THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

The relation between the supramolecular structure of cellulose and its hydrolysability

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Cover: Illustration of supramolecular structure of cellulose.

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ABSTRACT

The liberation of fermentable sugars from cellulosic biomass during enzymatic hydrolysis is often incomplete. One of the factors limiting the efficiency of enzymatic hydrolysis is the structural properties of cellulose. The aim of the work presented in this thesis was to increase our understanding of the relation between enzymatic hydrolysis and the structural properties of cellulosic substrates. The enzymatic hydrolysis of a number of cellulosic substrates derived from softwood preparations used in the pulp and paper industry, as well as model substrates, were studied. The differences in cellulosic substrates before and after enzymatic hydrolysis are described on the nanometre scale in terms of their supramolecular structure, i.e. the lateral dimensions of fibrils and fibril aggregates, the accessible surface area, the crystallinity and porosity, using solid-state nuclear magnetic resonance spectroscopy. The substrates were imaged and structural changes in the cellulosic substrates were characterized in real time on the micrometre scale in terms of their molecular density, ordering and autofluorescence, employing nonlinear optical microscopy. A strong correlation was found between the average pore size and the specific surface area of the starting material and the enzymatic conversion yield. The overall degree of crystallinity and the lateral dimensions of the fibrils increased in some samples as a result of hydrolysis. Avicel had a higher carbon–hydrogen bond density and a different pattern of ordered structures than the never-dried pulp fibres, possibly reflecting the collapse of the macromolecular structures during drying and rewetting. Monitoring of the substrates during enzymatic hydrolysis revealed substrate-characteristic hydrolysis pattern. The response of the most widely studied filamentous fungus for cellulase production, *Trichoderma reesei*, to cellulosic substrates with different supramolecular structures was studied. Substantial differences were found in the profile of the enzymes produced, despite the fact that there were only minor differences in the chemical composition of the cellulose-rich substrates. Culture filtrates from five filamentous fungi cultivations were evaluated regarding their ability to improve saccharification of the industrial cellulase cocktail Celluclast 1.5L. It was demonstrated that supplementing commercial cocktails with enzymes from carefully selected fungi can result in significantly more efficient saccharification of biomass.

Keywords: CARS, Cellulases, Cellulose I, SHG, CP/MAS ^{13}C-NMR, Imaging, MPEF, Secretomics, *Trichoderma reesei*

List of publications

This thesis is based on the work described in the following papers:

Paper I: **Peciulyte A**, Karlström K, Larsson P T and Olsson L.
Impact of the supramolecular structure of cellulose on the efficiency of enzymatic hydrolysis. *Biotechnol. Biofuels* 2015; 8:56.

Paper II: **Peciulyte A***, Kiskis J*, Larsson P T, Olsson L and Enejder A.
Visualization of structural changes in cellulosic substrates during enzymatic hydrolysis using multimodal nonlinear microscopy
Submitted

Paper III: **Peciulyte A**, Anasontzis G E, Karlström K, Larsson P T and Olsson L.
Morphology and enzyme production of *Trichoderma reesei* Rut C-30 are affected by the physical and structural characteristics of cellulosic substrates. *Fungal Genet. Biol.* 2014; 72:64-72.

Paper IV: **Peciulyte A**, Pisano M, de Vries R P and Olsson L.
Hydrolytic potential of different fungal crude extracts to enhance a commercial enzyme cocktail
Submitted

* These authors contributed equally to the study

Published paper not included in this thesis

Aldaeus, F, Larsson, K, Srndovic, J S, Kubat, M, Karlström, K, **Peciulyte, A**, Olsson, L, Larsson, P T. The supramolecular structure of cellulose-rich wood pulps can be a determinative factor for enzymatic hydrolysability. *Cellulose*, 2015; 22: 3991-4002.

The author's contributions

Paper I: I designed and carried out the experimental work apart from the CP/MAS ^{13}C-NMR analysis. I analysed the results and wrote the manuscript.

Paper II: I, together with Juris Kiskis, developed the idea and designed the experimental set-up. I was responsible for the enzymatic hydrolysis part and Juris Kiskis performed the microscopy measurements. I, together with Juris Kiskis, participated in all the experiments and the measurements, analysed the data, and wrote the manuscript.

Paper III: I designed and carried out the experimental work apart from the proteomic analysis; I analysed the results and wrote the manuscript.

Paper IV: I designed the work, supervised Maria Pisano who performed the screening of the fungal strains, and I performed the rest of the experiments. I analysed the results and wrote the manuscript.

Preface

This research was carried out according to the requirements for the degree of Doctor of Philosophy at the Department of Biology and Biological Engineering, Chalmers University of Technology, Sweden. The project was initiated in June 2011. The research was carried out under the supervision of Professor Lisbeth Olsson, Chalmers University of Technology, and Associate Professor Per Tomas Larsson, Innventia AB. Per Tomas Larsson provided expertise on high-resolution solid-state NMR and the supramolecular structure of cellulose. Lisbeth Olsson and I were responsible for the biological perspective, focusing on enzymes, fungal physiology and the enzymatic hydrolysis of cellulose.

The work described in this thesis was performed in collaboration with Innventia AB. The project was funded by the Swedish Research Council (*Vetenskapsrådet*) under the Programme for Strategic Energy Research under grant agreement № 621-2010-3788.

List of abbreviations

AA	Auxiliary activity
AFM	Atomic force microscopy
BG	β-glucosidase
CARS	Coherent anti-Stokes Raman scattering
CAZy	Carbohydrate-active enzymes
CBD	Cellulose-binding domain
CBH	Cellobiohydrolase
CBM	Carbohydrate-binding module
CD	Catalytic domain
DNS	Dinitrosalicylic acid
DCr	Degree of crystallinity
EG	Endoglucanase
GH	Glycosyl hydrolase
HPAEC-PAD	High-performance anion-exchange chromatography coupled with pulsed amperometric detection
LC	Liquid chromatography
LFD	Lateral fibril dimensions
LFAD	Lateral fibril aggregate dimensions
LPMO	Lytic polysaccharide monooxygenase
MPEF	Multiphoton excited fluorescence
MS	Mass spectrometry
QCM-D	Quartz crystal microbalance with dissipation
SHG	Second harmonic generation
SSA	Specific surface area

Contents

1 Introduction

Cellulose fibrils from wood samples several million years old have been shown to have the same structure as those from recent wood samples [1, 2], demonstrating that cellulose is highly recalcitrant. The natural resistance of cellulose to deconstruction to monomers is termed "recalcitrance" [3]. Cellulose is the most abundant organic polymer on earth, representing about 1.5×10^{12} tons of the total annual biomass production, and it is the raw material believed to be able to satisfy the increasing demand for sustainable and biocompatible products [4]. Apart from the production of paper and cardboard, use in building materials, cellulose has already found applications in biofuel production, pharmaceuticals, foodstuffs and medicine [4-7] and interest in cellulose is still growing. In most of the cellulose-based products of interest processes, enzymes play an important role.

The world is currently in a transition from a fossil-based economy towards a bio-based economy. There is much debate on how a sustainable bio-economy can be established, where resources such as plant biomass, land and water are used in the most efficient way. The population of the world is currently over 7 billion, and is predicted to reach 9 billion by 2050, which will place great demands on the resources available. Therefore, it is necessary to use renewable resources in a sustainable manner.

The biorefinery is a promising concept as an alternative to petro-based refineries [8, 9]. The term biorefinery was established in the 1990s [10]. According to the definition by the National Renewable Energy Laboratory, a biorefinery is "a facility that integrates biomass conversion processes and equipment to produce fuels, power, and chemicals from biomass". The Borregaard biorefinery in Sarpsborg, Norway, which is based on the pulp and paper industry, is one of the world's most successful biorefineries [11]. In Sweden, which is a densely forested country, forest products have been a cornerstone of the economy and a key export. Sweden is the world's third largest combined exporter of paper, pulp and sawn wood products (Swedish Forest Industries Federation, statistics from 2013). In a combined effort between the Swedish Government and various industrial sectors, a national research agenda [12] has been introduced to secure the future of the forest-based sector in Sweden through the development of products with a higher added value, using building blocks from forest-based biomass. In 2011, the Swedish Research

Council Formas, together with VINNOVA and the Swedish Energy Agency, prepared a national strategy for the establishment of a sustainable bio-based economy in which the forest industry was highlighted as an important area [13], and where renewable resources from other areas, such as agriculture and marine resources, were expected to play an important part in the longer term.

The enzymatic hydrolysis of cellulose is generally considered to be a sustainable means of obtaining monosaccharides that can be converted into a number of products via microbial fermentation [8]. Enzymes are often preferred over inorganic compounds with catalytic capacity because they are environmentally sustainable. Bioethanol is a prime example of the conversion of monosaccharides into renewable transportation fuels employing fermentation [14]. However, the enzymatic hydrolysis of cellulose is often incomplete, and we do not yet have a full understanding of the process. The interdependence of the enzyme–substrate interaction, changes in substrate morphology, and non-hydrolytic cellulose disruption remains elusive. It has been suggested that substrate-related factors predominantly affect the rate of hydrolysis of cellulose [15, 16]. The aim of the work presented in this thesis was to contribute to a better understanding of the supramolecular structure of cellulose and its relation to enzymatic hydrolysability. As the supramolecular structure of cellulose is one of the key factors determining the efficiency of enzymatic hydrolysis, this was studied with different methodologies that provided information on its structure on both the nano- and micro-scales.

Until recently, the types of enzymes required for the enzymatic hydrolysis of cellulose were divided into three major classes: (i) endoglucanases (EGs), which randomly break the cellulose chain, (ii) exoglucanases (or cellobiohydrolases (CBHs)), which liberate the D-glucose dimer cellobiose from the ends of the cellulose chain, and (iii) β-glucosidases (BGs) which release D-glucose from the soluble oligomeric breakdown products [17, 18]. It has recently been demonstrated that the inclusion of a novel class of enzymes in filamentous fungi, currently referred to as lytic polysaccharide monooxygenases (LPMOs) (formerly called GH61) greatly increases the performance of cellulases [19]. Reese et al. [20] were the first to suggested that undefined enzymes could play a major role in the disruption of the recalcitrant structure of cellulose, thus allowing attack by traditional cellulases (EGs, CBHs and BGs). LPMOs may be useful in this respect as they have been shown to render cellulose more accessible to traditional cellulases through their oxidative action on cellulose polymers, introducing nicks, which in turn create more ends

for cellulase action. The field of LPMO research is expanding rapidly, and they have been shown to act on various substrates such as chitin, cellulose, hemicellulose and starch. LPMOs are produced by a wide range of bacteria and filamentous fungi [19, 21-24]. Microorganisms, mainly bacteria and fungi, which are natural degraders of cellulose secrete a broad consortia of enzymes, among which many activities are still unknown, but may play an important role in biomass degradation.

Cellulose has a simple chemical structure, being composed of β-D-glucan polymer chains, but the spatial organization of these polymer chains makes the structure of cellulose very complex. In its solid state, the cellulose polymers are packed together, forming fibrils and fibril aggregates. The complexity of the structure of cellulose could be one of the reasons why different enzyme activities are required in the enzymatic hydrolysis of cellulose. It has been hypothesized that the recalcitrant structure of cellulose is the cause of its incomplete enzymatic hydrolysis. The structure of cellulose can be determined on the macro-, micro- and nano-scale, depending on the technique employed. Although researchers have devoted significant effort to investigating the structure of cellulose over several decades, this is not yet understood in sufficient detail.

A better understanding of the structure of cellulose may facilitate the identification of the conditions required for efficient enzymatic hydrolysis. The efficiency of the enzymatic hydrolysis of cellulose can be improved through different directions: (i) improvement of the performance of cellulolytic enzymes [25], (ii) improvement of the pretreatment technologies of cellulosic materials [26] and (iii) finding key structural determinants for efficient hydrolysis of cellulose. Figure 1 summarizes the topics covered in this thesis.

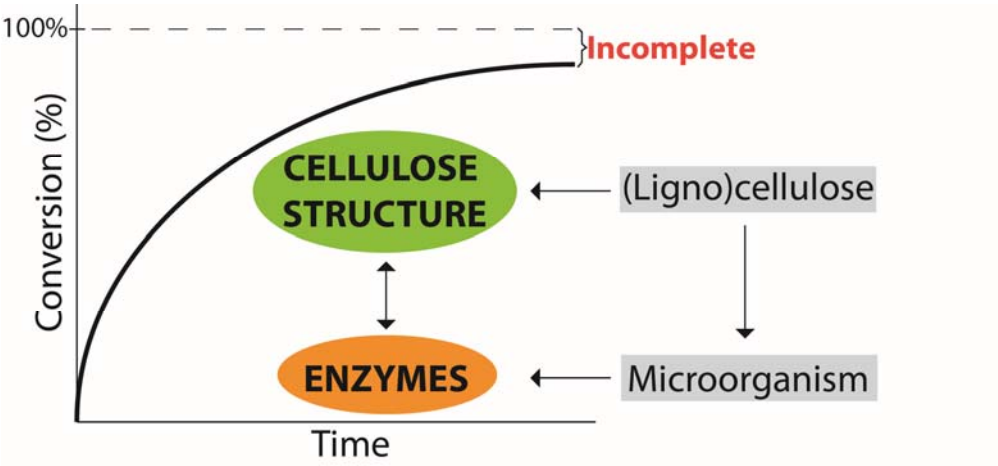

Figure 1. The main structural components of plant biomass are cellulose, hemicellulose and lignin present at varying amounts, depending on the plant species. Cellulose and hemicellulose can be hydrolysed using enzymes to form fermentable sugars. Plant biomass is very recalcitrant and pretreatment should be performed prior to enzymatic hydrolysis. The purpose of pretreatment is to partially break down the recalcitrant structure of cellulosic biomass to make the cellulose more accessible to enzymes. However, the enzymatic hydrolysis of cellulose is often incomplete. The overall aim of this work was to improve our knowledge on the structural determinants of cellulosic substrates that affect enzymatic hydrolysability.

The analysis of the structure of cellulose is challenging as native cellulose is insoluble. According to its chemical composition, it can be regarded as a homogeneous substrate, however it is a heterogeneous substrate at the level of its supramolecular structure, consisting of a complex assembly of fibrils and fibril aggregates, depending on the source and method of isolation. **The initial aim of this work was thus to utilize experimental methods to study the structure of cellulose and the influence of enzymatic action on the structure**. The structure of cellulose can be studied on different scales: macroscopic, microscopic and nanometre. **In Paper I, differences in cellulosic substrates are described on the nanometre scale in terms of their supramolecular structure, i.e. the lateral dimensions of fibrils and fibril aggregates, the accessible surface area, degree of crystallinity and porosity.** Solid-state cross-polarization magic-angle spinning carbon-13 nuclear magnetic resonance (CP/MAS ^{13}C-NMR) spectroscopy was used, which allowed measurements of the supramolecular structure of cellulose on the nanometre scale without prior sample treatment. In order to gain greater insight into the structural determinants of cellulose hydrolysis, a number of cellulosic substrates derived from softwood preparations used in the pulp and paper industry, as well as some model substrates such as Avicel and cotton, were enzymatically hydrolysed. Their conversion

was related to the cellulose structure in an attempt to identify the structural determinants that are important for efficient enzymatic hydrolysis.

Various microscopy techniques can be used to obtain direct visualization of cellulosic substrates. The resolution of these techniques extends from the macro- to the nanometre scale. Some techniques allow real-time imaging. Some of them provide information about the surface morphology of the sample, while in others spatial resolution and/or sample imaging is based on the intrinsic chemical and physical properties of the sample. Some techniques require sample preparation prior to analysis. **Paper II describes the investigation, on the micrometre scale, of cellulosic substrates in terms of their chemical and physical properties during enzymatic hydrolysis in real time. Optical nonlinear imaging techniques, namely coherent anti-Stokes Raman scattering, second harmonic generation and multiphoton excited fluorescence were used to characterize cellulosic substrates in terms of their molecular density, ordering and autofluorescence.** An advantage of these imaging techniques is that no sample preparation is required prior to analysis, and enzymatic hydrolysis could be performed under standard conditions (temperature, buffer solution). Furthermore, they provide three-dimensional images and semi-quantitative information.

