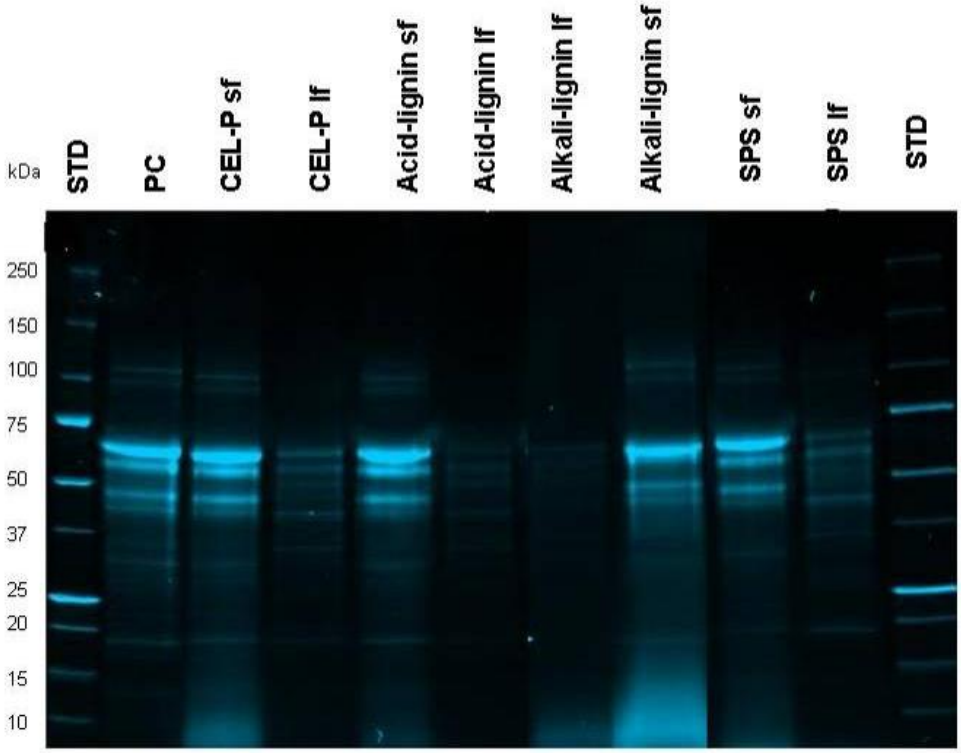

Fig. 30. Fractions obtained from adsorption experiment with Celluclast 1.5L cellulase preparation in SDS-PAGE gradient gel (4 to 20%). Criterion fluorescent imaging system was used for visualisation. The picture was constructed of two gel images. Abbreviations: solid fractions (sf), adsorption supernatants (lf), protein size standard (STD), positive control (PC). Positive control contains the original amoun of Celluclast proteins used in the adsorption experiment (0.5 mg/ml).

7.2.3 Adsorption of *Aspergillus niger* β-glucosidases (Novozym 188)

The SPS, acid-lignin and CEL-P samples were used to adsorb enzymes of *Aspergillus niger* enzyme preparation (Novozym 188) in the same adsorption conditions as in previous experiments. Novozym 188 preparation has high β-glucosidase activity and it was the only parameter measured from the adsorption supernatant. *Aspergillus* β-glucosidases showed substantially different adsorption behaviour from *Trichoderma* β-glucosidase: almost all of the initially added activity remained in the supernatant with SPS and CEL-P and acid-lignin showed only minor adsorption capacity (Figure 31).

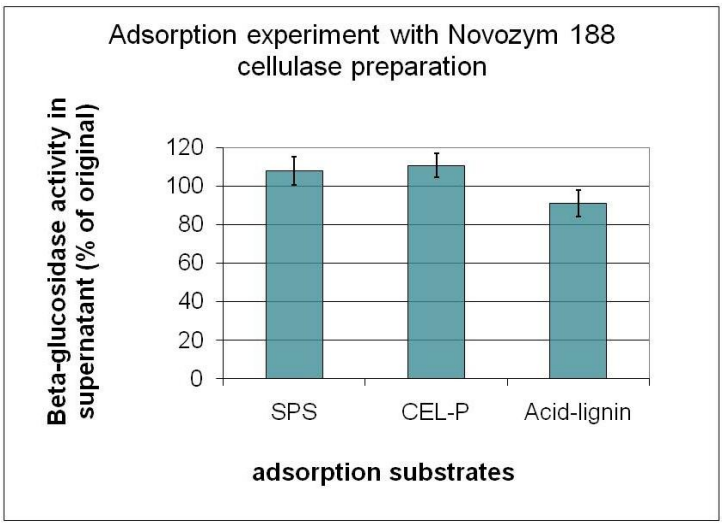

Fig. 31. Adsorption of Novozym 188 cellulase preparation with SPS and isolated lignin-rich residues, CEL-P and acid-lignin.

The results from activity measurements were confirmed also with SDS-PAGE and figure 32 shows the distribution of proteins between adsorption supernatant (lf) and solid fraction (sf) on a gel. The gel picture shows that the majority of Novozym-proteins remain soluble in liquid fraction, including the 137 kDa (Unno et al. 1993) major β-glucosidase of *A. niger*. In addition to β-glucosidase, the preparation contains also an array of other proteins and interestingly, only few of them seem to have affinity on the adsorption substrates.

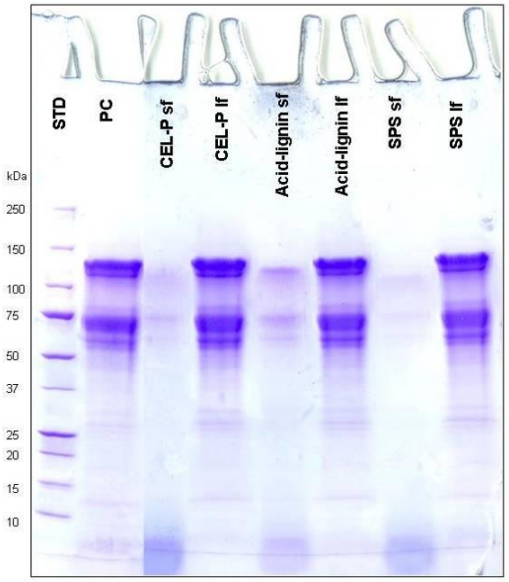

Fig. 32. Fractions obtained from adsorption experiment with Novozym 188 enzyme preparation in SDS-PAGE gradient gel (4 to 20%). Coomassie staining was used for visualisation. Abbreviations: solid fractions (sf), adsorption supernatants (lf), protein size standard (STD), positive control (PC).

7.2.4 Adsorption of bovine serum albumin (BSA)

Bovine serum albumin (BSA) was used in adsorption experiments with microcrystalline cellulose, SPS, acid-lignin and CEL-P. In general, BSA is used to prevent unspecific protein binding on hydrophobic surfaces. Since BSA lacks catalytic activity, protein content measurement was the only means to quantify BSA from the adsorption supernatants. BSA was found to have a high affinity on lignin-rich substrates and accordingly, protein content in the adsorption supernatants was under the detection limit of the used protein assay. On the contrary, BSA had significantly less affinity on cellulose-rich substrates, SPS and microcrystalline cellulose and the protein concentration could be measured from the adsorption supernatant (figure 33). However, all adsorption solids and supernatants were used for SDS-PAGE and the high affinity of BSA to lignin-rich substrates can be verified from the gel picture (figure 34).