Filamentous fungi are among the most potent producers of the enzymes that are used to break down plant cell walls in order to release monosaccharides serving as source of carbon and energy. *Trichoderma reesei* (teleomorph *Hypocrea jecorina*) is the main industrial source of these enzymes, referred to as cellulases, and has the capability to produce and secrete large amounts of enzymes. *T. reesei* has a long history of strain improvement. Among the improved mutants, the widely studied *T. reesei* Rut C-30 strain is a parental strain of many commercial strains used today. **Paper III describes a study on the response of *T. reesei* Rut C-30 to different cellulosic substrates with differences in their supramolecular structure, which would be reflected in the enzymes resulting from fungal growth. Cellulosic substrates have similar chemical compositions, so it could be expected that the enzymes produced by the fungus would be similar. The aim of this work was to explore this hypothesis.**

Novel enzyme-producing microorganisms are found in nature, and these may constitute an unutilized potential in the search for efficient enzyme mixtures. Filamentous fungi produce a very complex cocktail of enzymes which reflects both the individual fungus as

well as its growth substrate and conditions. **In the study presented in Paper IV, the goal was to evaluate the potential of the enzymes produced by five filamentous fungi to improve the saccharification of the industrial cellulase cocktail Celluclast 1.5L. The study demonstrated that supplementing commercial cocktails with enzymes from a careful selection of fungi could result in enzyme cocktails that are significantly more efficient in biomass saccharification.**

In nature, microorganisms grow freely on plant biomass and produce enzymes with activities best suited to hydrolyse a certain chemical bond in the plant biomass or disassemble a particular structure of cellulose. In the laboratory (and in industry), a complex enzyme mixture is loaded in one dose with the intention of obtaining as complete hydrolysis as possible in only a few days. In light of this, I wanted to emphasize that the structure of cellulose is complex, and to give an indication of the different ways in which it can be studied.

2 History of cellulose research

In 1838, the French chemist Anselme Payen determined the molecular formula of the resistant fibrous solid in all plant cell walls to be $C_6H_{10}O_5$. He introduced the term "cellulose" to describe this material [4, 27]. However, Payen was not aware that the material he was studying also included other carbohydrates. So what Payen called "cellulose", is now called "pulp" [28]. Cellulose already played an important role in history, being used for Egyptian papyri and as an energy source, in building materials and in textiles, long before its chemical composition was described. Before the chemical composition of 'cellulose' in plant cell walls had been determined, the English scientist Robert Hooke [29], who was one of the first inventors of the microscope, discovered plant cells while looking at cork in 1665 [30]. However, the observation of enzyme action on cellulose required a microscope with a higher resolution than that made by Hooke. The first direct study on the structural dynamics of enzymatic cellulose degradation on the cellulose surface was made using transmission electron microscopy, and was published in 1981 [31]. The crystalline structure of cellulose was first established by Carl von Nägeli in 1858 [32], and was later verified by X-ray crystallography. The introduction of solid-state CP/MAS ^{13}C-NMR in the early 1980s provided new insight into the structure of cellulose [33, 34].

Elwyn T. Reese was a pioneer in studying the systems of cellulolytic enzymes, and he proposed a two-step mechanism for cellulose hydrolysis in 1950 [20]. The first step (C_1), involving scission of the cross linkages in native cellulose by an unknown mechanism, was suggested to occur prior to the hydrolysis step (Cx). This provided a fundamental step towards our understanding of the synergistic action between the cellulose-degrading enzymes. The topics of cellulose research discussed in this chapter are summarized in Figure 2. However, despite all the research carried out and technical advancements, the structure of cellulose and its enzymatic hydrolysability is still not fully understood.

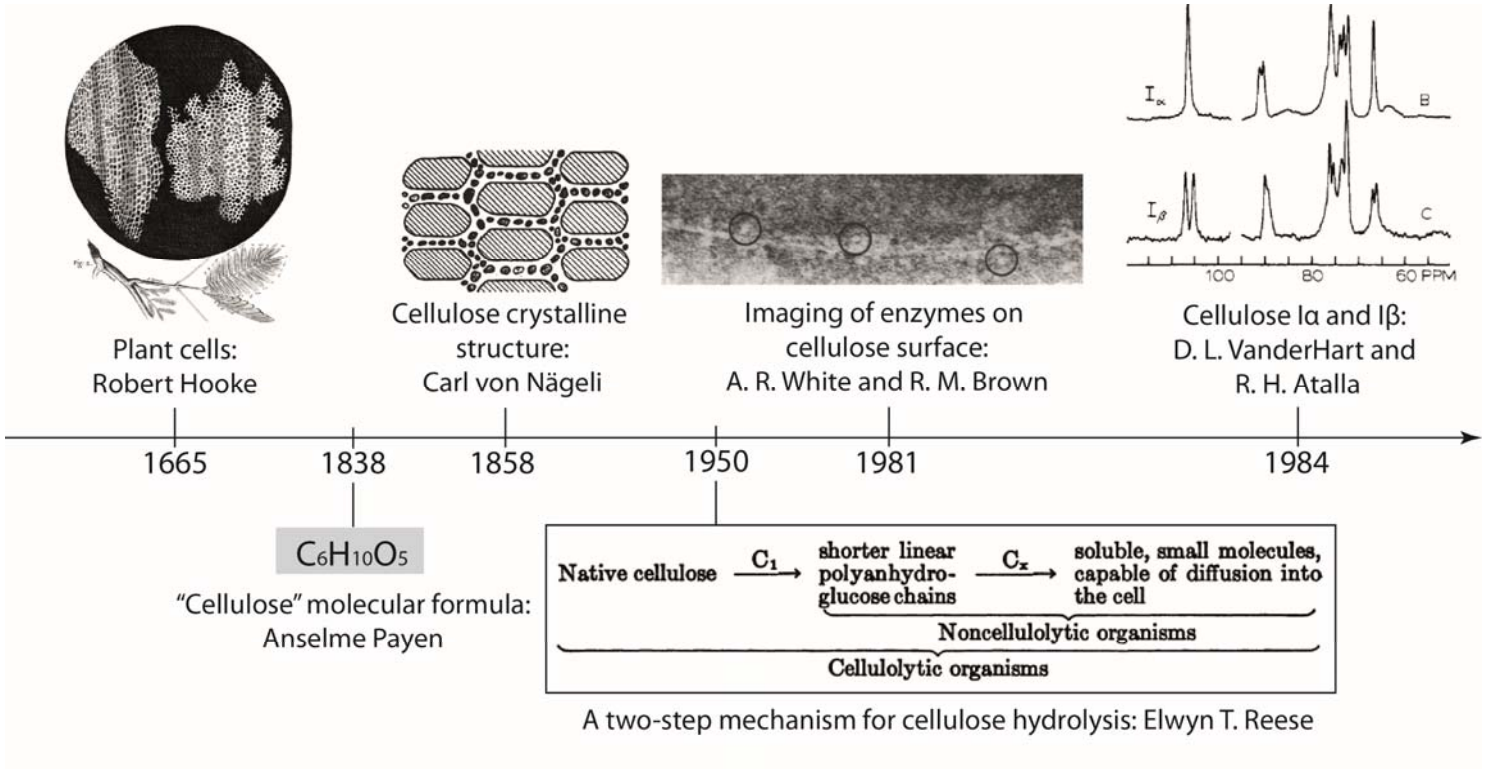

Figure 2. History of cellulose research. A view of a thin slice of cork in which Robert Hooke observed "empty" spaces contained by walls, which he termed cells (image from [30]). Anselme Payen purified the cell walls of various plant tissues and determined the molecular formula of the material, which he named "cellulose", to be $C_6H_{10}O_5$ [28]. The concept of the crystalline structure, called "micelle" or "micellen", was proposed by Carl von Nägeli (reprinted with permission from Macmillan Publishers Ltd: Nature [32], copyright 1961). The hypothesis presented by Elwyn T. Reese that the cellulases, presumed to convert native cellulose to sugars, consisted of at least two systems. During the first step (C_1), splitting of the cross linkages in native cellulose by an unknown mechanism was suggested to occur prior to the enzymatic hydrolysis of the β-1,4-glycosidic linkage (Cx) (reprinted with permission from [20]). A view of a cellulose microfibril bundle where the circles indicate possible cellulase enzyme molecules bound to the cellulose substrate (image from [31]). CP/MAS ^{13}C-NMR spectrum of two proposed crystalline forms of cellulose I, namely Iα and Iβ (reprinted with permission from [33], copyright 1984 American Chemical Society).

3 Sources of cellulose and its isolation

The major sources of cellulose are plants, where cellulose is usually embedded in a matrix of hemicellulose and lignin. The isolation of cellulose is important because it gives the opportunity to convert cellulose into useful products such as printing paper, board, textiles, cellulose nanoparticles [35] and paper-based biosensors [36]. It has also been suggested that cellulose-to-starch transformation been can address the food vs. biofuel dilemma [37]. Sixty years ago, Reese pointed out that the human diet depended heavily on starches, and asked, "Can we convert cellulose into starch, or starchlike food?" [38]. Today, we are not so distant from making it happen.

Depending on the source of cellulose and its subsequent application, it is important to consider during its isolation whether we want to preserve its structure or break it down, and how pure the cellulose must be. In the present work, the primary source of cellulose was softwood. The greatest amounts of softwood are found in Sweden, where Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*) account for about 41% and 39% of the total standing volume in Swedish forests, respectively. The cellulose fibres were isolated during the chemical pulping process, namely pre-hydrolysis soda cooking. Therefore, the main focus of the work described in this thesis is on pulp fibres obtained from softwood, and the pulping process is used as an example of cellulose isolation (see Section 3.2).

3.1 Sources of cellulose in nature

Cellulose production is mainly attributed to plants as they are the major sources of cellulose. However, a large variety of organisms apart from plants produce cellulose [39], including a variety of bacteria [40], fungi, algae and cyanobacteria (which is the most ancient form of life on earth) [41], and even animals, i.e., the tunicates (marine invertebrates) [42]. The green alga *Valonia ventricosa* is known to produce one of nature's most perfect crystalline forms of cellulose. It has been suggested that cellulose offered protection from dangerous ultraviolet radiation, and helped sustain early life forms in earth's harsh primitive atmosphere [39]. Some bacteria have evolved to produce

cellulose pellicles that keep the bacterium floating on the surface [35]. This diversity provides evidence of the ancient evolutionary process of cellulose production [39].

The cellulose in plants is made up of glucose, which is produced in the living plant cell during photosynthesis. In the oceans, most cellulose is produced by unicellular plankton or algae using the same type of carbon dioxide fixation found in the photosynthesis of terrestrial plants. Other cellulose-producing organisms that have no photosynthetic capacity require glucose or some organic substrate synthesized by a photosynthetic organism to form cellulose [43]. Cellulose provides structural support and tensile strength for plants to help them withstand wind, etc. Cellulose is surrounded by a matrix of hemicellulose and lignin in the secondary plant cell walls, which accounts for most of the carbohydrates in plant biomass; these three components are known collectively as lignocellulose [44]. Unlike other plants, cotton contains almost pure cellulose with small amounts of waxes and ash, and does not have a matrix of hemicellulose or lignin. Bacteria and algae also produce cellulose that is devoid of hemicellulose and lignin. Various cellulosic substrates originating from plants, bacteria and algae are used as model substrates in different kinds of studies (Table 1).

Table 1. Summary of the main characteristics of cellulosic model substrates

Substrate	Cellulose content (%)	DCr	SSA ($m^2 g^{-1}$)	Description	Reference
Avicel	97	56 ± 3[a]	113 ± 5[a]	Microcrystalline powder, particle size ~50 μm	Papers I, II, III and IV
Avicel + IL	n.d.	Reduced, depending on the % of Avicel and water mixed with IL	n.d.	A mixed amorphous-crystalline substrate	[45-47]
Cotton	98	65 ± 2[a]	90 ± 4[a]	Dried fibres	Paper I
Never-dried pulp fibres	97	57 ± 1[a]	153 ± 7[a]	Pre-hydrolysis soda cooking and oxygen delignification	Papers I, II, III and IV
Dried pulp fibres	97	57 ± 1[a]	94 ± 2[a]	Oven drying of fibres	Paper I
BC	n.d.	0.76–0.95[b]	200[c]	Chemical treatment to eliminate the cells	[48]
Whatman No. 1 filter paper	n.d.	~0.45[b]	n.d.	Has intact cellulose matrix and cell wall structure	[48]
PASC	n.d.	0[b]	240[c]	Prepared from cellulose powder by phosphoric acid treatment	[48]
Solka Floc	76	n.d.	n.d.	Powdered cellulose	[49]
Cellulose model films	n.d.	n.d.	n.d.	Studied by QCM-D technique	[50]

DCr: degree of crystallinity; SSA: specific surface area; IL: ionic liquid; BC: bacterial cellulose; PASC: phosphoric acid swollen cellulose; QCM-D: quartz crystal microbalance with dissipation; [a] measured with CP/MAS ^{13}C-NMR; [b] measured with wide-range X-ray diffraction; [c] measured with the Brunauer-Emmett-Teller method; n.d.: not determined.

These substrates have different structural characteristics and are not necessarily chemically pure cellulose. Cellulosic substrates differ in their average degree of polymerization, DCr and SSA. Microbial and plant celluloses differ in their crystal structures having different ratio of α and β forms. Cellulose Iα is dominant in bacterial and algal cellulose, and Iβ is dominant in higher plants [48].

3.2 Pulping processes

The world's first chemical (sulphite) pulp mill was established in Sweden in 1872, by the Swedish Engineer Carl Daniel Ekman [51]. Afterwards pulp and paper production started on a large scale in Sweden, and was an important component of Swedish industrialization [52]. Pulping is a process involving the liberation of lignocellulosic fibres from the plant matrix [51]. Pulping can be performed on both grasses and wood. Sweden is a country dominated by forests, mainly softwood. Therefore, sourcing fibres originating from softwood was a natural choice for this work. The part of wood used in pulp production is the longitudinal tracheids in softwoods, and these are referred to as "fibres" in the pulping process [51]. The fibres in wood are held together by the middle lamella, which consists mainly of lignin. Pulping can be done by mechanical or chemical means. During mechanical pulping the fibres are liberated by grinding, but no delignification occurs. In the chemical pulping process, chemical reactions degrade and dissolve lignin to liberate the wood fibres, while affecting the strength-bearing polysaccharides as little as possible [51]. Chemical pulping processes are further divided into kraft, sulphite and soda pulping [53].

The cellulose fibres used in this work were obtained by pre-hydrolysis soda pulping followed by oxygen delignification (Figure 3). During pre-hydrolysis, a substantial part of the hemicelluloses is removed by water at elevated temperature, i.e. autohydrolysis [54]. Delignification takes place during soda cooking, where the cooking agent is the hydroxide ion (OH^-). Lignin is removed in the following oxygen delignification step. This process results in a pulp with a cellulose content above 98%. The pre-hydrolysis step used before the addition of soda has been shown to open up the wood matrix [55]. This

was of importance in the present work as the pulp fibres were to be subjected to enzymatic hydrolysis, and an open cellulose structure provides a greater surface area for enzyme action.

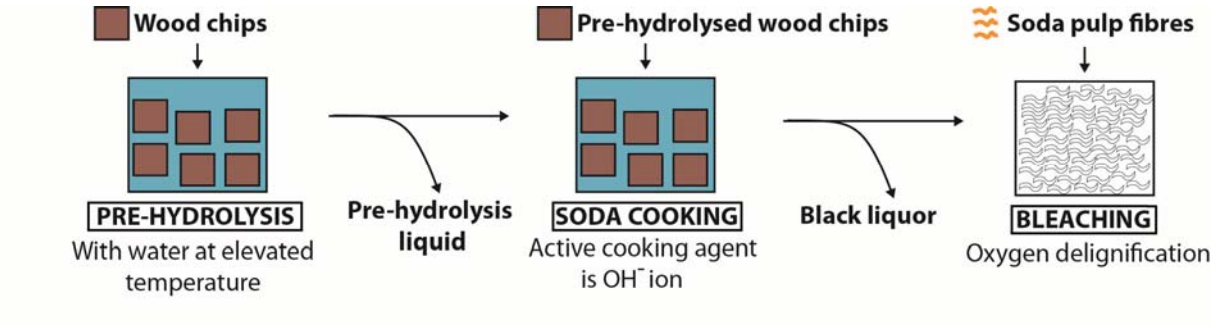

Figure 3. The main steps in a pre-hydrolysis soda cooking process.

The major application of the chemical pulping process is in the production of paper pulp and dissolving pulp, however there is considerable interest in the production of new added-value products using the biorefinery concept [55-58] (Figure 4).

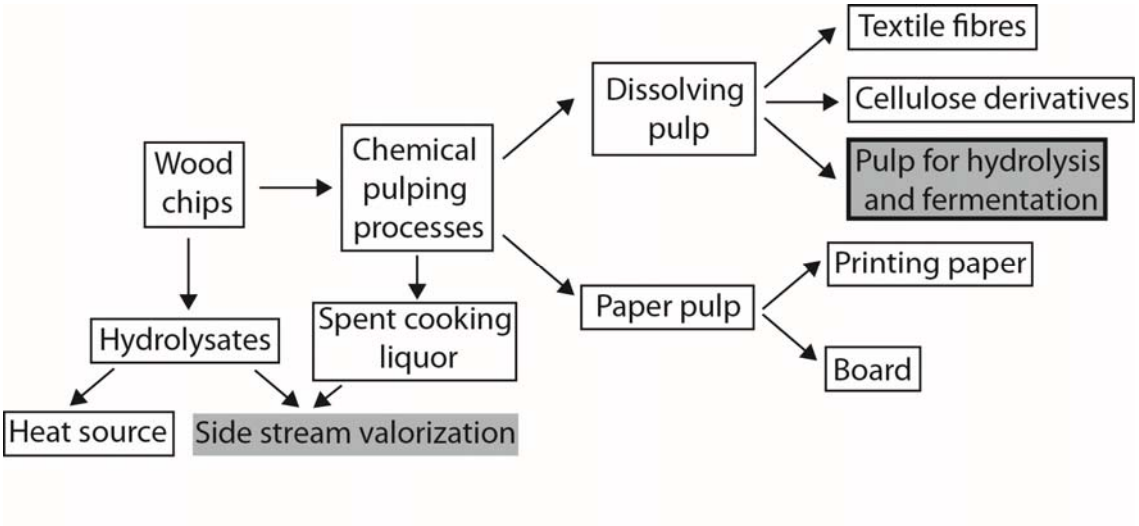

Figure 4. Major applications of the chemical pulping process. Existing processes and applications are given in white boxes, while those in grey-shaded boxes are areas where significant research is being devoted to finding novel uses of these streams. The area of research described in this thesis is indicated in the grey-shaded box with a heavy black boarder.

The development of new products from cellulosic materials is dependent on understanding the structure of cellulose that determines the properties of new materials. (Chapter 6 describes the structure of cellulose in greater detail.)

4 Cellulolytic enzymes

Many different enzymatic activities are involved in the hydrolysis of plant biomass. However, in this chapter only the enzymes involved in the hydrolysis of cellulose will be discussed. For other enzyme activities that are important for the complete hydrolysis of pretreated lignocellulosic material the reader is referred elsewhere [59-61]. The terms "cellulases" or "cellulolytic enzymes" commonly used in the literature are synonymous, and refer to multicomponent enzyme systems. Recently, the understanding of enzymatic hydrolysis of cellulose based on CBHs, EGs and BGs working in synergy has been complemented with a new family of enzymes, LPMOs. The hydrolysis of cellulose is distinct from most other enzymatic reactions because it involves soluble enzymes acting on an insoluble substrate. For the enzymatic hydrolysis of cellulose to be possible, the cellulases must first be adsorbed onto the surface of the insoluble substrate. Cellulases, having different structures and preferences for the binding sites on the substrate work in synergy to hydrolyse insoluble cellulose to the final product glucose. However, this is not an easy task for the enzymes.

4.1 Molecular structure and mechanisms of cellulolytic enzymes

Cellulases are modular enzymes composed of independently folded, structurally and functionally discrete units, referred to either as domains or modules. Most of the cellulolytic enzymes have a modular structure containing three separate structural elements, a catalytic domain (CD), a carbohydrate-binding module (CBM), and an interdomain linker [62]. CBMs were previously defined as cellulose-binding domains (CBDs) as the first examples of these protein domains bound crystalline cellulose as their primary ligand [63, 64]. The main proposed functions of CBMs are to concentrate enzymes on the polysaccharide substrates, maintaining the enzyme in the proximity of the substrate, helping to direct the CD to the substrate [64]. It has been shown that CBMs from two different families that were appended to the same CD exhibited different

capabilities to degrade crystalline cellulose, implying that CBMs can recognize distinct regions of this otherwise chemically invariant polysaccharide [65]. It has been suggested that the presence of a CBM is an advantage for enzyme activity at low substrate loads, but a disadvantage at high enzyme loads [66, 67]. The generally accepted paradigm is that the CBMs of cellulases are required for efficient saccharification of insoluble substrates. Based on sequence data, a large proportion of identified cellulases seem to lack CBMs, and this finding raised the question of the role of CBMs in nature [67]. The interdomain linkers are heavily glycosylated to protect them from proteolysis. Besides serving as a tether between the CBM and the CD, it has been suggested that the linkers bind directly to cellulose, which increases the binding affinity over the CBM alone [68]. This information was obtained from simulations of molecular dynamics, however, concerns could be expressed that binding of the linker to the substrate would restrict the movement of the enzyme on the cellulose surface.

Cellulolytic enzymes, glycosyl hydrolases (GHs), hydrolyse glycosidic bonds via the mechanism of general acid catalysis, which requires a proton donor and a nucleophile/base, denoted AH and B^-, respectively in Figure 5. Hydrolysis, as the name suggests, leads to the breaking of bonds by adding water. Hydrolysis occurs via two main mechanisms, giving rise to either the retention or inversion of the anomeric configuration [69]. During the hydrolysis of the β-glycosidic bond by an inverting enzyme, a product with the α-configuration is created, whereas with retaining enzymes the β-configuration is preserved.

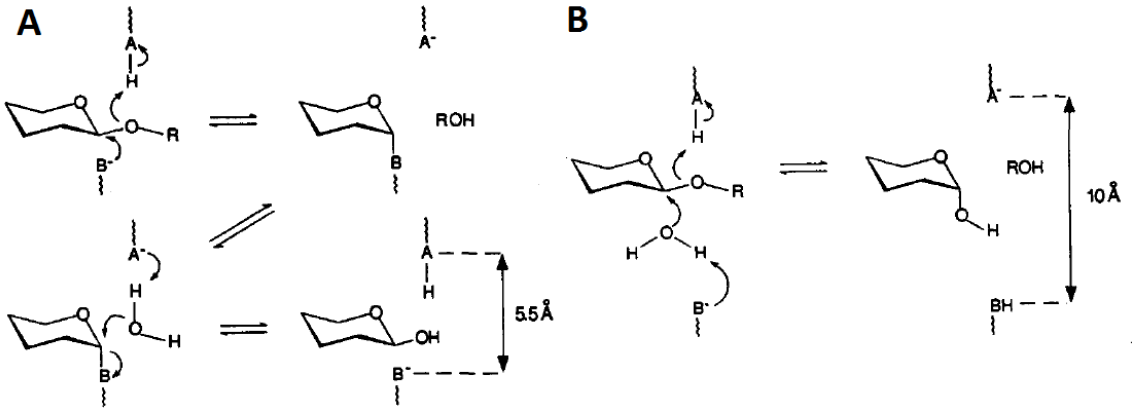

Figure 5. Schematic representation of the retaining (A) and inverting (B) mechanisms. The retaining mechanism proceeds in two steps. First, a covalently bound intermediate is formed through nucleophilic, B^-, attack. In the second step, a water molecule frees the hydrolysis product from the enzyme and recharges the proton donor, AH. During the inverting mechanism, protonation of the glycosidic oxygen and release of the hydrolysis product are accompanied by the concomitant attack by a water molecule that is activated by the base residue, B^-. (Reprinted from [69], copyright 1995, with permission from Elsevier).

CBHs and EGs act on insoluble substrates [70]. Three-dimensional structures of the cellulases Cel6A (CBH II) [71] and Cel7A (CBH I) [72] have shown that the active sites of CBHs are located inside the tunnel. In Cel7A (CBH I), this tunnel is 50 Å long, while in Cel6A (CBH II) it is shorter, 20 Å. These tunnels have proved to be essential for the progressive action of CBHs to cleave cellulose chains from the reducing, Cel7A (CBH I), or non-reducing ends, Cel6A (CBH II), and to release cellobiose as a major product [70]. CBHs with shorter active site tunnels may exhibit some degree of EG activity. The structure of EG I [73] has revealed the presence of an open substrate-binding cleft rather than a tunnel. EGs cleave at random at internal disordered sites in the cellulose polysaccharide chain, producing oligosaccharides of various lengths, and consequently new chain ends [74]. In *T. reesei* Cel6A (CBH II) and Cel7A (CBH I) dominate over other cellulases. The action of CBHs includes 'pulling' the cellulose chain away from its neighbouring chains, which is considered difficult. In addition, CBHs perform simultaneous multiple hydrolysis reactions without dissociating from the substrate. The task of EGs is less demanding as they hydrolyse within the available chain and then dissociate [75]. BGs act on cellobiose and short soluble oligosaccharides. BGs are essential for the efficient hydrolysis of cellulosic biomass as they relieve the inhibition of the CBHs and EGs by reducing end product (i.e., cellobiose) inhibition [76]. *T. reesei*, the organism commonly used for the production of industrial cellulase cocktails, naturally

secretes only low amounts of BGs into the culture broth. Commercial cellulase mixtures are therefore often supplemented with BG activity originating from other microorganisms.

4.2 Molecular structure and mechanism of lytic polysaccharide monooxygenases

Copper-dependent LPMOs were recently found to be broadly spread in both the bacterial and fungal kingdoms [19, 24]. This discovery constituted a breakthrough in the understanding of the fundamental mechanisms of biomass utilization [21, 24, 77-79]. Initially, it had been thought that LPMOs acted on highly crystalline substrates, such as cellulose and chitin (a nitrogen-containing polysaccharide, chemically related to cellulose). However, LPMOs acting on water-soluble cellulose-derived oligosaccharides [80], hemicellulose [21] and starch [23] have recently been described. LPMOs promote the efficiency of cellulases by cleaving glycosidic bonds in polysaccharide chains through oxidative action, thus rendering the substrate more susceptible to hydrolysis by other cellulases. Many LPMOs contain CBMs [79], which suggests that LPMOs have specific and not random recognition of the substrate. LPMOs have been shown to form products oxidized in the C1 position [19, 24] and/or C4 position [80-82] of the sugars, and oxidation at the C6 position has also been debated in the literature. LPMOs use copper-oxygen species as opposed to classical acid/base-facilitated hydrolysis (Figure 5) to initiate and promote polysaccharide breakdown. An external electron donor is also required for LPMOs to be active [78].

Although it is only a few years since the discovery of LPMOs, they already play a central role in commercial enzyme preparations, e.g. the Cellic CTec enzyme products produced by Novozymes A/S. The roles of GHs and LPMOs have mostly been described in the context of biomass conversion. There is an emerging body of literature in which LPMOs have been associated with the pathogenicity of bacteria [22]. The range of action of LPMOs remains to be elucidated.

4.3 Classification of cellulolytic enzymes

Cellulases can be classified in two main ways, based either on substrate specificity or on the structural similarities between the enzymes. The International Union of Biochemistry and Molecular Biology classifies enzymes based on the nature of the reactions that they catalyse, and the EC number system is used. All cellulolytic enzymes belong to the *O*-glycosyl hydrolases (EC 3.2.1.x) [69]. Traditionally, cellulases have been classified into two distinct classes such as EGs (EC 3.2.1.4) and CBHs (EC 3.2.1.91). BGs (EC 3.2.1.21) are sometimes classified as cellulases, but they are not 'real' cellulases because they act on soluble oligosaccharides.

It has been noted that the sequence-based families of GHs grouped together enzymes with different specificities. Therefore, the Carbohydrate-Active Enzymes (CAZymes) database (http://www.cazy.org), which uses sequence-based family classification for the enzymes that assemble, modify and break down oligo- and polysaccharides, has been proposed [83, 84]. The CAZy classification was accepted and, as a consequence, the *T. reesei* cellulases were renamed. For example, CBH II is now called Cel6A, where "Cel" denotes "cellulase", "6" the GH family and "A" that this was the first reported family 6 cellulase from this organism. LPMOs are classified into auxiliary activity class AA9 (formerly GH61), AA10 (formerly CBM33), AA11 and AA13 in the CAZy database [85-87]. LPMOs puzzled scientists for a while. They were originally classified based on measurements of very weak EG activity in one family member [88]. It took more than 10 years after the GH61 family had been first recognized, for the reaction mechanism to be reported [19, 79].

4.4 Enzymatic hydrolysis of cellulose

Successful degradation of native cellulose requires the cooperative action of a multicomponent enzyme system. Originally, Reese et al. [20] suggested that undefined enzymes could play a major role in the step prior to hydrolysis (Figure 2). LPMOs are promising candidates for the unidentified enzymes responsible for the C_1 step. LPMOs

have been shown to help overcome the recalcitrance of cellulose by disrupting its structure, followed by the action of cellulases [78]. LPMOs work in synergy with the classical cellulases discussed in Section 4.1. Non-hydrolytic proteins called expansin-like proteins, such as swollenin [89] and loosenin [90], have recently been suggested to function by enhancing hydrogen bond disruption and aiding in the amorphogenesis of cellulose. Cip proteins (Cip 1 and Cip 2) have also been suggested as relevant proteins in the enzymatic hydrolysis of plant biomass [91]. An idealized picture of enzymatic hydrolysis of cellulose is shown in Figure 6.

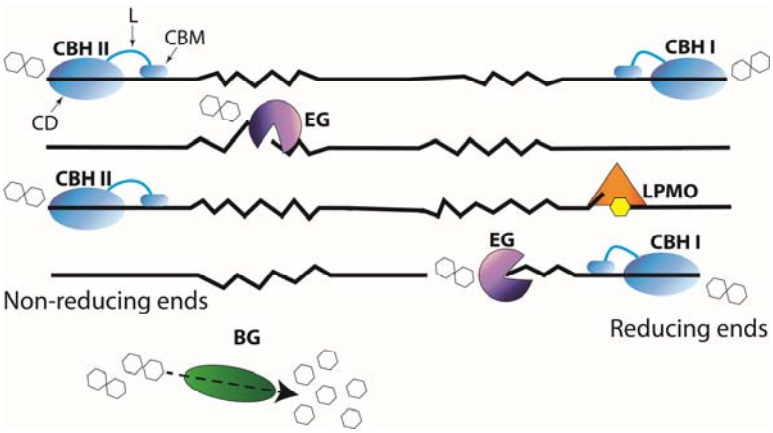

Figure 6. Many enzymes are involved in the enzymatic hydrolysis of cellulose. Cellulose has crystalline (ordered) and non-crystalline (disordered) regions. Cellobiohydrolases (CBHs) work progressively from the reducing end (CBH I) and non-reducing end (CBH II) of cellulose, releasing cellobiose. Most of the cellulolytic enzymes have a modular structure containing a catalytic domain (CD), a carbohydrate-binding module (CBM), and an interdomain linker (L). Endoglucanases (EGs) introduce random cuts in the amorphous regions of cellulose. β-glucosidase (BG) hydrolyses cellobiose and soluble oligosaccharides (up to a degree of polymerization of 6). Lytic polysaccharide monooxygenases (LPMOs) cleave glycosidic bonds in polysaccharide chains through oxidative action.

For efficient enzymatic hydrolysis it is essential that enzymes work in cooperation, or synergistically. Synergy between cellulolytic enzymes occurs when the combined action of the enzymes leads to a higher rate of action than the sum of their individual actions [45, 74]. One of the possible reasons for the production of multiple cellulases for the hydrolysis of only one type of bond present in cellulose, the β-1,4 linkage, is that the supramolecular structure of cellulose is rather complex, as will be discussed in more detail in Chapter 6. Enzymes cover a large surface area during their action on cellulose.