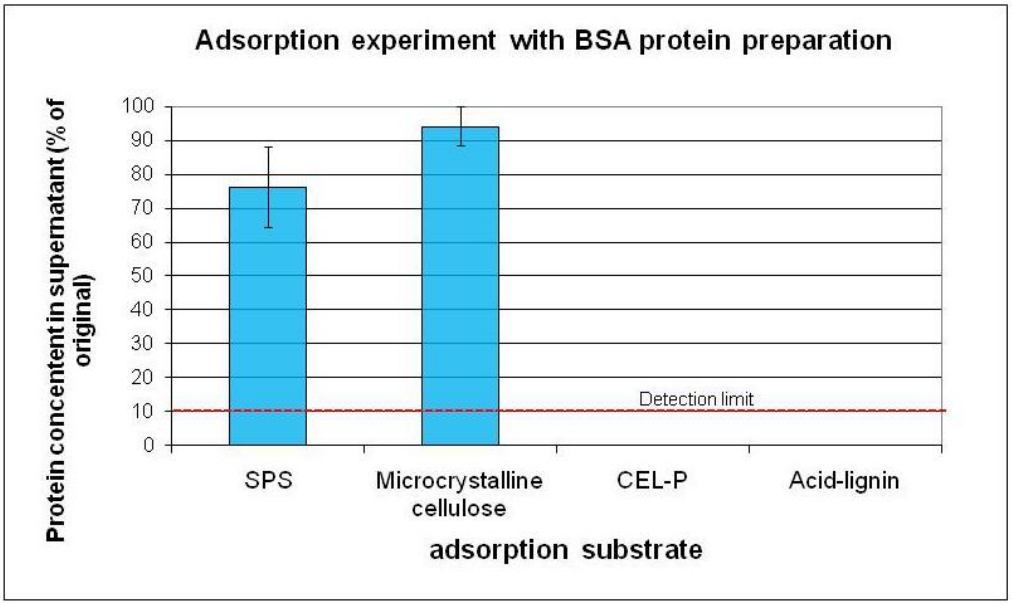

Fig. 33. The adsorption of bovine serum albumin (BSA) on cellulose-rich adsorption substrates (SPS and microcrystalline cellulose) and lignin substrates (CEL-P and acid-lignin). Protein concentration was under the detection limit with lignin adsorption substrates.

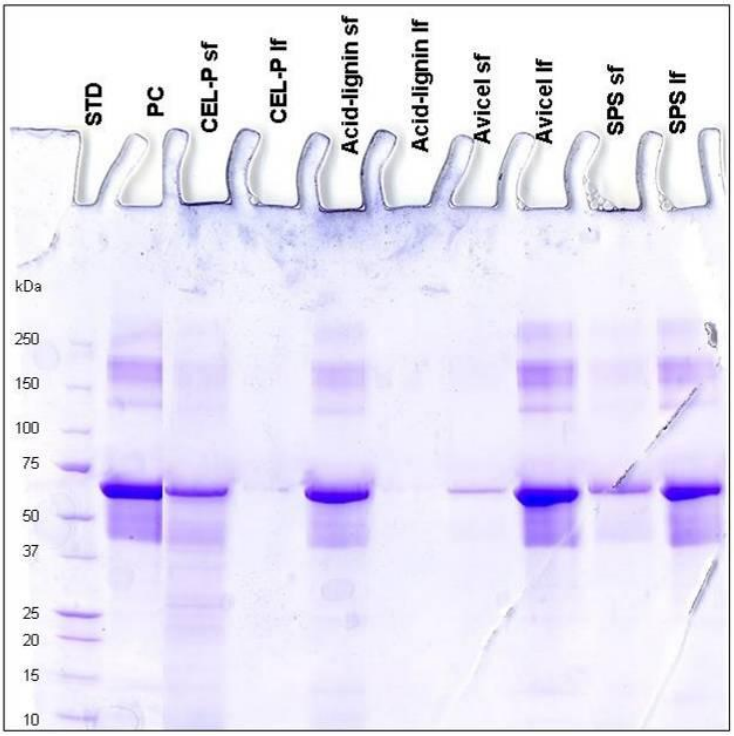

Fig. 34. Fractions obtained from adsorption experiment with BSA in SDS-PAGE gradient gel (4 to 20%). Coomassie staining was used for visualisation. Abbreviations: solid fractions (sf), adsorption supernatants (lf), protein size standard (STD), positive control (PC), Avicel (microcrystalline cellulose).

7.3 Activity and hydrolysis capacity of solid-bound Trichoderma reesei cellulases

7.3.1 Activities of solid-bound *Trichoderma reesei* cellulases

The wet CEL-P and alkali-lignin samples were chosen as model sorbents to test the activity of solid-bound *Trichoderma* cellulases from the Celluclast 1.5L preparation. First, adsorption experiments were carried out as previously, but the endoglucanase and CBHI enzyme activities were measured from three fractions after the experiment: 1. Solid fraction, 2. Adsorption supernatant and 3. Washing buffer from adsorption solids. The results from endoglucanase and CBHI activity measurements are shown in figures 35 and 36, respectively. In the figures, enzyme activities from all the three fractions are combined into one bar, and the 100% level on the value axis shows the activity which was initially added in the adsorption experiment. Interestingly, over 100% of the originally added endoglucanase activity could be tracked when the three fractions were combined (figure 35).

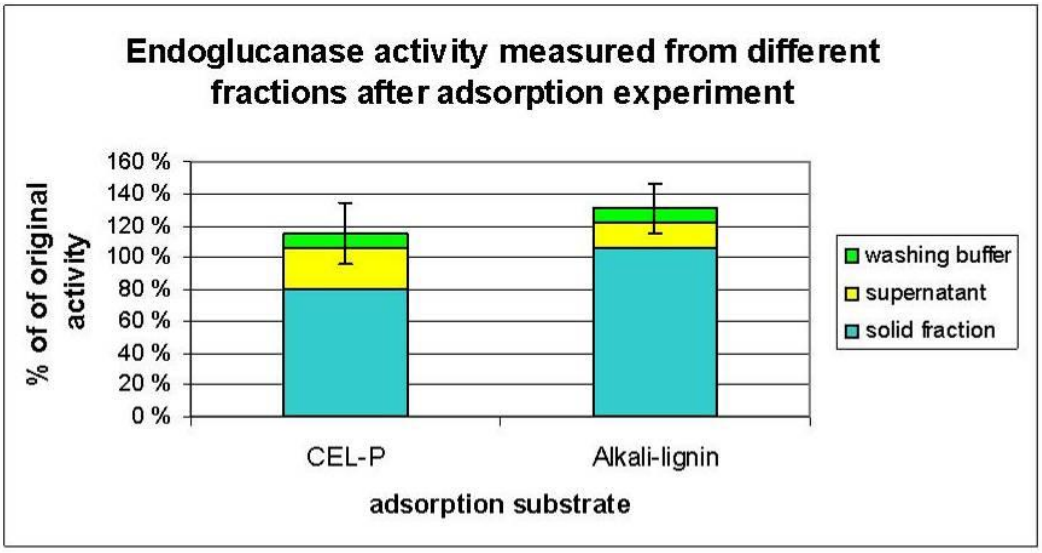

Fig. 35. Endoglucanase activities measured from the three fractions after an adsorption experiment with Celluclast cellulase preparation: (■) washing buffer of adsorption solids, (■) adsorption supernatant and (■) washed adsorption solids.

On the contrary, summing up CBH I activities from the three fractions added up only to 60-80% of the original CBHI exoglucanase activity (figure 36). Comparison of figures 35 and 36 reveals that activities measured from the solid residue were proportionally lower in CBHI measurements than in endoglucanase measurements. This suggests that solid-bound CBHI type exoglucanases loose some of their activity towards MUL substrate or that the activity measurement fails to give reliable results from the solid-bound enzymes.