The catalytic domain of Cel7A (CBH I) has been shown to cover 10 sugar units [92]. Although the GHs are “optimized” to hydrolyse β-1,4 linkages in cellulose polymers, they encounter numerous challenges. Steric hindrance of bound enzymes, called “traffic jams”, have been shown to reduce the hydrolytic efficiency of cellulase on the cellulose surface [16]. Cellulose is a heterogeneous substrate when it comes to its structure, and obstacles on its surface hinder enzyme action [93]. The efficiency of enzymatic hydrolysis also depends on the pH, mixing and temperature [66]. LPMOs also require copper, oxygen and a reducing agent to be active [78, 81]. Addition of non-ionic surfactants have also been shown to increase the glucose yield obtained from enzymatic saccharification of cellulosic substrates [94].

5 Cellulase production and analysis

Much can be gained by designing more efficient enzyme cocktails for the hydrolysis of plant biomass. Bacteria and filamentous fungi produce enzymes for biomass degradation in order to have a competitive advantage in their natural environment. Filamentous fungi are well known for their ability to decompose organic matter in general, and cellulosic substrates in particular. Generally, only a few bacterial species are cellulolytic. Filamentous fungi have been studied extensively with regard to the production of enzyme mixtures for biomass hydrolysis [74]. Among them, *T. reesei* is in widespread industrial use for enzyme production. It has been claimed that there is a correlation between the composition of the enzyme mixture produced by filamentous fungi and the composition of the carbon source [95].

Proteomics can be used for accurate determination of the protein composition of several hundred protein components produced by filamentous fungi. Enzymes produced by filamentous fungi for biomass degradation are mainly secreted outside the cell, therefore, the type of proteomics used to study secreted proteins is called “secretomics”. Studies of the enzymes produced by filamentous fungi in response to the carbon source may provide important information, making several improvements possible. Examples of these are: (i) the detailed characterization of an enzymatic cocktail reflecting the potential of the fungus studied, (ii) the improvement of industrial cellulase mixtures with complementing enzymatic activities, and (iii) the identification of novel enzymatic activities. Identification of LPMOs through secretomic analysis demonstrates the enormous potential of enzyme discovery [96]. In this work, the potential of enzymes produced by different filamentous fungi was evaluated to improve the saccharification ability of the investigated industrial cellulase cocktail (**Paper IV**), therefore attention will be focussed on filamentous fungi as cellulase producers. Enzyme production was studied by employing secretome analysis of *T. reesei* during its growth on different cellulosic substrates (**Paper III**). Therefore, *T. reesei* will be described in more detail in this chapter.

5.1 Cellulase producers in nature

Microorganisms play an important role in the global carbon cycle by hydrolysing cellulose in plant cell walls. Cellulose serves as a carbon and nutrient source for the microorganisms. However, it is not readily available as a nutrient source because it exists as highly ordered linear β-(1,4)-D-glucan polymers bundled together in fibrils. Filamentous fungi secrete cellulases which hydrolyse β-(1,4)-D-glucan polymers to release glucose.

Generally, aerobic microorganisms secrete individual cellulases from the cell. Many anaerobic microorganisms have evolved to degrade plant cell walls by the formation of a large extracellular enzyme complex called the cellulosome. The cellulosome consists of a non-enzymatic scaffolding protein and many bound cellulases. Cell-free and cell-bound cellulosomes have been described [61, 97]. The anaerobic rumen bacterium, *Fibrobacter succinogenes*, has recently been shown to be specialized for growth on cellulose, as it uses an array of hemicellulose-degrading enzymes only to gain access to cellulose, and it lacks many of the genes necessary to transport and metabolize the hydrolytic products of non-cellulose polysaccharides. Adherence of the bacterium to a solid cellulose substrate appears to be a requirement, it does not possess cellulosomes, and little cellulase activity is detected in culture medium [98]. Another unusual anaerobic soil bacterium, *Cytophaga hutchinsonii*, has been suggested to use EGs attached to the cell surface to attack insoluble cellulose while gliding along the fibres [99]. Nature exhibits a vast diversity of microorganisms whose mechanisms for degrading cellulose are not yet fully understood.

Fungi employ different mechanisms when attacking wood biomass [60] (Table 2). Wood decay fungi have historically been classified into white rot, which degrades all components of wood cell walls, or brown rot, which leaves the lignin largely intact. Lignin-degrading phenol oxidases are the key enzymes of white-rot fungi [60]. Brown-rot fungi have evolved from white-rot fungi [100]. It has been suggested that brown-rot wood decay involves initial non-enzymatic attack on the wood cell wall, generating hydroxyl radicals (·OH) extracellularly via the Fenton reaction. Fenton systems include mechanisms for extracellular H_2O_2 generation and for the reduction of Fe^{3+} to Fe^{2+}, which might be accomplished by extracellular fungal metabolite such as hydroquinone or by extracellular enzymes such as cellobiose dehydrogenase. However, it is not understood

how·OH targets wood cell wall components [100, 101]. Moreover, a recent sequencing of the brown-rot fungus *P. placenta*, which has been suggested to use the Fenton reaction showed a number of AA9 (formerly GH61) genes [100], which raises questions about the actual mechanisms of wood degradation by brown-rot fungi. Little is known about the degradation of lignocellulose by soft-rot fungi. Soft-rot fungi typically attack materials with higher moisture, and lower lignin content [60]. The soft-rot fungus *T. reesei*, which is a model organism for cellulose and hemicellulose degradation, has been shown to have laccase activity [102], suggesting that it may have the ability to degrade lignin. A new categorization of rot types has been suggested based on the improved understanding of the genomics and biochemistry of wood decay [103].

Table 2. Comparison of white-rot, brown-rot, and soft-rot fungi

Mechanism	Enzymes	Parts of wood degraded	Examples of fungi	Reference
White rot	Hydrolytic enzymes and ligninolytic enzymes	All parts of plant biomass; complete degradation of lignin to carbon dioxide	*Phanerochaete chrysosporium, Pycnoporus cinnabarinus*	[104, 105]
Brown rot	Mainly non-enzymatic attack and relatively few cellulases	Hemicellulose is hydrolysed first, then cellulose; lignin is modified	*Postia placenta, Serpula lacrymans*	[100, 101]
Soft rot	Cellulases and hemicellulases	Carbohydrate polymers with low lignin content	*T. reesei, Xylaria longipes*	[106]

Filamentous fungi are important for the production of biomass-degrading enzymes in industry. Fungi have also been used to modify wood to produce a superior kind of wood which resembles that of a Stradivarius violin [106]. There is unutilized potential in the rich diversity of enzymes produced by filamentous fungi that could be used to design efficient enzyme mixtures for cellulose hydrolysis. Filamentous fungi produce a very complex cocktail of enzymes that are specific to the individual fungus and to its growth conditions. Fungal genome sequencing has also accelerated in recent years, an example is "1000 Fungal Genomes" project [107]. Emerging fungal genomes are revealing a large number of putative genes. The percentage of genes in fungal genomes without known or predicted function is around 30-45% with, on average, a higher percentage in

basidiomycetes than ascomycetes (R.P. de Vries, personal communication). Although *in silico* annotations of fungal genomes provide extensive amounts of information, experimental analyses are necessary to better understand the complex mixture of enzymes secreted in response to inducers. The study presented in **Paper IV** showed that the production of cellulolytic enzymes is strongly dependent on the nature of the carbon source. Enzymes from carefully selected fungi can result in enzyme cocktails that are significantly more efficient in plant biomass saccharification.

5.2 *Trichoderma reesei*

The research of cellulases started with the isolation of the filamentous fungus strain *T. reesei*, which caused considerable problems for the US army during World War II as it rotted their cotton fabrics. The advantage of the outstanding cellulolytic activity of this fungus was soon recognized [108]. *T. reesei* (teleomorph *Hypocrea jecorina*) was first identified as *T. viride* QM6a, but was later shown to be different from *T. viride*, and was therefore renamed *T. reesei* in honour of Elwyn T. Reese [17, 108, 109]. Interest in enzyme production by *T. reesei* increased during the oil crisis in the 1970s, when saccharification of cellulose to glucose and its subsequent conversion to ethanol for use as a renewable fuel became economically attractive. Among the improved mutants, a widely studied *T. reesei* strain, Rut C-30, was developed after three rounds of mutagenesis of wild-type QM6s [110, 111], which is still considered one of the best producers of cellulolytic enzymes in the public domain (Figure 7). Treatment of QM6a with UV light and selection for the ability to hydrolyse cellulose under carbon catabolite repression led to strain M7. ("Carbon catabolite repression" arises when the end product of cellulose hydrolysis, glucose, inhibits further enzyme synthesis.) The M7 strain was further mutagenized by chemical treatment under carbon catabolite repression. This led to the isolation of strain NG14, which showed a significant increase in secreted protein and cellulase activity, but still exhibited considerable catabolite repression. NG14 was subjected to another round of UV light irradiation, and screening for elevated cellulose hydrolysis levels and resistance to 2-deoxyglucose to eliminate carbon catabolite repression led to the identification of Rut C-30 [112]. The resulting strain produces twice

as much extracellular protein as its parental strain NG14, reaching more than 30 g L^{-1} in industrial fermentation, and it also exhibits catabolite derepression [17].

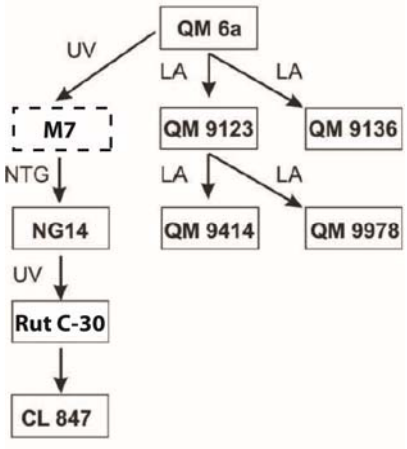

Figure 7. Pedigree of important *T. reesei* strains derived from the original isolate QM 6a by classical mutagenesis, including irradiation by linear particle accelerators (LA), or exposure to UV light (UV) or N-methyl-N′-nitro-N-nitrosoguanidine (NTG). All the strains except CL 847 have been sequenced. Strain M7 is shown in the box with a dashed line as it is no longer available. (Figure from [17]).

So far, numerous other mutations have been identified, in addition to the three mutations in *T. reesei* Rut C-30 found previously: 1) a truncation of the *cre1* gene, which renders the strain carbon catabolite derepressed; 2) a frameshift mutation in the glucosidase II alpha subunit gene *gls2α* involved, which in turn increases protein secretion by an unknown mechanism; and 3) an 85-kb deletion that eliminated 29 genes, including transporters, transcription factors, and primary metabolic enzymes [112].

T. reesei represents a paradigm for the production of enzymes that hydrolyse biomass polysaccharides [17, 108, 113]. *T. reesei* produces two CBHs (Cel7A and Cel6A), five EGs (Cel7B, Cel5A, Cel12A, Cel61A and Cel45A), and two BGs (Cel3A and Cel1A) [114]. Numerous genes encoding biosynthetic pathways for secondary metabolites may promote the survival of *T. reesei* in its competitive soil habitat. Genome analysis provided little mechanistic insight into its extraordinary capacity for protein secretion [113].

5.3 Influence of the insoluble carbon source on the production of cellulases in *T. reesei*

Various natural lignocellulosic substrates are able to induce the fungal production of secreted enzymes suitable for degrading specific combinations of polysaccharides and chemical bonds found in the carbon source [95]. Mary Mandels and E. T. Reese raised the question of the induction of cellulases by cellulose in the 1960s [115]: "The inducing substrate, cellulose, is insoluble. How then does the induction occur?", and the answer has eluded researchers since then. Cellulosic substrates cannot cross the cell membrane due to their insoluble nature. Several explanations have been suggested, most of which involved the formation of a soluble, low molecular weight inducer from cellulose [116]. The generally accepted hypothesis for the induction of cellulases is that the low levels of constitutively expressed cellulases first hydrolyse cellulose to soluble sugars. These sugars are presumably converted into true inducers which enter the cell directly. Sophorose (two β-1,2-linked glucose units) is the most potent soluble inducer in *T. reesei*, and has for many years been considered to be the natural inducer of cellulases in *T. reesei*. The fact that sophorose formation requires the action of BGs has been discussed [95]. The regulation of cellulases is driven by specific transcriptional factors that bind to cellulase gene promoters acting either inductively or repressively. Among them, at least three transcriptional activators XYR1, ACE2 and HAP2/3/5, and the two repressors CRE1 and ACE1, are involved in the regulation of different cellulase genes in a coordinated way [95].

Both the amounts of enzymes produced and which enzymes are produced by fungi depend on the cultivation conditions. Different temperatures, pH values and agitation rates have been investigated when evaluating the enzyme production potential of *T. reesei* Rut C-30 during growth on Avicel as the carbon source. It has been found that not only were the protein levels influenced by the fermentation conditions, but the expression profile was also influenced. This profile had a profound effect on the performance of the enzyme mixtures during the hydrolysis of biomass [91]. It has been suggested by some studies that application of enzymes to hydrolyse the same substrate as was used for enzyme production could be advantageous [49, 117], as reported in **Paper IV**. However, other studies revealed no such advantage [118].

Numerous studies have been carried out in which insoluble cellulosic substrates were used as the carbon source for enzyme production by fungi, however, few attempts have been made to relate the enzyme profile of the fungus to the structural characteristics of cellulosic substrate. The hypothesis tested in the study described in **Paper III** was that the supramolecular structure of cellulose would have an effect on the profile of the enzymes secreted by *T. reesei* Rut C-30. One of the unique aspects of this study was the effect of the structural properties of cellulosic substrates on fungal response. Structural differences in the cellulose-rich substrates were found to cause *T. reesei* to produce different titres of proteins and exhibit different extracellular enzyme profiles.

5.4 Secretome of *T. reesei* and its analysis

Secretomic analysis (secretomics) refers to the systematic identification and quantification of all proteins (the secretome) of a biological system at a specific point in time. Apart from providing information on fungal physiology, secretome analysis has recently shifted towards the characterization of enzyme mixtures, in order to facilitate the development and optimization of specific enzymatic cocktails for the more efficient hydrolysis of biomass. In proteomic research, proteins are usually identified by the mass-to-charge ratio of their peptides and fragments using mass spectrometry (MS) in combination with database searches. MS-based proteomics is characterized by a great variety of techniques and instrumentation [119]. Sample separation prior to MS analysis is generally required to reduce the biological complexity of the sample, in order to reduce the risk of ambiguous identifications. Biological samples often contain large numbers of proteins at highly varying concentrations. The risk is that abundant species may conceal less abundant ones if the sample is not pretreated. The separation techniques commonly used in proteomics can be divided into gel-based and gel-free approaches [120]. The currently most popular method among gel-based approaches is 2D difference gel electrophoresis, in which proteins from different samples are labelled with different fluorescent probes, enabling quantification of proteins from different samples in the same gel [121]. In the gel-free approach, protein separation by multidimensional liquid chromatography (LC) is combined with MS, the method often being denoted LC-MS/MS. Gel-free quantitative proteomics can be broadly categorized into label-based and label-

free methods. The most common *in vitro* labelling method in multiple samples relies on chemical labelling, using isobaric mass tags such as iTRAQ® (Isobaric Tags for Relative and Absolute Quantitation) or TMT® (Tandem Mass Tag) [122]. Secretome analysis using TMT was used in the study presented in **Paper III**. Isobaric tags are applied after enzyme digestion of the protein samples to covalently label the peptides of different samples. The isobaric mass tags have different isotopic substitutions so, as the tags are cleaved off the peptides in the MS/MS mode, the result is reporter ions of different weight, thus enabling quantification of each protein from different samples [122]. These techniques provide only relative quantification of proteins, and cannot be used to determine absolute protein abundance in samples. Accurate estimation of protein abundance in multiple samples using MS-based proteomic strategies remains difficult [123]. No single method can provide complete information on all the protein components in a complex mixture, and different methodologies are usually required to provide detailed quantitative information [120].

Chundawat et al. [124] explored the protein composition of several commercial enzyme preparations from *T. reesei* using a proteomic approach. They demonstrated that in Celluclast 1.5L the major enzyme was Cel7A (CBH I), followed by Cel6A (CBH II). Interestingly, considerable amounts of accessory proteins, such as swollenin (4%) and Cip proteins (5%) were present. Proteins Cip1 and Cip2, which were found in *T. reesei* cultures by Lehmann et al. [91] were identified as relevant proteins for the hydrolysis of biomass, and were therefore suggested to study further [91]. Very little is known about Cip1 except that it has a secretion signal peptide and a CBM. Extracellular proteins secreted by *T. reesei* have been studied during fungal growth on lactose, cellulose and more complex lignocellulose substrates [91, 123, 125-131] (Table 3). Although *T. reesei* is known to be a poor producer of BG, its production has been shown to be favoured during growth in a lactose-based medium [128]. *T. reesei* is known to produce high levels of proteases [132], according to secretome analysis [123]. It has also been shown that a number of oxidative enzymes are formed by *T. reesei* during growth on cellulose [127]. In some proteomic studies several intracellular enzymes were detected in the culture filtrate. This indicates that enzyme secretion by *T. reesei* is accompanied by considerable autolysis or mycelial fragmentation, the possible roles for high enzyme production which have not yet been investigated [126]. Secretome analysis is usually performed on the culture filtrate. The tendency of the enzymes to adsorb onto the substrates productively,

due to the presence of CBMs, and non-productive adsorption on lignin [133] further complicates the picture, as the enzyme levels in the fungal culture supernatants might not give a true picture of the real enzyme production.

Table 3. Distribution of cellulase components in crude extracts of *Trichoderma*

Enzyme	**Celluclast 1.5L** [a]	**A**	**B**
Cel3A (BG)	3	2	2
Cel7A (CBH I)	35	1	1
Cel6A (CBH II)	13	14	8
Cel7B (EG I)	6	n.d.	1
Cel5A (EG II)	6	34	3
Cel12A (EG III)	0.5	0.4	0.5
Cel45A (EG V)	0.1	1	2
AA9 (GH61A and GH61B)	1	1	3
Hemicellulases and enzymes acting on starch	21	23	37
Other proteins	13	24	43

n.d.: not determined; [a] % protein composition from Chundawat et al. [124]; A: relative abundance (%) from cultivation D from Lehmann et al. [91]; B: relative abundance (%) from cultivation Avicel, L from **Paper III**.

5.5 Microorganism morphology

The morphology exhibited by the filamentous fungus during growth will determine its enzyme production capacity. Filamentous fungi can grow as freely dispersed mycelium, aggregates of mycelium and very dense clumps also referred to as pellets, which can be up to several mm in diameter in submerged fermentation [134-136]. In the present work, *T. reesei* Rut C-30 formed pellets that consisted of hyphae and pulp fibres during growth on the pulp fibres (**Paper III**). When the fibres were hydrolysed, the hyphae grew in a dispersed manner. Which of these macroscopic morphologies dominates in a given

submerged cultivation is determined by several factors, e.g. the strain used, the stirrer speed, the spore concentration of the inoculum and the pH during germination. Low inoculum concentrations of *T. reesei* Rut C-30 have been shown to result in pellet morphology, while increasing the inoculum led to pulpy growth with high concentrations of mycelium [137]. Dispersed hyphal growth is the preferred macroscopic morphology for enzyme production, as it allows almost all the individual hyphal elements to be in contact with the medium. However, this could be related to the specific organism and/or strain, rather than being a general feature of filamentous fungal morphology. In the case of pellet growth, the hyphal elements will be inside the pellets, resulting in problems in the diffusion of substrates and products. The major drawback of freely dispersed growth is the resulting high viscosity of the broth. This may also prevent the cultures from being well oxygenated during large-scale enzyme production.

Not only the macroscopic morphology is important for the level of enzyme production by filamentous fungi, but also the microscopic one [138]. The microscopic morphology refers to the morphology of the individual hyphal elements, i.e., the diameter and length of the hyphal elements and the number of tips on an individual hyphal element. It has been suggested that there is a correlation between the number of tips and the enzyme secretion capacity [139, 140]. However, data on the actual secretion efficiency and tip density are not yet conclusive [140]. It is well-established that the majority of secreted proteins are secreted through the growing hyphal tips [141].

6 Cellulose composition and structure

The structure of wood can be studied at different levels: (i) the macroscopic level, on the scale of millimetres and above, which includes large tissue elements; (ii) the microscopic level, on the scale of micrometres, which includes the main elements of the cell wall; (iii) the supramolecular structure (sometimes also referred to as the ultrastructure), on the scale of nanometres, which includes the assembly formed by fibrils and fibril aggregates; and (iv) the molecular level [142]. Regardless of the source, cellulose is always composed of β-(1,4)-D-glucan polymers arranged into fibrils and fibril aggregates, which form a complex assembly. How these β-(1,4)-D-glucan polymers assemble and their further arrangement into complex networks depends on the source, method of isolation and sample treatment prior to analysis.

Cellulose I is the crystalline cellulose that is produced naturally. Native cellulose I can undergo reversible and/or irreversible conversion during various treatments, to form other polymorphs, such as cellulose II, III and IV, which are non-native forms of cellulose. The work described in this thesis was focussed on native cellulose I and, therefore, only cellulose I will be discussed in this chapter. The model of supramolecular structure of cellulose used in this work is described in Section 6.2. Findings from the study on supramolecular structure of cellulose during enzymatic hydrolysis, some of which are presented in **Paper I**, are discussed in Section 6.3.

6.1 Native cellulose

Cellulose I exists in the forms of fibrils, which are bundles of β-(1,4)-D-glucan polymer chains. Each β-(1,4)-D-glucan polymer chain is composed of anhydroglucopyranose units joined via β-(1→4)-glucosidic linkages. The repeating unit in the β-(1,4)-D-glucan polymer is cellobiose, which is composed of two anhydroglucose residues linked via β-(1,4)-glucosidic bonds (Figure 8). The degree of polymerization in β-(1,4)-D-glucan polymer chains varies from 100 to over 15,000 depending on the cellulose source [48]. Since a molecule of water is lost during the formation of a β-(1,4)-glucosidic bond, the

glucose units in β-(1,4)-D-glucan polymer are referred to as anhydroglucose units. The C1-OH end of the β-(1,4)-D-glucan polymer has reducing properties, while the C4-OH end is an alcohol group which has non-reducing properties.

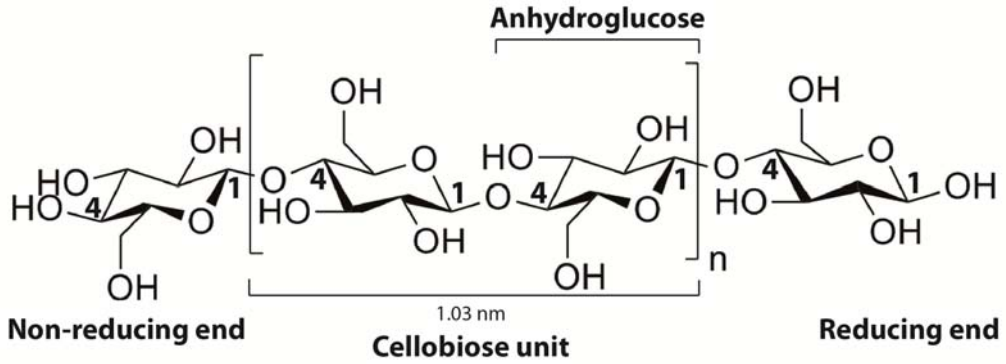

Figure 8. The β-(1,4)-D-glucan polymer has a reducing and a non-reducing end. It is composed of repeating units of cellobiose (length 1.03 nm), which consist of two anhydroglucose residues linked via β-(1,4)-glucosidic bonds.

Only at the reducing end of the polymer chain can the final ring open to expose an aldehyde end-group. Different chemical and enzymatic reactions (Figure 6) have a preference for the reducing or non-reducing end of the β-(1,4)-D-glucan polymer. Degradation of cellulose during pulping with alkali involves an undesired peeling reaction at the reducing end [51]. One of the most common reducing sugar assays used to evaluate the extent of enzymatic hydrolysis of cellulose is the dinitrosalicylic acid (DNS) method [143]. DNS reacts with the reducing end of glucose and the product is measured spectrophotometrically [144].

6.2 Supramolecular structure of cellulose

During biosynthesis, van der Waals forces and intermolecular hydrogen bonds between hydroxyl groups and oxygen atoms of adjacent molecules promote parallel stacking of multiple β-(1,4)-D-glucan polymers forming fibrils and fibril aggregates. The parallelism of polymers in cellulose I is also the result of biosynthetic constraints. Such parallel stacking is not seen, for example, in non-native cellulose (cellulose II), which suggests that it is not only the polymer interactions that are responsible for the parallelism (P.T.

Larsson, personal communication). There has been much discussion recently that hydrogen bonds cannot fully explain the insoluble nature of cellulose [145, 146]. Native cellulose I consists of two crystalline forms, called cellulose Iα and cellulose Iβ [33] which are present at various proportions depending on the source of the cellulose. The β-(1,4)-D-glucan polymers are oriented parallel in cellulose I, where all the reducing ends are on one side and the non-reducing ends are on the other side (Figure 6). In cellulose I the β-(1,4)-D-glucan polymers are arranged to form the nanostructure known as a fibril. Fibrils and fibril aggregates are often called microfibrils or elementary fibrils, and macrofibrils, respectively. The dimensions of the fibrils vary according to source, for example, 3.5 x 3.5 nm in cotton, 6 x 6 nm in bacterial cellulose and 10 x 20 nm in algae cellulose, according to CP/MAS ^{13}C-NMR measurements [33]. CP/MAS ^{13}C-NMR spectroscopy and X-ray diffraction methods are commonly used to study the structural characteristics of cellulose I and are dependent on conceptual models, which include some assumptions affecting the interpretation of recorded data [147]. Therefore, before comparing data from different studied, one should be aware how the data were obtained.

Cellulose fibrils contain ordered (crystalline) and disordered (non-crystalline) components [148-150]. It is not clear exactly how the ordered and disordered regions are distributed within the fibril, nor the extent to which they occur. In recent years, a fibril has commonly been represented as 36-chain fibril model has been proposed based on atomic force microscopy (AFM) imaging [149]. Recently a "rectangular" fibril model with 18–24 chains has been suggested for softwood, based on information from X-ray scattering measurements [150], where it was seen that the degree of disorder in chain packing increased outwards from the fibril centre. Thomas et al. [151] also concluded that 18–24 chain models of the primary wall of celery collenchyma were most likely to fit the results; a 24-chain cross section being more likely. *Valonia* cellulose crystals have been suggested to have an irregular hexagonal shape with two narrow hydrophobic faces, to which enzymes preferentially bind [152, 153]. Molecular dynamics simulations have shown that the surface structure of cellulose is different from that of bulk crystalline cellulose [154]. When water is sorbed to cellulose its properties differ considerably from those of bulk water [155]. Cellulosic substrates have been shown to constrain water to different degrees which had an effect on enzymatic hydrolysis studied by low-field NMW relaxometry [156]. Water constraint by model cellulose-rich substrates has been shown to be an advantage for enzymatic hydrolysis [156], however greater water constraint by

hemicelluloses has been shown to be inhibitory for cellulases [157]. Therefore, the picture of enzymatic hydrolysis of cellulose is more complicated than cellulose–enzyme interaction. Fibrils have a strong tendency to form aggregates due to hydrogen bonding interactions between the fibrils, and it has been shown that fibril surfaces within fibril aggregates are inaccessible to solvents [148], which in turn suggests that enzymes cannot access the surfaces of the fibrils within the fibril aggregate.

The models discussed above refer to the architectural organization of β-(1,4)-D-glucan polymers within a single fibril and fibril aggregates of cellulose. However, fibril aggregates assemble into a network. Figure 9 depicts the model used in **Paper I** to interpret the results of CP/MAS ^{13}C-NMR measurements on cellulosic substrates prior to and after enzymatic hydrolysis. This aim of this model is to describe the possible arrangement of fibrils and the complexity of the supramolecular structure of cellulose.

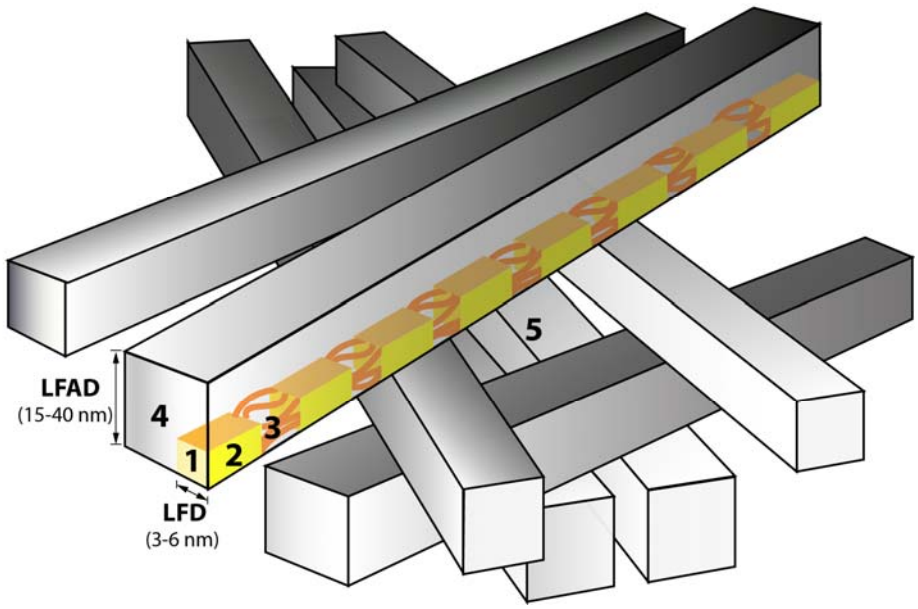

Figure 9. Schematic representation of the model used in the present work to represent the supramolecular structure of cellulose, where fibril aggregates are shown as having square cross-sections with the following key elements: a fibril which consists of a bundle of β-(1,4)-D-glucan polymers (1), which is a mixture of structures with a high degree of three-dimensional order (crystalline) (2) and disordered (non-crystalline) domains (3); a fibril aggregate (4), which is a structural element of cellulose composed of a bundle of fibrils; and a pore (5), which is a cavity between fibril aggregates. Modified from **Paper I** with permission.

The arrangement of cellulose fibrils varies depending on the layer in the plant cell wall [142, 158]. Thus, the model in Figure 9 does not refer to a particular layer in the plant

cell wall. This model incorporates knowledge obtained from the literature: (i) a highly porous fibre wall morphology, which has been shown to be the result of the removal of non-cellulosic material [159]; (ii) ordered (crystalline) and disordered (non-crystalline) regions [160-162]; and (iii) refinement of the model over the years by several researchers, including Iversen, Wickholm and Larsson [148, 162], who used spectral fitting of signal components from CP/MAS ^{13}C-NMR spectra to extract information on the relative amounts of cellulose allomorphs (Iα and Iβ), the average lateral dimensions of fibrils and fibril aggregates (LFD and LFAD), specific surface area (SSA), degree of crystallinity (DCr) and average pore sizes [163]. As the surface of cellulosic substrates must be accessible to enzymes for efficient enzymatic hydrolysis, the size of a typical fibre wall pore must be greater than the typical size of the enzyme molecules, which is around 10 nm [152].

6.3 Supramolecular structure of cellulose during enzymatic hydrolysis

The supramolecular structure of cellulose I may change as the result of sample history, e.g. isolation procedure, drying, depolymerization reactions, etc. In the study described in **Paper I** three cellulose-rich substrates, never-dried pulp fibres, cotton and Avicel, were evaluated with respect to changes in the cellulose supramolecular structure during drying, acid treatment and treatment with sodium hydroxide, as illustrated in (Figure 10). Sodium hydroxide treatment was performed in an attempt to increase the SSA of the sample, and was performed in such way that no detectable amounts of cellulose II were formed. Since the substrates used in this study had a cellulose content exceeding 96%, the main differences between the substrates were interpreted as being of structural nature. For a more detailed discussion the reader is referred to **Paper I**.