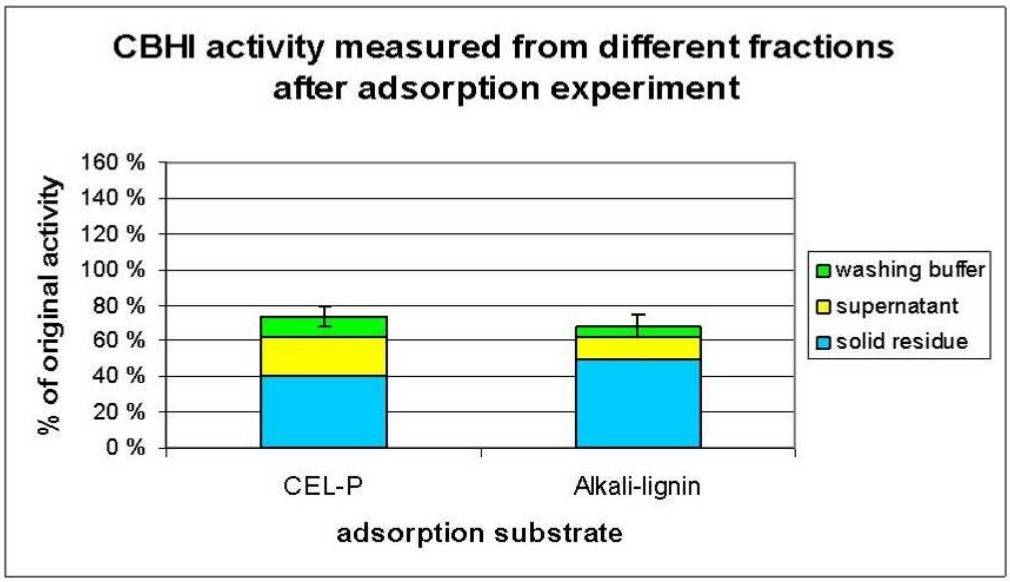

Fig. 36. Cel7A activities measured from the three fractions after an adsorption experiment with Celluclast enzyme preparation: (■) washing buffer of adsorption solids, (■) adsorption supernatant and (■) washed adsorption solids.

7.3.2 Hydrolysis of microcrystalline cellulose with lignin-bound *Trichoderma reesei* cellulases

As shown in the previous chapter, lignin-bound endoglucanases and CBH I enzymes sustain majority of their catalytic activity towards soluble substrates, and thus lignin-bound cellulases were tested for their ability to degrade crystalline cellulose in a 72 hour hydrolysis. Reactions were supplemented only with a β-glucosidase preparation (Novozym 188) since Celluclast 1.5L preparation has a low β-glucosidase activity.

The course of hydrolysis with the bound enzymes is presented in figure 37. The rate of hydrolysis was rapid for the first hours, but soon it decreased dramatically and remained slow. The hydrolytic capacity of lignin-bound enzymes was compared to that of free enzymes: the enzymes bound on alkali-lignin reached an average 17% hydrolysis yield, whereas CEL-P-bound enzymes performed slightly better with an average of 24% yield.

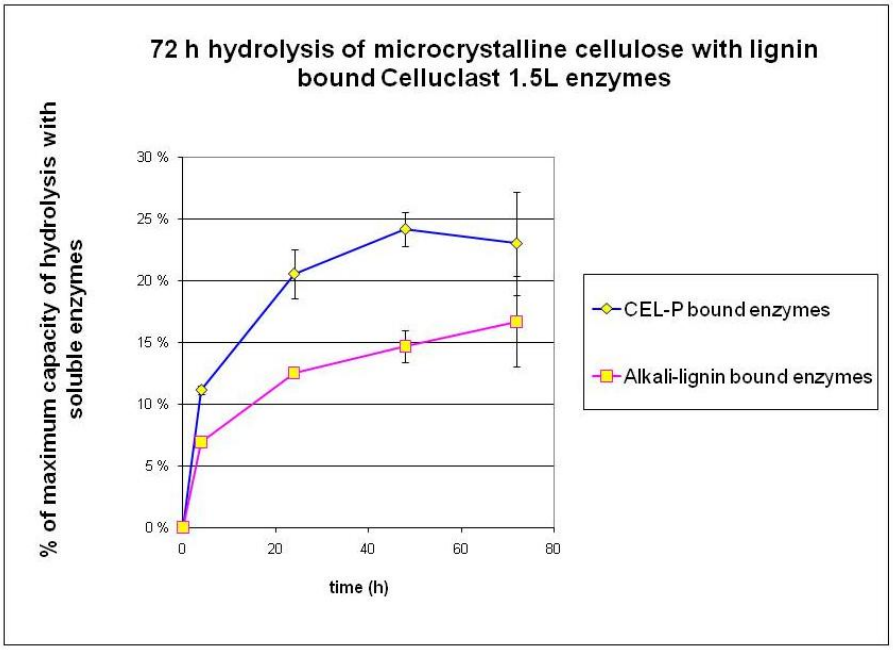

Fig. 37. The hydrolytic capacities of CEL-P-bound enzymes and alkali-lignin-bound enzymes. The hydrolytic capacity of solid-bound enzymes is compared to that of equal amount of free Celluclast 1.5L enzymes.

8 Discussion

8.1 Isolation and characterisation of adsorption substrates

Steam pretreated spruce (SPS) was chosen as starting material for lignin extraction since *Trichoderma* cellulases are known to have a high affinity on SPS-derived lignins. If there are common interactions that drive enzyme adsorption on lignin, they are strongly present if SPS-derived sorbents, which makes SPS suited for adsorption studies. However, it should be remembered that the softwood origin and pretreatment methods have an impact on the lignin structure and there may be structures that are not present in any other lignocellulosic biomass. Accordingly, enzymes that bind on SPS-derived lignin may behave differently with some other substrate.

As previously mentioned, lignin isolation is far from simple and the suitability of the isolation process should be carefully evaluated if the isolated lignin is used in adsorption studies. The first aim of this study was to evaluate the pros and cons of extraction methods and the suitability of different samples for adsorption studies. This ranking is summarised in table 8. Despite of the residual, contaminating carbohydrates and the tedious extraction and purification protocols, protease purified CEL was considered as the best alternative for further adsorption studies. Carbohydrate degradation of CEL-P sample was carried out in mild conditions when compared with acid hydrolysis. Mild isolation procedure is important in preserving the chemical structure of lignin. Furthermore, particles of CEL-P are very likely to be similar with those that are present during enzymatic hydrolysis of SPS, which is desirable in some research applications. Alkali-lignin was relatively easy to extract, but the fraction represented only about 15% of total lignin in SPS. In addition to several advantages, the use of CEL-P holds certain drawbacks which are discussed next.

Table 8. Comparison of the isolation methods of lignin. Assessment is based on the fact that the material will be used for adsorption or screening purposes

Lignin sample	Advantages	Disadvantages
CEL (enzymatic hydrolysis residue)	• similar structures are present in process conditions • represents total lignin of the wood sample • carbohydrate degradation with enzymes preserves the chemical structure of lignin	• tedious extraction process • purification of the residue is essential to remove solid-bound enzymes prior to adsorption experiments • sample includes residual carbohydrates
Acid-lignin (acid hydrolysis residue)	• represents total lignin of the wood sample	• strong acids are likely to alter the lignin structure • possibly contaminated with carbohydrates
Alkali-lignin (dissolved lignin)	• easy extraction process • low amount of carbohydrate contaminants	• represents only a small fraction of the total lignin in the wood sample • drying alters adsorption properties of the material

The CEL-P sample was shown to be more contaminated with carbohydrates than alkali-lignin. Most probably it is also more contaminated than acid-lignin. This raises the question, weather cellulases were bound to carbohydrates rather than to lignin during the adsorption experiments. However, the data from adsorption experiments showed (Figure 29) that enzyme activities in supernatant followed the same adsorption pattern in all three lignin samples: acid-lignin, alkali-lignin and CEL-P. Accordingly, it can be assumed that enzyme-lignin interactions dominate cellulase binding to CEL-P.