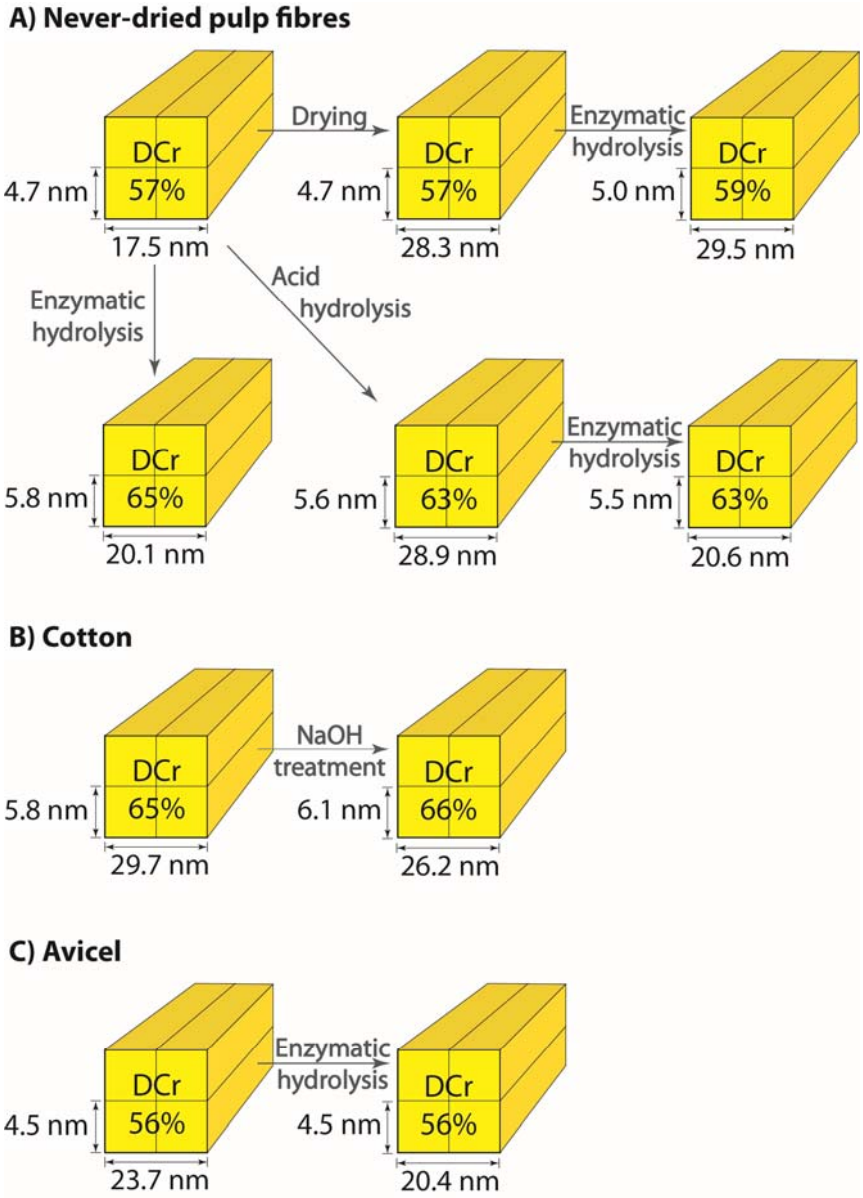

Figure 10. Illustration of the study on the changes in the supramolecular structure of three cellulosic substrates: (A) never-dried pulp fibres, (B) cotton and (C) Avicel, after different treatments. The never-dried pulp fibres were dried, and then subjected to acid hydrolysis and enzymatic hydrolysis. Cotton was treated with sodium hydroxide (NaOH). Avicel was subjected to enzymatic hydrolysis. The fibril aggregates are modelled as four fibrils, shown as rectangular yellow boxes (note that these are not drawn to scale). The dimensions of the fibrils and fibril aggregates are indicated in nm. The estimated degree of crystallinity (DCr) is given as percentages. The results depicted in this figure are from CP/MAS ^{13}C-NMR measurements summarized from **Paper I**.

The first observation was that never-dried pulp fibres had the largest SSA, i.e. the smallest LFAD, and cotton had the smallest SSA, i.e. the largest LFAD. Cotton and Avicel were

dried substrates. When never-dried pulp fibres were dried and rewetted, the LFD remained unchanged (4.7 nm) while the LFAD increased (from 17.5 to 28.3 nm), i.e. an increase in the degree of aggregation was observed (Figure 10A). Such a permanent increase in the LFAD has been observed in previous studies using CP/MAS ^{13}C-NMR [164, 165]. It has been suggested that a process called hornification takes place in the fibre cell walls, traditionally measured as an irreversible reduction in the water-binding capacity of pulp [166]. In papermaking, the reduction in wet fibre flexibility caused by hornification has been shown to decrease the tensile strength of the paper. However, the molecular mechanism behind hornification is not fully understood. Co-crystallization has been suggested as one of the mechanisms behind hornification, based on the results of CP/MAS ^{13}C-NMR studies [167]. For co-crystallization to occur, the fibril aggregates must be parallel over a sufficient distance, there should be no obstruction by non-cellulosic components, and the environment must be sufficiently plastic for the crystalline domains to be rotated or displaced so that adjacent surfaces can be brought together. There is a thermodynamic advantage, in that co-crystallization lowers the surface energy of crystalline domains [167]. An increased preference for the aggregation of surfaces with higher hydrophobicity in cellulose fibrils has also been suggested [168].

One common explanation of the slowing down or cessation of enzymatic hydrolysis of cellulose is that after the cellulose that is more easily accessible to the enzymes has been converted into sugars, the cellulose remaining, i.e. the crystalline, cellulose, is recalcitrant to enzymatic hydrolysis. For details on the various techniques used to measure the crystallinity of cellulose, the reader is refereed to Park et al. [161]. In some studies, crystallinity has been found to have no significant effect on the hydrolysability of cellulosic substrates [169-171]. In **Paper I** it was found that DCr increased in some substrates and remained constant in others after enzymatic hydrolysis, and no correlation was found between DCr and hydrolysability.

The LFD increased during both acid and enzymatic hydrolysis. The LFD of never-dried pulp fibres increased from 4.7 nm to 5.6 nm during acid hydrolysis, and from 4.7 nm to 5.8 nm during enzymatic hydrolysis (Figure 10A). This increase was significant. Both NMR and wide-angle X-ray scattering have shown similar trends, i.e., increasing LFD/crystallite size as a result of reinforced prehydrolysis conditions used to produce pulp fibres [147]. The mechanism behind this is still unknown. During the biosynthesis of cellulose I fibrils in plant cell walls, the LFD is believed to be fairly monodisperse

within a plant species. Therefore, an explanation in terms of alterations to the underlying distribution of LFD, e.g. by preferential hydrolysis of fibrils with smaller LFD, is unlikely since this would require a significant initial fraction of fibrils as large as, or larger than, those remaining after hydrolysis. The observed increase in the LFD seems to contradict the assumption of initially monodisperse LFD, alternatively, some other hitherto unexplained mechanism is responsible for the increase in LFD.

A tentative interpretation of these findings is that a mechanism similar to the well-established Ostwald ripening process [172] may occur during crystal growth, where larger crystals increase in size at the expense of smaller ones (P.T. Larsson, personal communication) (Figure 11). As enzymatic hydrolysis proceeds and the fibres rich in cellulose I are successively converted into shortened cellulose particles, two major changes occur. The mechanical restrictions imposed by the fibre wall morphology are lifted, and the average fibril aggregate length (degree of polymerization) is decreased. Enzymatic cleaving of the fibril aggregates yields cellulose I particles with an increased number of polymer ends. After initially transferring a polymer end from one fibril to a neighbouring fibril, such a process could propagate throughout the entire length of the polymer, transferring a polymer between neighbouring fibrils with only a small part of the polymer being in a "dissolved" state at any given instant.

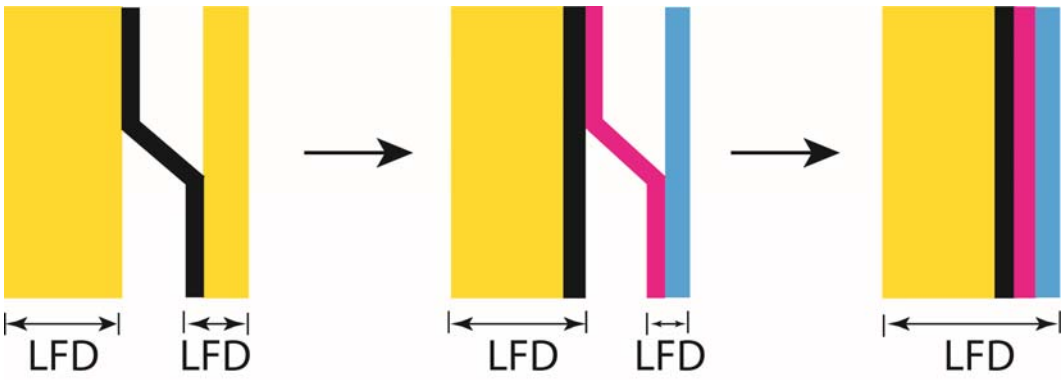

Figure 11. Proposed interpretation of the observed increase in lateral fibril dimensions (LFD) observed during acid and enzymatic hydrolysis of cellulosic substrates. Larger fibrils are suggested to grow at the expense of smaller ones. The yellow areas represent adjacent fibrils, and the black, magenta and blue lines represent the transfer of a β-1,4-D-glucan polymer from one fibril to a neighbouring fibril.

In such a situation, successive migration of surface polymers from one fibril to its neighbour may take place, thermodynamically driven by a decrease in the surface area. If such a process proceeds to the point where some fibrils have been completely

consumed to the benefit of their neighbours, an increase in the degree of crystallinity and the average LFD would be observed. It is conceivable that polymer migration could be kinetically favoured by the close proximity of fibrils in a fibril aggregate and an abundance of polymer ends, in agreement with the observations on hydrolysed samples. If such a successive polymer migration mechanism exists, it raises some interesting questions about the lateral size and cross-sectional shape of fibrils in isolated cellulose I materials, since both lateral fibril size and cross-sectional shape may be a consequence of the isolation procedure, rather than reflecting the properties of the native cellulose fibril.

As discussed above, for enzymatic hydrolysis to occur, the surface of cellulose must be accessible to enzymes and the pore should be larger than the enzyme molecules. Pore size has proven to be an important determinant in enzymatic hydrolysis, and as drying reduces the size of the pores in cellulose, the enzymatic hydrolysability is also reduced (**Paper I**). The importance of the pore size for the initial rate of hydrolysis has been demonstrated previously [171], and is in agreement with a recent study by our group [169]. After the removal of non-cellulosic components, the pores in the never-dried fibre wall remain in the water-swollen state, however, they may collapse upon drying [173]. Different methods have been used to measure pore sizes [174-178]. These usually require either sample treatment and/or assumptions regarding the shape of the pores. In the study presented in **Paper I** a recently developed method was applied which allowed the measurement of the average pore size of the samples in the water-swollen state employing CP/MAS ^{13}C-NMR and the fibre saturation point [163, 173].

7 Analysis of enzymatic hydrolysis of cellulose

Generally, carbohydrates are recalcitrant and highly polar, have the properties of weak acids, have isobaric mass, are stereoisomers, have low water solubility, are insoluble above a degree of polymerization of 6, and are transparent in the UV and visible wavelength range. The chemical and physical properties of carbohydrates place constraints on the methods that can be used for analysis. During enzymatic hydrolysis soluble carbohydrates, i.e. glucose, and other oligosaccharides are released from cellulose into solution. High-performance anion-exchange chromatography (HPAEC) coupled with pulsed amperometric detection (PAD) provides high detection sensitivity for carbohydrates, monosaccharides and soluble oligosaccharides, provided standards are available for quantification. However, monitoring solubilized products, is likely to miss certain enzyme activities that function through modifying insoluble (ligno)cellulosic substrates, including polysaccharide oxidases, LPMOs and several lignin active enzymes [179].

Little is currently known about the role of progressive cellulose deconstruction in hydrolysis [15]. The analysis of a solid substrate prior to and during enzymatic hydrolysis can provide new insights into the relationship between the structure of cellulose and enzymatic efficiency. Various analytical techniques which have been used to characterize (ligno)cellulose after or during enzyme treatment could be categorized as: (i) primarily imaging techniques, (ii) physicochemical techniques, and (ii) micro-spectroscopy techniques [179]. The supramolecular structure of cellulose can be studied at nanometre resolution using CP/MAS ^{13}C-NMR (discussed in Section 7.2). Imaging techniques used to study insoluble cellulosic substrates and cellulose-cellulase interaction are discussed in Section 7.3. More emphasis is put on nonlinear microscopy techniques used in **Paper II**. Cellulose can be characterized on the micrometre scale using nonlinear optical microscopy, such as coherent anti-Stokes Raman scattering (CARS) second harmonic generation (SHG) and multiphoton excited fluorescence (MPEF).

7.1 HPAEC-PAD

Only soluble hydrolysis products can be analysed using chromatographic techniques. The separation mechanism is based on the weakly acidic properties of sugar molecules. The pKa values of typical sugars are in the range 12–14. The ionization of the hydroxyl groups of the saccharides is achieved at a highly alkaline pH, yielding negatively charged species. The detection employs the ability of gold electrode surface to catalyze the oxidation of polar compounds in alkaline media [180]. In the present work, soluble hydrolysis products were analysed with HPAEC-PAD using a CarboPac PA 1 column (Dionex). A typical chromatogram of sugar analysis of plant biomass is shown in Figure 12.

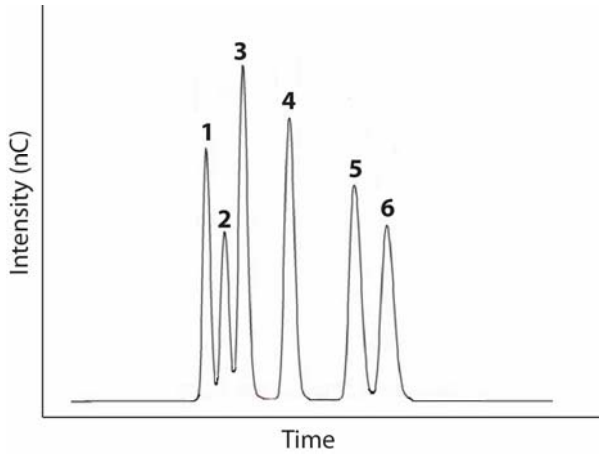

Figure 12. Typical chromatogram showing a separation of different sugars by HPAEC-PAD. Based on the retention time and peak area of the standard sugars, peaks in the sample can be identified and quantified. Note that, although each sugar is present at the same concentration, the height and the area of the peaks are not the same. Arabinose (1), rhamnose (2), galactose (3), glucose (4), xylose (5), mannose (6).

Although HPAEC-PAD has numerous advantages, it also has some limitations. Highly alkaline conditions may cause chemical modification of the compounds being analysed, and esterification is not stable under alkaline conditions, therefore, acetylated and non-acetylated compounds (found in hemicellulose) cannot be separated.

7.2 CP/MAS ^{13}C-NMR

NMR techniques make use of the magnetic properties of atomic nuclei to provide information about the dynamics and structure of a sample. Some isotopes have a magnetic moment, which is utilized in NMR. The atomic nucleus in a molecule is magnetically shielded by the surrounding electron distribution, which varies at different positions in the molecule, yielding magnetic responses of different energies, hence a spectrum. One advantage of NMR is that distinct magnetically active isotopes have different magnetic moments, making it possible to study a single isotope, such as ^{13}C, separately. In solid organic samples such as cellulose, the abundance and spatial distribution of protons form a network of strongly coupled spins. This is due to the nature of the solid samples, where the molecules have little or no mobility. In solid samples, anisotropic, i.e. orientation-dependent, interactions between the nuclei are not averaged by rapid molecular motions, as is the case in solution-state NMR. This lack of rapid averaging is a source of line broadening in solid-state NMR spectra. In order to achieve high-resolution spectra from nuclei such as ^{13}C in solids, three problems must be overcome: (i) broadening due to dipolar interactions between ^{13}C and ^{1}H in the cellulose sample, (ii) low sensitivity mainly due to the low natural abundance of ^{13}C, and (iii) broadening due to chemical shift anisotropy [181].