Nitrogen analysis revealed that the protease treatment was more efficient in removing solid-bound enzymes than the urea treatment. This result was consistent with results from adsorption experiments - CEL-P was a better sorbent than CEL-U or unpurified

CEL. Despite extensive protease treatment, some protein seemed to remain bound on the CEL-P residue which is likely to reduce protein binding during adsorption experiments. For this reason, protease treatment should be further optimised in order to achieve even better purification results. Difficulties in enzyme removal from CEL demonstrated that the protein-lignin interactions are exceptionally strong, but majority of them are not covalent as the SDS-treatment and electrical currency were able to detached proteins from lignin during the SDS-PAGE.

The use of proteases in CEL purification raises the question whether the CEL-P residue is contaminated with protease activity that would cause cellulase degradation during adsorption experiments. Consequently, protease activity was assayed from CEL-P adsorption supernatant and none could be detected.

When carbohydrate analysis results were interpreted, variation in mannose contents of CEL-type samples was observed. Because the mannose content of SPS was low, it was obvious that mannose had enriched to CEL-type hydrolysis residues. Most probably mannose was originated from glycosylated enzymes of *T. reesei*, because protein removal resulted in lower mannose contents for CEL-U and CEL-P samples. Enzymes of filamentous fungi are known to carry high-mannose N-glycans (Maras 1999). This gave rise to the idea that the extent of protein contamination could be directly estimated from the mannose content. Figure 38 compares nitrogen and mannose contents of purified samples (CEL-P and CEL-U) with unpurified CEL-sample. The higher proportion of nitrogen in CEL-U sample may be caused by urea contaminants if washing of CEL-U was inadequate after the urea treatment. In this case, mannose content may serve as a better means to estimate the effect of purification on the amount of solid-bound protein. It is more troublesome, however, to use the mannose content in the determination of exact amounts of bound proteins, because the amount of enzyme glycosylation in an enzyme preparation is dependent on the used production strain and growth conditions. Glycosylation of *T. reesei* enzymes Cel6A, Cel7B and Cel5A were reported to account for 12-24% of the enzyme's molecular mass (Hui et al. 2002). If the amount of protein in CEL sample is calculated based on these figures, CEL residue would contain 34-68 mg enzyme/g of CEL, which is not an accurate result but the order of magnitude is reasonable which indicates that the mannose is originated from enzyme glycosylations. As a conclusion, the results from nitrogen and mannose content analysis were consistent

and together they can be used to estimate the amount of solid-bound protein in the sample.

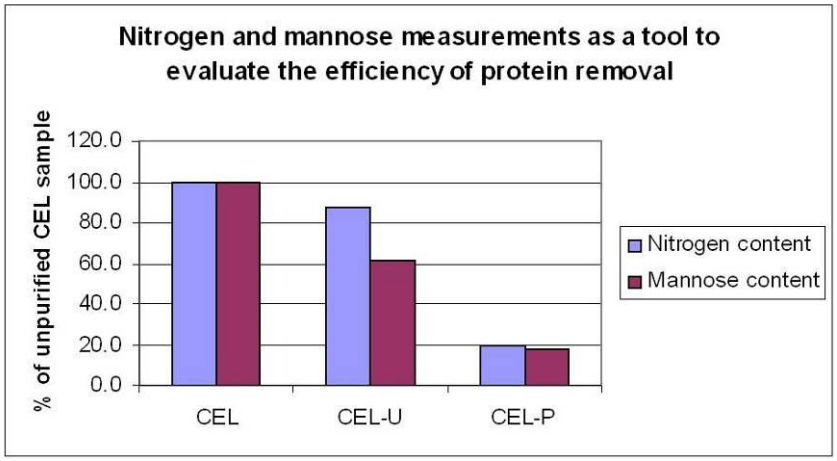

Fig. 38. Nitrogen and mannose contents of purified samples, CEL-U and CEL-P, were compared to those of unpurified CEL.

The microscopic pictures revealed major differences in particle sizes and structures even though the samples appeared similar to the naked eye. The hydrolysis residues, CEL and acid-lignin, sustained the shapes of wood cell structures and accordingly, variation in particle shapes and sizes in the samples was overwhelming. After the Safranin/Alcian Blue dye treatment, the colouring of hydrolysis residues was more red than purple, which indicates that lignin had exposed to particle surfaces. Freeze-drying did not have any strong influence on the structures or adsorption capacities of hydrolysis residues, whereas dissolved alkali-lignin responded very differently to drying. The wet alkali-lignin sample had a syrup-like sticky consistency, whereas drying caused agglomeration. Alkali-lignin samples could be stored dry, but had to be dissolved in alkaline conditions and precipitated with acid prior to adsorption experiments. Presumably, drying has always some effect on the structure of biomass, whether it is visible in microscope or not. For this reason, only wet substrates were used in further studies.

In this study, acid-lignin was found to adsorb *T. reesei* cellulases, whereas Palonen et al. (2004) reported that pure Cel7A and Cel5A enzyme components of *T. reesei* did not adsorb on similarly prepared acid-lignin. In the study of Palonen et al. (2004), acid-lignin was dried in +105ºC oven prior to adsorption studies, which had most probably destroyed the adsorption capacity of the substance. This observation shows that in addi-

tion to drying, lignin-rich samples should not be treated in high temperatures if they will be used for adsorption experiments.

Microscopic pictures of adsorption substrates revealed great variation in particle sizes and shapes. In addition to visual characterisation, particle sizes were also analysed with laser equipment, but reliable results could not be obtained due to the particle shape heterogeneity. However, when the particles are used in adsorption experiments special attention should be paid to the interpretation of the results. According to data in figure 29, it seems that alkali-lignin is a better enzyme sorbent than SPS. However, such conclusions can only be drawn if the available surface area for enzyme adsorption is similar in both adsorption substrates. Next section will discuss how to avoid misinterpretation of the adsorption results.

8.2 Competitive adsorption of Trichoderma reesei cellulases

Enzyme activity measurements were employed to study competitive adsorption of cellulases on different adsorption substrates. It was attempted to find out if enzyme activity measurements could be used to study differences in the adsorption behaviour of different enzyme activity classes. Comparison of *T. reesei* enzyme adsorption was carried out with lignin-rich (CEL-P, acid-lignin and alkali-lignin) and cellulose-rich samples (SPS and crystalline cellulose). Interestingly, highly distinctive pattern of adsorption was revealed between lignin-rich and cellulose-rich particles.

The lignin-rich samples adsorbed considerable amounts of proteins, and particularly notable was that almost equal proportions of endoglucanase, Cel7A (CBH I) and β-glucosidase activities had remained in supernatant after the adsorption experiment. Accordingly, it can be concluded that enzymes, contributing to enzyme activity levels, had similar affinities to lignin. As previously mentioned, the pattern was considerably different with cellulose-rich surfaces: higher proportion of Cel7A activity was adsorbed when compared to endoglucanase and β-glucosidase activities. This result is consistent with a recent study that showed the pure Cel7A (CBH I) to have higher affinity on cellulose surface than the major endoglucanase Cel7B of *T. reesei* (Orelma 2008).

Nevertheless, the use of enzyme activity measurements as a tool to measure enzyme adsorption is not straightforward. Several enzymes, present in different amounts, may

be active on the same substrate, but their structure and adsorption behaviour may differ significantly. For this reason, activity measurements reflect the adsorption behaviour of the most abundant enzymes that respond to a certain activity measurement and activities of minor enzyme components are masked. Accordingly, enzyme activity assays are not suited for detailed studies of adsorption dynamics, but more general conclusions, concerning major enzyme components in the mixture, can be drawn of cellulase adsorption.

Adsorption supernatants and adsorption solids were analyzed with SDS-PAGE gels in order to find out whether certain enzymes are enriched to the liquid or solid phase. Unfortunately, the major cellulases of *T. reesei* are of similar size (50 to 70 kDa) and separate bands could not be detected for band intensity analysis. Nevertheless, SDS-PAGE proved as a good tool to verify which fraction contained the majority of proteins. It was somewhat surprising that the SDS-PAGE could be carried out successfully also with solid fractions after adsorption experiment; electrical current detached the solid-bound enzymes from particles and they were distributed to the gel according to their molecular size. Unfortunately, gel lanes that contained samples of solid lignin fractions were clearly smearier than the lanes with liquid fraction samples. The background was probably caused by low molecular weight particles that had dissolved from the solids during the run.

8.3 Comparison of adsorption between *Aspergillus niger* and *Trichoderma reesei* β-glucosidases

Interestingly, *A. niger* β-glucosidase enzymes did not show affinity on lignin- or cellulose-rich surfaces which was confirmed with β-glucosidase activity assays as well as SDS-PAGE analysis. The SDS-PAGE gel picture in figure 32 revealed, that majority of proteins in Novozym 188 preparation remained in the liquid phase after the adsorption experiment. Proteins in Novozym 188 preparation may have low affinity on lignin- and cellulose-rich surfaces or the preparation contains substances, such as surfactants, that prevent enzyme binding. However, previous studies support the fact that Novozym 188 enzymes have reduced affinity on lignaceous hydrolysis residues (Tu et al. 2007a).

8.4 BSA adsorption on different adsorption substrates

The BSA preparation was found to have high affinity on all tested lignin-rich samples (CEL-P and acid-lignin) and, on the other hand, BSA was found to adsorb considerably less on SPS and very little on crystalline cellulose. These results are consistent with previous studies (Yang & Wyman 2006, Kawamoto et al. 1992). BSA is generally known to adsorb especially on hydrophobic surfaces and these results indicate that both surfaces (CEL-P and acid-lignin) are hydrophobic which drives BSA adsorption.

8.5 Enzymatic activities of lignin-bound T. reesei endoglucanases and CBHI (Cel7A) exoglucanase

Solid fractions of CEL-P and alkali-lignin were subjected to enzyme activity measurements to find out if solid-bound enzymes were still active. This experiment showed that endoglucanase activity remained high despite of immobilisation, whereas Cel7A type exoglucanase showed decreased CBHI activity when enzymes were bound on the lignin particles.

Over 100% of originally added endoglucanase activity could be traced back from the three fractions obtained after adsorption experiment and the same result was obtained with alkali-lignin and CEL-P as the protein sorbent. A plausible explanation for the activities exceeding 100% is the magnetic stirring which was used during the activity measurements. Stirring was applied only to the samples that contained solid lignin particles in order to avoid particle sedimentation during the activity measurement, whereas mixing was not included to the positive control. Sometimes, however, immobilisation may enhance the enzyme activity. Uraki et al. (2001) immobilized a commercial cellulase mixture on lignin derivative particles and found out that filter paper activity and stability of the mixture was enhanced after immobilisation.

When it comes to Cel7A type exoglucanase enzymes, clear activities were measured from the solid fractions, but the originally applied activity could not be achieved and the same result was obtained from enzymes incubated with CEL-P and alkali-lignin. Binding may interfere with the catalytic site (Figure 39A) or alternatively, lignin interferes with the colorimetric activity assay by masking the actual enzymatic activity. Lignin

caused background to the assay, which was taken into account in the calculations, but the linearity of the assay could be further studied by using internal standards.

These results show, however, that enzyme binding on lignin does not denature the enzyme structure completely because the catalytic activity is preserved. A probable explanation for this is that the studied enzymes bind mainly from their CBM-domains, which has been shown by Palonen et. al (2004) with the full-length Cel7A and Cel5A enzymes. CBM-mediated binding may allow the catalytic core to function normally on soluble substrates.

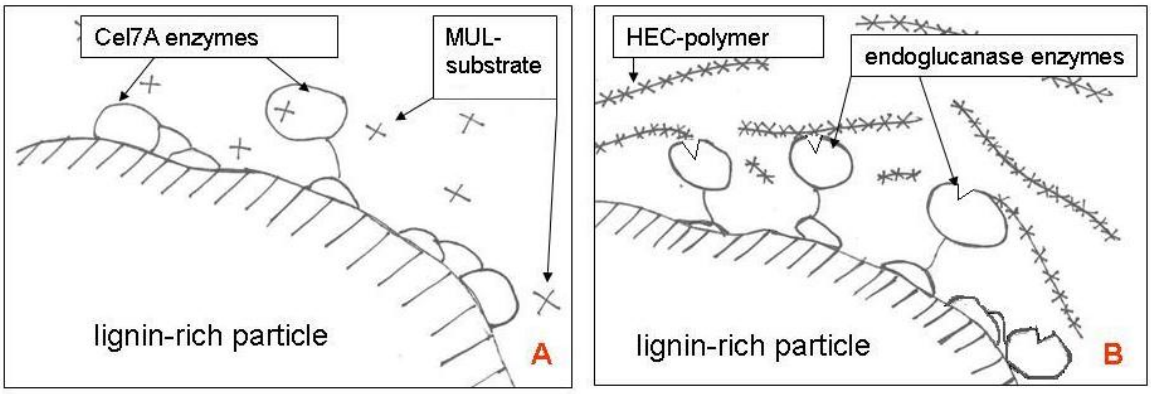

Fig. 39. Difference in cellulase binding on lignin-rich particles A) exoglucanase Cel7A binding and B) endoglucanase binding

Protein binding on hydrophobic surface is generally thought to denature the protein structure, which intensifies the protein-surface interaction and results in irreversible binding. Lignin surfaces are hydrophobic and it can be speculated that CBM-domains may undergo denaturation on lignin surface, which anchors the protein irreversibly but allows catalytic core to function normally. The Cel7A core may have more interaction with lignin (figure 39A) than endoglucanase cores (figure 39B) which could result in reduced catalytic activity. This theory is not supported by previous findings; contrarily Palonen et al. (2004) found that pure Cel7A core did not bind on isolated lignins whereas endoglucanase Cel5A core domain was found to bind on alkali-lignin.

8.6 Hydrolysis of crystalline cellulose with solid-bound cellulases

Solid-bound enzymes from CEL-P and alkali-lignin samples were also used in a hydrolysis of microcrystalline cellulose. The rate of hydrolysis was shown to decrease

sharply after a few hours and the sugar yield after 72 hours was about 80% lower than the corresponding yield with soluble enzymes. It is generally accepted that efficient hydrolysis of solid cellulose requires enzymes to bind on the cellulosic surface and a plausible reason for the poor hydrolysis capacity of lignin-bound enzymes is their incapability to desorb and readsorb on cellulose in hydrolysis conditions. Girard & Converse (1993) got similar results when they mixed fresh steam pretreated wood with lignaceous residue that contained adsorbed cellulases. Only 15% to 20% of expected hydrolysis activity could be detected from adsorbed enzymes. Readsorption of enzymes from cellulosic hydrolysis residue to fresh substrate has also been studied, and it was found that enzymes can quickly redistribute between cellulosic hydrolysis residue and fresh substrate (Lee et al. 1995). The same study demonstrated that recovery of hydrolysis activity was inversely dependent on the lignin content of the hydrolysis residue. In other words, lignin restricted desorption and readsorption of enzymes on fresh cellulosic substrates.