In solid samples, molecules are normally oriented randomly with respect to the external magnetic field, and a superposition of all possible chemical shifts is observed for each nucleus. This gives rise to broad signals. Chemical shift anisotropy can be experimentally reduced by a process called magic angle spinning (MAS) [182], which involves rapid rotation of the sample at an angle of 54.7 degrees relative to the external magnetic field. Cross polarization (CP) is needed to compensate for the low natural abundance of the ^{13}C isotope, which is only 1.1%. The main isotope of carbon, ^{12}C, does not have a magnetic moment and can therefore not be detected with NMR. The low abundance of ^{13}C results in reduced sensitivity of the NMR spectra and a lower signal-to-noise ratio. In the CP technique, signal enhancement is achieved by first exciting the ^{1}H spins and then transferring the magnetization to the ^{13}C spin system [183]. Proton decoupling is needed to remove dipolar interactions between ^{13}C and ^{1}H in order to achieve high resolution of the ^{13}C nuclei. CP in combination with MAS and proton decoupling provides spectra with

high resolution. Cellulose-rich samples are usually soaked in deionized water prior to packing in a MAS rotor before recording spectra.

A typical CP/MAS ^{13}C-NMR spectrum from cellulose I contains six signals from the different anhydroglucose unit carbon atoms split into fine structure clusters due to the supramolecular structure of cellulose I fibrils [33, 148] (Figure 13A). The amount of information contained in this fine structure is high, but the accessibility of the information is hampered by severe overlap of the signals [184]. In order to obtain quantitative information on the supramolecular structure of cellulose, spectral fitting is therefore needed. Spectral fitting of the C4 region of CP/MAS ^{13}C-NMR spectra has provided a detailed picture of the cellulose I supramolecular structure [148, 162]. Among the various regions of the spectrum of cellulose, the C4 region is distributed over a wider range of chemical shift than the signals from the other regions [148], therefore spectral fitting was applied to the C4 region. Spectral fitting of the C4 region consists of seven distinct lines (Figure 13B). Two signals (at about 84.3 and 83.3 ppm) are assigned to C4 atoms in β-(1,4)-D-glucan polymers that constitute the cellulose I fibril surfaces accessible to bulk water. Analysis of cellulose–water interfaces has shown that the C4 atoms (two signals from accessible fibril surfaces in Figure 13B) in cellulose chains located above different crystallographic planes have different mobilities [185]. These surfaces should also be accessible to the enzymes, provided there is no obstacle to their diffusion caused by the spatial distribution of fibril aggregates in the fibre cell wall (Figure 9). Another C4 signal (at about 83.8 ppm) is assigned to C4 atoms in β-(1,4)-D-glucan polymers that constitute the cellulose I fibril surfaces inaccessible to bulk water, formed either by interior distortions or aggregation of fibrils. As the enzyme molecules are larger than water molecules, fibril surfaces inaccessible to bulk water are also considered inaccessible to the enzyme molecules. Three C4 signals arising from the crystalline fibril interior (87-91 ppm) originate from the cellulose I polymorphs Iα, Iβ and a common overlapped signal I(α+β), in agreement with previous findings [33]. Polymers in a highly ordered state, but with conformations suggested to be intermediate between those of crystalline polymers and surface polymers have been interpreted as a para-crystalline form of cellulose giving rise to a signal the 87-91 ppm range, broader that the typical cellulose Iα and Iβ signals. The spectral behaviour of para-crystalline cellulose indicates that it has greater mobility than the crystalline cellulose [148].

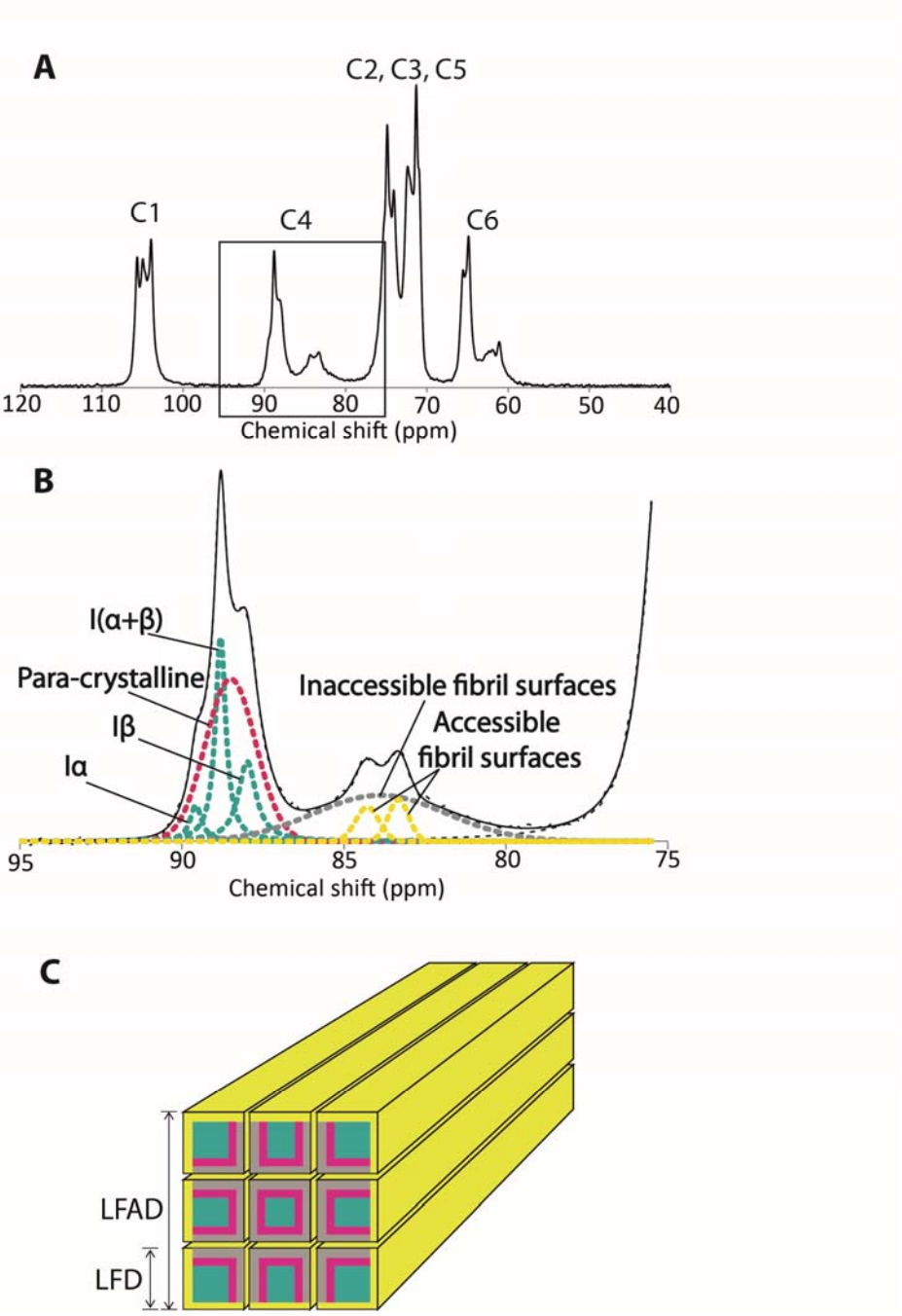

Figure 13. A representative CP/MAS ^{13}C-NMR spectrum from cotton (A), together with a typical spectral fitting result, showing an enlargement of the C4 region of cotton cellulose (B). To determine the supramolecular structure of cellulose, the C4 region is fitted with a set of mathematical functions representing the signals originating from C4 carbons in cellulose Iα, cellulose Iβ, cellulose I(α + β), para-crystalline cellulose, C4 carbons in polymers on inaccessible fibril surfaces and C4 carbons in polymers on accessible fibril surfaces. (C) illustrates the model of the aggregated cellulose I fibrils used in calculations of the lateral fibril aggregate dimensions (LFAD) and lateral fibril dimensions (LFD). The model differentiates between crystalline regions (turquoise), para-crystalline regions (magenta), accessible surface areas (yellow) and inaccessible surface areas that result from the close proximity of fibrils in a fibril aggregate (grey). Modified from **Paper I** with permission.

A CP/MAS ^{13}C-NMR spectrum recorded from cellulose, and subjected to spectral fitting of the C4 region, provides quantitative data on the nanostructure of fibre wall cellulose. Spectral fitting must be performed on lignin- and hemicellulose-free cellulose samples (glucose content > 95%) since interference from hemicellulose and lignin signals influences the calculations. Average values of the parameters describing the supramolecular structure (LFD, LFAD, SSA and DCr) present in cellulosic samples can be determined with high statistical precision using CP/MAS ^{13}C-NMR. The cumulative length of a fibril (with a cross-section of 4 nm × 4 nm) in 150 mg of cellulose (typical sample mass used for measurements), averaged during one measurement, corresponds to 8 round-trips to the moon (P.T. Larsson, personal communication).

7.3 Imaging of cellulose

Various imaging techniques can be used to study cellulose. For a more thorough description of the methods used to characterize the structure of cellulose and cellulose–cellulase interactions the reader is referred elsewhere [15, 179, 186-189]. In order to obtain a realistic picture of the enzymatic hydrolysis of cellulose, it is important to perform the study under relevant conditions [190]. Unfortunately, advanced imaging techniques often involve quite harsh conditions, such as vacuum chambers, low or extreme cryogenic temperatures, dry surfaces, or intense sample processing. AFM is a particularly powerful option as it allows direct assessment of enzyme activity in aqueous environments and ambient conditions [15]. A recent application of high-speed AFM provided a real-time information of cellulase action directly on cellulose fibrils [16]. In general, high-resolution imaging techniques (on the nanometre scale) can only be used to scan a small area. In order to study dynamic processes, such as enzymatic hydrolysis of cellulose, imaging speed is increased at the expense of spatial resolution and chemical resolution. Although the throughput of imaging techniques is relatively low, image analysis of microarrays of plant-derived oligosaccarides is an important exception with ability to screen enzyme activity on characterized oligosaccharides [191]. Summary of techniques allowing assessment of cellulase action on (ligno)cellulosic substrates is shown in Figure 14.

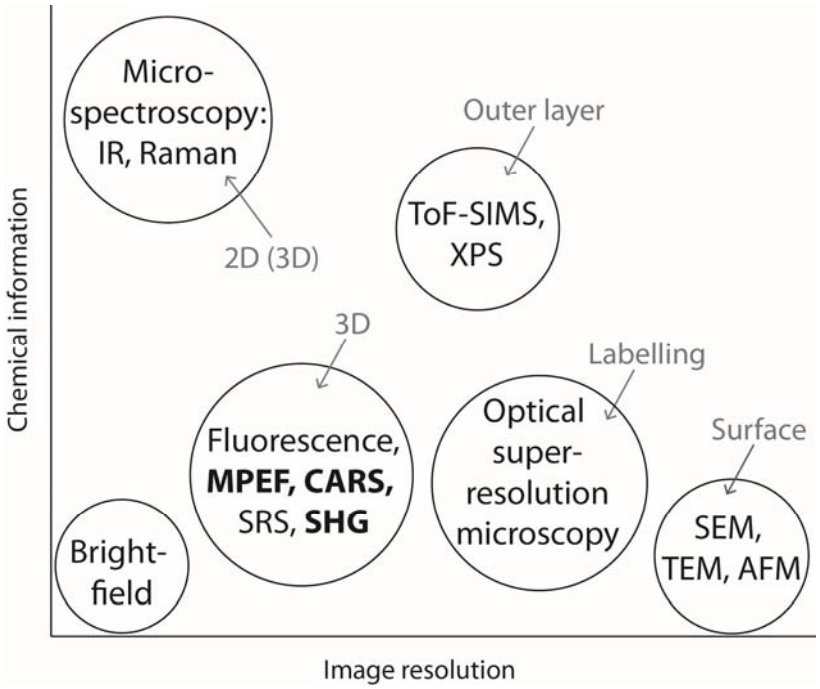

Figure 14. Comparison of imaging techniques that have been used to assess enzyme action on insoluble (ligno)cellulosic substrates. IR: infrared; ToF-SIMS: time–of–flight secondary ion mass spectrometry; XPS: X-ray photoelectron spectroscopy; MPEF: multiphoton excited fluorescence; CARS: coherent anti-Stokes Raman scattering; SRS: stimulated Raman scattering; SHG: second harmonic generation; SEM: scanning electron microscopy; TEM: transmission electron microscopy; AFM: atomic force microscopy; 2D: two–dimensional; 3D: three–dimensional. Imaging techniques used in **Paper II** are written in bold.

Complementary imaging techniques are often combined with other methods to study the enzymatic hydrolysis of cellulose. An overview of imaging studies on (ligno)cellulose and cellulose–cellulase interactions are summarized in Table 4.

Table 4. Overview of imaging techniques employed to study cellulose and cellulose–cellulase interactions

Technique	Information obtained	Substrate	Enzymes investigated	Reference
HS-AFM	Movement of single enzyme molecules on cellulose surface, processivity of enzymes	Algal cellulose (Cellulose Iα) and cellulose III	Cel7A and Cel6A	[16]
Optical microscopy, CLSM, two-colour SRS, AFM	Different mechanisms of fungal cellulases and cellulosomes to deconstruct plant cell walls	Corn stover	Commercial cellulase mixture (Cellic CTec2) and mixture of cellulosomes from *Clostridium thermocellum*	[190]
CLSM, AFM, HPAEC-PAD	Demonstration of LPMO action on cellulose surface	Avicel + IL	LPMO, CBH I, CBH II, EG	[45]
AFM, QCM-D	Morphological changes in cellulose films; dynamics of cellulases	Cellulose model films	Commercial cellulase mixture	[50]
FE-SEM	Changes occurring in the surface of cellulose during kraft cooking	Spruce fibres	-	[159]
Electron microscopy	Enzyme synergy	Bacterial cellulose	CBH I, EG IV	[31]
Electron microscopy, AFM	Preferential binding of enzymes to the hydrophobic phase of cellulose crystal	*Valonia* cellulose	CBH I	[152, 192]
Electron microscopy	CBH II acts on non-reducing end of cellulose	*Valonia* cellulose	CBH II	[193]

Table 4 - continued

Electron microscopy	CBH I acts on reducing end of cellulose	*Valonia* cellulose	CBH I	[194]
PLM, CLSM	Dislocations within cellulose fibres are of crystalline nature	Filter paper fibre	EG	[195]
MCARS	Semi-quantitative imaging of cellulose, hemicellulose and lignin	Different wood samples	-	[196]
STXM, ToF-SIMS	Role of laccase in promoting xylanase activity	Wood sections of aspen	Laccase, xylanase, commercial cellulase mixture	[197]
STORM, TIRF, 3D-SIM	Cell wall architecture	Epidermis cells of onion	-	[198]
SHG	Monitoring reduction of crystallinity of cellulose	Avicel + IL	-	[47]
CARS, SHG, MPEF	Characterization of the substrates in terms of their molecular density, ordering and autofluorescence; real-time imaging of enzymatic hydrolysis	Avicel, never-dried pulp fibres	Commercial cellulase mixture	Paper II

HS-AFM: high-speed atomic force microscopy; CSLM: confocal laser scanning microscopy; SRS: stimulated Raman scattering; IL: ionic liquid; HPAEC-PAD: high-performance anion-exchange chromatography coupled with pulsed amperometric detection; QCM-D: quartz crystal microbalance with dissipation; FE-SEM: field emission-scanning electron microscopy; TIRF: total internal reflection fluorescence; PLM: polarized light microscopy; MCARS: multiplex coherent anti-Stokes Raman scattering; STORM: stochastic optical reconstruction microscopy; 3D-SIM: three-dimensional structured illumination microscopy; SHG: second harmonic generation; STXM: scanning transmission X-ray microscopy; ToF-SIMS: time-of-flight secondary ion mass spectrometry; MPEF: multi photon excited fluorescence.