In this work, CEL-P-bound enzymes seemed to be slightly more efficient to hydrolyze microcrystalline cellulose than alkali-lignin-bound enzymes. This effect could be subjected to the higher carbohydrate content of CEL-P: in the beginning of hydrolysis, there may be more of enzymes that can desorb and start up the hydrolysis. Presumably, the enzymes that are bound to carbohydrates desorb more easily than the lignin-bound enzymes. Soon, however, the rate of hydrolysis decreases sharply which may be caused by enzyme inactivation or readsorption on lignin.

9 Conclusions and future prospects

This work suggests that protease purified enzymatic hydrolysis residue (CEL-P) is best suited lignin-rich substrate for adsorption studies that aim at e.g. enzyme screening applications. Most importantly, CEL-P particles resemble particles that are present in process conditions and the sample has sorbent properties similar to those of other lignin candidates. Purification was shown to be essential to restore the adsorption capacity of the residue, and protease treatment was found more efficient than urea treatment in removing solid-bound enzymes. However, protease treatment will need further optimisation in order to achieve even better level of purity. During the course of this study, a new strategy to monitor cellulase content in the sample was observed. Mannose content

of the residue seemed to give an estimate of the amount of protein in the sample, because of mannose-rich glycosylations of fungal cellulases. Accordingly, carbohydrate analysis would offer an estimate of residual carbohydrates as well as solid-bound cellulases, but the potential of this approach should be studied in more detail to find out if it is also applicable to mannose-rich biomass samples.

Cellulase activity measurements revealed that *T. reesei* cellulases adsorbed on lignin- and cellulose-rich particles with highly distinctive adsorption patterns. The major endoglucanase, exoglucanase and β-glucosidase components had very similar affinities on lignin-rich particles, whereas affinity on cellulose-rich particles had much more variation: Cel7A (CBHI) exoglucanase showed more affinity on cellulose-rich particles than endoglucanases and β-glucosidases. In this study, *T. reesei* and *A. niger* β-glucosidases showed considerably different affinities on lignin: *A. niger* β-glucosidase did not show affinity on lignin-rich residues, whereas *T. reesei* β-glucosidase behaved the opposite way. Further studies with purified enzymes could clarify the reasons that result in this major difference in their adsorption behaviour. Furthermore, this kind of approach would have potential to reveal details of enzyme-lignin interactions.

Lignin-bound *T. reesei* endoglucanases were found to be active towards soluble HEC-substrate in the activity measurements, whereas the activity of lignin-bound Cel7A (CBH I) had declined. In further studies, lignin-bound enzymes were not capable of efficiently hydrolysing crystalline cellulose and it was concluded that enzymes are not able to desorb from lignin and start up the hydrolysis on cellulose crystals. Most probably, major *T. reesei* endo- and exoglucanases are anchored on lignin particles from their cellulose binding domains. This scenario would explain the efficient hydrolysis of soluble substrates, as well as inefficient hydrolysis of insoluble cellulose. In future work, it could be beneficial to study the lignin-CBM interactions in more detail and find out how much there is variation in adsorption behaviour among the fungal CBM family. Currently, the roles of pH (ionic interactions) and temperature in enzyme-lignin interactions are being studied. In my opinion, future work should focus on finding new lignin-tolerant cellulase preparations and on explaining the differences in enzyme adsorption on lignin.

10 References

Adler, E. 1977. Lignin chemistry - past, present and future. Wood Science and Technology 11: 169-218.

Arai, T. & Norde, W. 1990. The behaviour of some model proteins at solid-liquid interfaces. 1. Adsorption from single protein solutions. Colloids and Surfaces 51: 1-15.

Bailey, M.J. & Linko, M. 1990. Production of beta-galactosidase by *Aspergillus oryzae* in submerged bioreactor cultivation. Journal of Biotechnology 16: 57-66.

Bailey, M.J. & Tähtiharju, J. 2003. Efficient cellulase production by *Trichoderma reesei* in continuous cultivation on lactose medium with a computer-controlled feeding strategy. Applied Microbiology and Biotechnology 62: 156-162.

Barr, B.K., Hsieh, Y.L., Ganem, B. & Wilson, D.B. 1996. Identification of two functionally different classes of exocellulases. Biochemistry 35: 586-592.

Berlin, A., Balakshin, M., Gilkes, N., Kadla, J., Maximenko, V., Kubo, S. & Saddler, J. 2006. Inhibition of cellulase, xylanase and beta-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.

Bernfeld, P. 1955. Amylases, Alpha and Beta. Methods in Enzymology 1: 149-158.

Björkman, A. 1956. Studies on finely divided wood. Part 1. Extraction of lignin with neutral solvents. Svensk papperstidning 59: 477-485.

Boerjan, W., Ralph, J. & Baucher, M. 2003. Lignin biosynthesis. Annual Review of Plant Biology 54: 519-546.

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.

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.

Bradford, M.M. 1976. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Analytical Biochemistry 72: 248-254.

Brash, J.L. & Horbett, T.A. 1995. Proteins at Interfaces II - an overview. In: Horbett T.A & Brash, J.L. (Eds.). Proteins at Interfaces II. ACS Symposium Series 602. Washington DC: ACS. 1-23 p.

Brunow, G., Kilpeläinen, I., Sipilä, J., Syrjänen, K., Karhunen, P., Setälä, H. & Rummakko, P. 1998. Oxidative coupling of phenols and the biosynthesis of lignin. In: Lignin and lignan biosynthesis. Ed. Lewis, N.G. & Sarkanen, S. Washington: American Chemical Society. 131-147 p.

Brunow, G., Lundquist, K. & Gellerstedt, G. 1998. Lignin. In: Sjöström, E. & Alén, R. (Eds.). Analytical methods in wood chemistry, pulping and papermaking. Berlin: Springer. 77-120 p.

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. & Linder, M. 1999. Widely different off-rates of two closely related cellulose-binding domains from *Trichoderma reesei*. European Journal of Biochemistry 262: 637-643.

Chang, V.S. & Holtzapple, M.T. 2000. Fundamental factors affecting biomass enzymatic reactivity. Applied Biochemistry and Biotechnology 84-6: 5-37.

Compton, S.J. & Jones, C.G. 1985. Mechanism of dye response and interference in the Bradford protein assay. Analytical Biochemistry 151: 369-374.

Côté, W.A. 1967. Wood ultrastructure; an atlas of electron micrographs. Seattle: University of Washington Press. 31 p.

Cutler, D.F., Botha, C.E.J. & Stevenson, D.W. 2008. Plant Anatomy - an applied approach. Oxford: Blackwell Publishing. 302 p.

Donaldson, L.A. 1992. Lignin distribution during latewood formation in *Pinus radiata*. Iawa Bulletin 13: 381-387.

Erickson, M., Larsson, S. & Miksche, G.E. 1973. Gas-chromatographic analysis of lignin oxidation products. 7. Improved method for characterization of lignins by methylation and oxidative degradation. Acta Chemica Scandinavica 27: 127-140.

Eriksson, T., Börjesson, J. & Tjerneld, F. 2002. Mechanism of surfactant effect in enzymatic hydrolysis of lignocellulose. Enzyme and Microbial Technology 31: 353-364.

Fengel, D. & Wegener, G. 1984. Wood: Chemistry, Ultrastructure, Reactions. Berlin: Walter de Gruyter. 613 p.

Fergus, B.J., Procter, A.R, Scott, J.A.N. & Goring, D.A.I. 1969. Distribution of lignin in sprucewood as determined by ultraviolet microscopy. Wood Science and Technology 3: 117-138.

Freudenberg, K. & Grion, G. 1959. Contribution to the mechanism of formation of lignin and of the lignin-carbohydrate bond. Chemische Berichte-Recuil 6: 1355-1363.

Freudenberg, K. & Harkin, J.M. 1960. Models for the linkage of lignin to carbohydrates. Chemische Berichte-Recuil 93: 2814-2819.

Ghose, T.K. 1987. Measurement of cellulase activities. Pure and Applied Chemistry 59: 257-268.

Girard, D.J., Converse, A.O. 1993. Recovery of cellulase from lignaceous hydrolysis residue. Applied Biochemistry and Biotechnology 39: 521-533.

Henrissat, B. 1991. A classification of glycosyl hydrolases based on amino-acid-sequence similarities. Biochemical Journal 280: 309-316.

Hui, J.P.M., White, T.C. & Thibault, P. (2002). Identification of glycan structure and glycosylation sites in cellobiohydrolas II and endoglucanases I and II from *Trichoderma reesei*. Glycobiology 12: 837-849.

IPCC Intergovernmental Panel on Climate Change. 2007. Contribution of Working Groups I, II and III to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Geneva, Switzerland: IPCC. Available online http://www.ipcc.ch/ipccreports/ar4-syr.htm (6.5.2009).

Jervis, E.J., Haynes, C.A. & Kilburn, D.G. 1997. Surface diffusion of cellulases and their isolated binding domains on cellulose. Journal of Biological Chemistry 272: 27016-24023.

Jørgensen, H., Eriksson, T., Börjesson, J., Tjerneld, F. & Olsson, L. 2003. Purification and characterization of five cellulases and one xylanase from *Penicillium brasilianum* IBT 20888. Enzyme and Microbial Technology 32: 851-861.

Jørgensen, H., Kristensen J.B., & Felby, K. 2007. Enzymatic conversion of lignocellulose into fermentable sugars: challenges and opportunities. Biofuels, Bioproducts & Biorefining 1: 119-134.

Kawamoto, H., Nakatsubo, F. & Murakami, K. 1992. Protein-adsorbing capacities of lignin samples. Mokuzai Gakkaishi 38: 81-84.

Kosikova, B. Joniak, D. & Skarnla, J. 1972. Lignin-carbohydrate bonds in beech wood. Cellulose Chemistry and Technology 6: 579-588.

Koutaniemi, S. 2007. Lignin biosynthesis in Norway spruce: from a model system to the tree. Doctoral thesis. Department of Applied Biology, University of Helsinki. 57 p. Available online http://ethesis.helsinki.fi

Kuhls, K., Lieckfeldt, E., Samuels, G.J., Kovacs, W., Meyer, W., Petrini, O., Gams, W., Borner, T. & Kubicek, C.P. 1996. Molecular evidence that asexual industrial fungus *Trichoderma reesei* is a clonal derivative of the ascomycete *Hypocrea jecorina*. Proceedings of the National Academy of Sciences 93: 7755-7760.

Kumar, R. & Wyman C.E. 2008. An improved method to directly estimate cellulase adsorption on biomass solids. Enzyme and Microbial Technology 42: 426-433.

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

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.

Lever, M. 1972. New reaction for colometric determination of carbohydrates. Analytical Biochemistry 47: 273-279.

Lewis, N.G. & Davin L.B. 1999. Lignans: Biosynthesis and function. In: Barton, D., Nakanishi, K., Meth-Cohn, O. & Sankawa, U. Comprehensive natrural products chemistry Vol 1. Amsterdam: Elsevier. 1007 p.

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 93: 12251-12255.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193: 265-275.

Lu, Y.P., Yang, B., Gregg, D., Saddler, J.N. & Mansfield, S.D. 2002. Cellulase adsorption and an evaluation of enzyme recycle during hydrolysis of steam-exploded softwood residues. Applied Biochemistry and Biotechnology 98: 641-654.

Maras, M., deBruyn, A., Vervecken, W., Uusitalo, J., Penttilä, M., Busson, R., Herdewijn, P. & Contreras, R. 1999. In vivo synthesis of complex N-glycans by expression of human N-acetylglucosaminyltransferase I in the filamentous fungus *Trichoderma reesei*. FEBS letters 452: 365-370.

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.

Norde, W. & Anusiem, A.C.I. 1992. Adsorption, desorption and readsorption of proteins on solid surfaces. Colloids and Surfaces 66: 73-81.

Norde, W. & Haynes, C.A. 1995. Reversibility and the mechanism of protein adsorption. In: Horbett, T.A. & Brash, J.L (Eds.). Proteins at interfaces II. ACS Symposium Series 602. Washington: ACS. p 26-40.

Norgren, M., Notley, S.M., Majtnerova, A. & Gellerstedt, G. 2006. Smooth model surfaces from lignin derivatives. I. Preparation and characterization. Langmuir 22: 1209-1214.

Ooshima, H., Sakata, M. & Harano, Y. 1986. Enhancement of enzymatic hydrolysis of cellulose by surfactant. Biotechnology and Bioengineering 28: 1727-1734.

Orelma, H. 2008. Lignosellolyyttisten entsyymien adsorptiotutkimus QCM-D tekniikalla. Master's thesis in the Helsinki University of Technology, Faculty of Chemistry and Material Sciences. 80 p.

Palonen, H. 2004. Role of lignin in the enzymatic hydrolysis of lignocellulose. Espoo. VTT Publications 520. 80 p. + app. 62 p.

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.

Plomion, C., Leprovost, G. & Stokes, A. 2001. Wood formation in trees. Plant Physiology 127: 1513-1523.

Pan, X.J., Xie, D., Gilkes, N., Gregg, D.J. & Saddler, J.N. 2005. Strategies to enhance the enzymatic hydrolysis of pretreated softwood with high residual lignin content. Applied Biochemistry and Biotechnology 121: 1069-1079.

Pan, X.J., Zhang, X., Gregg, D.J. & Saddler, J.N. 2004. Enhanced enzymatic hydrolysis of steam-exploded Douglas fir wood by alkali-oxygen post-treatment. Applied Biochemistry and Biotechnology 113: 1103-1114.

Pan, X.J. 2008. Role of functional groups in lignin inhibition of enzymatic hydrolysis of cellulose to glucose. Journal of Biobased Materials and Bioenergy 2: 25-32.

Rabe, T.E & Tilton, R.D. 1993. Surface diffusion of adsorbed proteins in the vicinity of the substrate glass-transition temperature. Journal of Colloid and Interface Science 159: 243-245.

Ralph, J., Lundquist, K., Brunow, G., Lu, F., Kim, H., Schatz, P.F., Marita, J.M., Hatfield, R.D., Ralph, S.A., Christensen J.H. & Boerjan, W. 2004. Lignins: Natural polymers from oxidative coupling of 4-hydroxyphenylpropanoids. Phytochemistry Reviews 3:29-60.

Reinikainen, T. 1994. The cellulose-binding domain of cellobiohydrolase I from *Trichoderma reesei*. Interaction with cellulose and application in protein immobilization. Espoo 1994. VTT Publications 206. 115 p.+ app. 46 p.

Saeki, S., Kuwahara, N., Nakatan, M. & Kaneko, M. 1976. Upper and lower critical solution temperatures in poly(ethyleneglycol) solutions. Polymer 17: 685-689.

Saloheimo, M., Nakari-Setälä, T., Tenkanen, M. & Penttilä M. 1997. cDNA cloning of a *Trichoderma reesei* cellulase and demonstration of endoglucanase activity expression in yeast. European Journal of Biochemistry 249: 584-591.

Sasaki, S., Nishida, T. & Tsutsumi, Y. 2004. Lignin dehydrogenative polymerization mechanism: a poplar cell wall peroxidase directly oxidizes polymer lignin and produces in vitro dehydrogenative polymer rich in β-*O*-4 linkage. FEBS Letters 562: 197-201.

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.

Shapiro, A.L., Vinuela, E. & Maizel J.V. 1967. Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. Biochemical and Biophysical Research Communications 28: 815-820.

Shevchenko, S.M., Beatson, R.P. & Saddler, J.N. 1999. The nature of lignin from steam explosion enzymatic hydrolysis of softwood - Structural features and possible uses. Applied Biochemistry and Biotechnology 77-9: 867-876.

Sjöström, E. 1993. Wood chemistry: fundamentals and applications**.** 2nd ed. San Diego, California: Academic Press. 293 p.

Ståhlberg, J., Johansson, G. & Pettersson, G. 1993. *Trichoderma reesei* has no true exocellulase - all intact and truncated cellulases produce new reducing end groups on cellulose. Biochimica et Biophysica Acta 1157: 107-113.

Stenberg, K., Tengborg, C., Galbe, M. & Zacchi, G. 1998. Optimisation of steam pretreatment of SO_2- impregnated mixed softwoods for ethanol production. Journal of Chemical Technology and Biotechnology 71: 299-308.

Tammelin, T., Österberg, M., Johansson, L.S. & Laine, J. 2006. Preparation of lignin and extractive model surfaces by using spincoating technique - application for QCM-D studies. Nordic Pulp & Paper Research Journal 21: 444-450.

Tamminen, T. & Hortling, B. 1999. Isolation and Characterization of Residual Lignin. In Argyropoulos, D.S (Ed.). Advances in Lignocellulosics Characterization. Atlanta: Tappi Press. 382 p.

Tan, L.U.L., Chan, M.K.H. & Saddler J.N. 1984. A modification of the Lowry method for detecting protein in media containing lignocellulosic substrates. Biotechnology Letters 6: 199-204.

Tenkanen, M. & Siika-Aho, M. 2000. An alpha-glucuronidase of *Schizophyllum commune* acting on polymeric xylan. Journal of Biotechnology 78: 149-161.

Tilbeurgh van, H., Loontiens, F.G., deBruyne, C.K. & Claeyssens, M. 1988. Fluorogenic and chromogenic glycosides as substrates and ligands of carbohydrases. Methods in Enzymology A 160: 45-59.

Tolan, J.S. 2002. Iogen's process for producing ethanol from cellulosic biomass. Clean Technologies and Environmental Policy 3: 339-345.

Tu, M.B., Chandra, R.P. & Saddler, J.N. 2007a. Evaluating the distribution of cellulases and the recycling of free cellulases during the hydrolysis of lignocellulosic substrates. Biotechnology Progress 23: 398-406.

Tu, M.B., Chandra, R.P. & Saddler, J.N. 2007b. Recycling cellulases during the hydrolysis of steam exploded and ethanol pretreated lodgepole pine. Biotechnology Progress 23: 1130-1137.

Unno, T., Ide, K., Yazaki, T., Tanaka, Y., Nakakuki, T. & Okada G. 1993. High recovery purification and some properties of a beta-glucosidase from *Aspergillus niger*. Bioscience, Biotechnology, and Biochemistry 57: 2172-2173.

Uraki, Y., Ishikawa, N., Nishida, M. & Sano, Y. 2001. Preparation of amphiphilic lignin derivative as a cellulase stabilizer. Journal of Wood Science 47: 301-307.

Wood, T.M. 1992. Fungal cellulases. Biochemical Society Transactions 20: 46-53.

Xu, F., Ding, H.S., 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.

-

11 Appendices

Appendix 1: Monosaccharide analysis with anion exchange chromatography

Equipment
Dionex ICS-3000 liquid chromatograph
ICS-3000 dual pump model DO-1
ICS-3000 ED electrochemical detector
ICS-3000 AS autosampler
CHROMELEON software 6.8

Columns
Dionex CarboPac PA-1 guard column
Dionex CarboPac +PA-1 analytical column

Liquids used during the run
A: Water
B: 100 mM NaOH
C: 300 mM NaAc/ 100 mM NaOH
D: 300 mM NaOH
Post column reagent 300 mM NaOH

Run conditions
Flow: 1 ml/min
Post column reagent 0.3 ml/min
Column oven temperature: +30ºC
Detector temperature: +30
Cooled autosampler: +15ºC
Injection volume: 25µl (full loop injection)

Scheme of the chromatographic run

Time		%A	%B	%C	%D
-15,00	delay	85	15		
0.00	injection	85	15		
2.00		85	15		
2.10		100			
36.00		100			
36.10	Acq.OFF			100	
39.00				100	
39.10					100
43.00					100
43.10	end	85	15		

Appendix 2: Solutions for SDS-PAGE

Sample buffer for SDS-PAGE (x4)

1. 1 M Tris-HCl pH 6.8: 5 ml
    - (60.6 g Trizma base (Sigma, T1503), 500 ml of distilled water, pH adjusted to 6.8 with 5 N HCl, stored at + 4ºC)
2. Glycerol (87%): 11.6 ml
3. SDS: 2 g
4. β-mercaptoethanol: 5 ml
5. Bromophenol blue: 10 mg
6. Distilled water 24 ml

Reagents are mixed until they are properly dissolved and volume of the solution is fixed to 50 ml with distilled water in a volumetric flask. Solution is aliquoted (0.5 ml) and stored at -20ºC.

Running buffer for SDS-PAGE (x10)

1. 30.3 g/l Trizma base (Sigma, T1503) (0.25 M)
2. 144.2 g/l glycine (1.92 M)
3. 10 g/l SDS (1%)

Reagents are dissolved to distilled water. pH should be 8.3 and it is not adjusted. Solution is kept at + 4ºC and diluted 1:10 with distilled water prior to use.

Coomassie Brilliant Blue R-250 stain

1. 500 mg Coomassie Brilliant Blue R-250 (Serva, Germany)
2. 150 ml ethanol (analytical grade)
3. 50 ml acetic acid (analytical grade)
4. 300 ml distilled water

Destain solutions

1. destain 1: 40% ethanol and 10% acetic acid (v/v)
2. destain 2: 5% ethanol and 7.5% acetic acid (v/v)

Appendix 3: Preparation of dinitrosalicylic acid (DNS) reagent and the protocol to measure reducing sugars with the DNS method

Reference: Bernfeld, P. 1955**.** Amylases, Alpha and Beta. Methods in Enzymology 1: 149-158.

1. Dissolve 80 g of NaOH to 3 litres of distilled water. Warm up to 35-45 ºC
2. Add gradually a mixture which includes
   - 1500 g of $C_4H_4KNaO_6$
   - 50 g of 3,5-dinitrosalicylic acid
3. Mix in magnetic stirring until everything has dissolved
4. Add distilled water up to 5 litres

Store the reagent in a brown glass bottle and filter the reagent through a filter prior to use.

1. Mix 2 ml of water-diluted sample to 3 ml of DNS reagent.
2. Heat the mixture for 5 minutes in a vigorously boiling water bath
3. Cool down the mixture in a cold water bath and measure the formed colour at 540 nm
4. Prepare the standard curve (0,1-0,5 g/l) from glucose and treat the standard samples in the same manner
5. Prepare the blank sample into distilled water