Nonlinear microscopy techniques, CARS, SHG and MPEF, were used in the present work to assess the dynamics of enzymatic hydrolysis of three cellulosic substrates (**Paper II**). These techniques were chosen as the cellulosic substrates could be characterized without any sample preparation, as the recorded signals are derived from the intrinsic chemical and physical properties of the sample. The experimental set-up allowed enzymatic hydrolysis in an aqueous environment and incubation to be monitored. A comparison of the images of cellulosic substrates obtained from scanning electron microscopy and nonlinear microscopy techniques is shown in Figure 15.

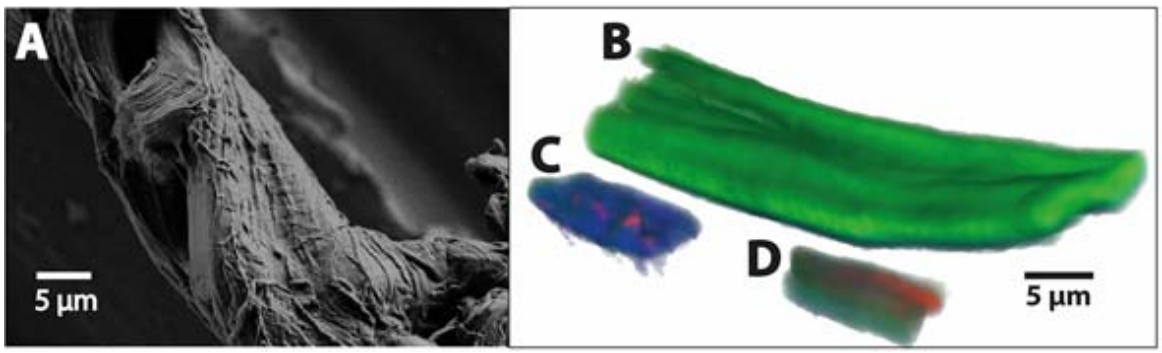

Figure 15. A dried pulp fibre imaged by scanning electron microscopy (A). A never-dried pulp fibre (B), Avicel (C) and acid-hydrolysed pulp fibre (D) imaged simultaneously with coherent anti-Stokes Raman scattering (blue), second harmonic generation (red) and multiphoton excited fluorescence (green) (**Paper II**).

SHG can be used to probe non-centrosymmetric structures, and has been used to image cellulose in cotton [199, 200] and rayon fibres [200], cellulose derived from the bacterium *Acetobacter xylinum* [201, 202] and the alga *Valonia ventricosa* [202]. MPEF can be used to probe the intrinsic fluorescence of the cellulosic substrate, i.e. lignin [203]. CARS probes molecular vibrations, allowing chemically sensitive imaging, for example, to image carbon–hydrogen bond density in cellulose. CARS microspectroscopy has been used to study the effects of hydration on cotton and rayon fibres [200] and the chemically resolved structure of wood samples [196]. In **Paper II**, the regularity of low- and high-intensity regions and carbon-hydrogen density, allowed to distinguish between never-dried and dried cellulosic substrates. Monitoring of the substrates during enzymatic hydrolysis in real-time revealed the substrate-specific hydrolysis pattern. As the follow-up of the study, it would be interesting to investigate in more detail the structure in the areas of the substrates which are more and less susceptible to enzymatic hydrolysis.

8 Conclusions

In my thesis the complexity of the supramolecular structure of cellulose was demonstrated. The structural dynamics of cellulose during enzymatic hydrolysis were demonstrated, and it was shown that the structure of cellulose is an important factor in enzyme production. Avicel and cotton, which are considered to be model substrates, and softwood pulp fibres were studied. The pulp fibres closely resemble the native structure of cellulose due to the intact fibre wall morphology.

In **Paper I** it was shown that drying of the substrates causes increased aggregation of the fibrils into larger fibril aggregates, accompanied by a decrease in the average pore size. Both enzymatic and acid hydrolysis were found to significantly increase LFD and the DCr through a mechanism which is not yet fully understood. One plausible explanation is that successive migration of surface polymers from one fibril to the neighbouring one may take place, thermodynamically driven by a decrease in surface area.

For efficient enzymatic hydrolysis, it is desirable that as much surface area as possible is accessible to the enzymes, and the pores of cellulose-based materials should be larger than the enzyme molecules. The SSA and pore size were found to be important determinants of the enzymatic hydrolysability of cellulose, whereas the DCr of the substrate was not (**Paper I**).

In **Paper II**, it was shown using CARS that, before enzymatic hydrolysis, Avicel had the higher carbon–hydrogen bond density than the never-dried pulp fibres. The regularity of low- and high-intensity regions (measured from the SHG signal) allowed to distinguish between never-dried and dried cellulosic substrates. Avicel showed a less regular pattern than the pulp fibres, possibly reflecting the collapse of the macromolecular structures during drying and rewetting. Monitoring of the substrates during enzymatic hydrolysis in real-time revealed substrate-characteristic hydrolysis pattern. A double exponential SHG decay for the never-dried pulp fibres was observed, indicating two phases of the process. The hydrolysis of Avicel was more than an order of magnitude slower than that of the fibres.

The filamentous fungus *T. reesei* Rut C-30 was shown to secrete enzymes with different profiles when cellulosic substrates with different structures were used as the energy and carbon source for the fungus (**Paper III**). The cellulosic substrates had similar chemical compositions, so it could be expected that the enzymes produced would be similar if the chemical composition was the only determining factor for enzyme production. The most recalcitrant substrate to enzymatic hydrolysis studied in **Paper I** and **Paper II**, induced the highest enzyme titre in the fungus in **Paper III**.

To explore the biodiversity of cellulose hydrolysis in nature, enzymes produced by five filamentous fungi were studied with the aim of improving the investigated industrial cellulase cocktail. The study described in **Paper IV** demonstrated that careful selection of fungi can result in enzyme cocktails that are significantly more efficient in plant biomass saccharification.

9 Future perspectives

Analytics plays an important role in the study of the enzymatic hydrolysis of cellulose. The system of enzymatic hydrolysis consists of a solid substrate, water, enzymes and soluble products released from the solid substrate. The ultimate goal is to find the relation between cellulose structure and the enzymatic cocktail needed to provide complete conversion of plant biomass in a few days. All components of the enzymatic hydrolysis system are important. However, the analysis of solid substrates is laborious, and it would be easier to render the substrate soluble and then analyse the soluble analytes, using powerful and sensitive techniques such as HPAEC-PAD and MS. However, this will only provide information about the chemistry of the analyte. Studies of insoluble heterogeneous cellulosic substrates could provide details about the chemical properties and/or the structure on different scales.

The conversion of cellulose to glucose slows down with time, but it is not fully understood why. In some of the substrates used in this work, an increase in the average lateral dimensions of the cellulose fibrils was observed by CP/MAS ^{13}C-NMR spectroscopy during enzymatic and acidic hydrolysis. However, the mechanism is not understood. The increase in the average lateral dimensions of the fibrils was approximately 1 nm, about the thickness of two glucan polymers, roughly a 25% increase in the average lateral dimensions of wood based cellulose fibrils. Direct verification of this by microscopy was not possible, and new ways of studying cellulose structure would have to be developed to confirm the tentative explanation. Nevertheless, CP/MAS ^{13}C-NMR is a powerful technique for taking measures with nanometre or sub-nanometre precision, making it a state-of-the-art tool for the study of the supramolecular structure of cellulose.

The recent discovery of LPMOs, which have been shown to have a considerable effect on the enzymatic hydrolysis of biomass, requires new methods to be developed to study their impact on the supramolecular structure of cellulose. It has been suggested that LPMOs oxidize the C1 and/or C4 carbons of the glucose molecule in cellulose with almost no release of any soluble product. In CP/MAS ^{13}C-NMR, the C4 signal, where changes introduced by LPMOs are expected to occur, is particularly well resolved. Therefore, this technique could allow information to be obtained on the effects of LPMOs on the supramolecular structure of cellulose. A study by Eibinger et al. [45] is worth mentioning,

in which they demonstrated the direct action of LPMOs on cellulose for the first time, using a combination of fluorescent dye adsorption and confocal laser scanning microscopy. In the coming years it is expected that the application of label-free coherent Raman imaging methods which offer chemical specificity will increase significantly [188]. I believe that improved spectral range of fast coherent Raman imaging holds much promise for dynamic measurements to study the action of enzymes on insoluble substrate. Advances in quantitative analysis of coherent Raman imaging data will lead to ability to capture smaller changes in chemical composition of the substrate.

The choice of model cellulosic substrates for studies of the enzymatic hydrolysis of cellulose requires attention. According to the literature, a variety of cellulases have been tested on a large number of substrates. Avicel, which is often used as a model substrate for native cellulose is supplied as a powder in which the morphology of the plant cell walls has been destroyed. A more representative substrate for native cellulose would be one in which the fibre wall morphology is retained. Materials referred to as cellulosic substrates in the literature are often composed of cellulose with residual amounts of hemicellulose and possibly lignin. Thus, caution should be exercised when interpreting the data and comparing the results to those from other studies.

Enzymatic cocktails produced by fungi are very complex, and may contain over a hundred components, many of which have no specific role. The percentage of genes in fungal genomes with no known/predicted function is around 30-45% with, on average, a higher percentage in basidiomycetes than ascomycetes (R.P. de Vries, personal communication). It is thus desirable to find enzymes that play a key role in the enzymatic hydrolysis of plant biomass. One possible approach could be to fractionate a complex enzyme mixture and perform enzymatic hydrolysis with the enzyme mixtures lacking a particular fraction to evaluate which fraction contains the key enzymes. This approach has been successively implemented in the discovery of LPMOs [96] and offers an applied potential in finding new enzymatic activities complementing the known ones. Proteomic studies allow identification of proteins with high accuracy, provided the protein is available in the database. Large sequencing efforts of fungal genomes are expected to offer new possibilities of enzyme discovery.

During secretome analysis, which is commonly employed to study the enzymes secreted by fungi, a whole pool of enzymes is analysed at once. However, fungi produce the

enzymes they need at any particular time. More detailed studies on specific enzymatic activities produced by fungi at specific times may help us understand which enzymatic activities are needed at any particular time during enzymatic hydrolysis to improve the process.

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References

1. Fengel D: **Aging and fossilization of wood and its components**. *Wood Sci and Technol* 1991, 25(3):153-177.
2. Fengel D: **Polysaccharide in fossilen Hölzern**. *Naturwissenschaften* 1974, **61**(10):450-451.
3. Beckham GT, Matthews JF, Peters B, Bomble YJ, Himmel ME, Crowley MF: **Molecular-level origins of biomass recalcitrance: decrystallization free energies for four common cellulose polymorphs**. *J Phys Chem B* 2011, 115(14):4118-4127.
4. Klemm D, Heublein B, Fink HP, Bohn A: **Cellulose: fascinating biopolymer and sustainable raw material**. *Angew Chem Int Edit* 2005, 44(22):3358-3393.
5. Schnepp Z: **Biopolymers as a flexible resource for nanochemistry**. *Angew Chem Int Edit* 2013, 52(4):1096-1108.
6. Petersen N, Gatenholm P: **Bacterial cellulose-based materials and medical devices: current state and perspectives**. *Appl Microbiol Biot* 2011, **91**(5):1277-1286.
7. Paulová L, Patáková P, Branská B, Rychtera M, Melzoch K: **Lignocellulosic ethanol: technology design and its impact on process efficiency**. *Biotechnol Adv* 2014, 33(2):1091-1107.
8. Bozell JJ, Petersen GR: **Technology development for the production of biobased products from biorefinery carbohydrates—the US Department of Energy's "top 10" revisited**. *Green Chem* 2010, 12(4):539-554.
9. Ragauskas AJ, Williams CK, Davison BH, Britovsek G, Cairney J, Eckert CA, Frederick WJ, Hallett JP, Leak DJ, Liotta CL: **The path forward for biofuels and biomaterials**. *Science* 2006, 311(5760):484-489.
10. Kamm B, Gruber PR, Kamm M, Eds: **Biorefineries–industrial processes and products.** In: *Ullmann's Encyclopedia of Industrial Chemistry*, vol. 5: Wiley-VCH: Weinheim, Germany; 2007: 659-688.
11. Rødsrud G, Lersch M, Sjöde A: **History and future of world's most advanced biorefinery in operation**. *Biomass Bioenerg* 2012, 46:46-59.
12. National research agenda **www.nra-sweden.se** (retrieved November, 2015)
13. **Swedish research and innovation strategy for a bio-based economy;** Report: R3:2012.
14. Otero J, Panagiotou G, Olsson L: **Fueling industrial biotechnology growth with bioethanol**. In: *Biofuels.* Edited by Olsson L, vol. 108: Springer Berlin Heidelberg; 2007: 1-40.
15. Bubner P, Plank H, Nidetzky B: **Visualizing cellulase activity**. *Biotechnol Bioeng* 2013, 110(6):1529-1549.
16. Igarashi K, Uchihashi T, Koivula A, Wada M, Kimura S, Okamoto T, Penttila M, Ando T, Samejima M: **Traffic jams reduce hydrolytic efficiency of cellulase on cellulose surface**. *Science* 2011, 333(6047):1279-1282.
17. Seiboth B, Ivanova C, Seidl-Seiboth V: ***Trichoderma reesei*: a fungal enzyme producer for cellulosic biofuels**. In: *Biofuel Production-Recent Developments and Prospects*. Dr. Marco Aurelio Dos Santos Bernardes (Ed.) InTech; 2011.
18. Yang B, Dai Z, Ding S-Y, Wyman CE: **Enzymatic hydrolysis of cellulosic biomass**. *Biofuels* 2011, 2(4):421-450.
19. Harris PV, Welner D, McFarland KC, Re E, Navarro Poulsen JC, Brown K, Salbo R, Ding H, Vlasenko E, Merino S, Xu F, Cherry J, Larsen S, Lo Leggio L: **Stimulation of lignocellulosic biomass hydrolysis by proteins of glycoside hydrolase family 61: structure and function of a large, enigmatic family**. *Biochemistry* 2010, 49(15):3305-3316.
20. Reese ET, Siu RGH, Levinson HS: **The biological degradation of soluble cellulose derivatives and its relationship to the mechanism of cellulose hydrolysis**. *J Bacteriol* 1950, 59(4):485-497.

21. Agger JW, Isaksen T, Várnai A, Vidal-Melgosa S, Willats WG, Ludwig R, Horn SJ, Eijsink VG, Westereng B: **Discovery of LPMO activity on hemicelluloses shows the importance of oxidative processes in plant cell wall degradation**. *Proc Natl Acad Sci USA* 2014 111(17):6287-6292.
22. Paspaliari DK, Loose JS, Larsen MH, Vaaje-Kolstad G: **Listeria monocytogenes has a functional chitinolytic system and an active lytic polysaccharide monooxygenase**. *FEBS journal* 2015, 282(5):921-936.
23. Vu VV, Beeson WT, Span EA, Farquhar ER, Marletta MA: **A family of starch-active polysaccharide monooxygenases**. *Proc Natl Acad Sci USA* 2014, 111(38):13822-13827.
24. Vaaje-Kolstad G, Westereng B, Horn S, Liu Z, Zhai H, Sorlie M, Eijsink V: **An oxidative enzyme boosting the enzymatic conversion of recalcitrant polysaccharides**. *Science* 2010, 330(6001):219-222.
25. Percival Zhang YH, Himmel ME, Mielenz JR: **Outlook for cellulase improvement: screening and selection strategies**. *Biotechnol Adv* 2006, 24(5):452-481.
26. Chandra RP, Bura R, Mabee WE, Berlin A, Pan X, Saddler JN: **Substrate pretreatment: the key to effective enzymatic hydrolysis of lignocellulosics?** *Adv Biochem Eng Biotechnol* 2007, 108:67-93.
27. Payen A: **Memoir on the composition of the tissue of plants and of woody material**. *CR Biol* 1838, 7:1052-1056.
28. Zugenmaier P: **Crystalline cellulose and derivatives: characterization and structures**. Berlin: Springer; 2008.
29. Ford BJ: **Antony van Leeuwenhoek—Microscopist and visionary scientist**. *J Biol Educ* 1989, 23(4):293-299.
30. Hooke R: **Micrographia, or, Some physiological descriptions of minute bodies made by magnifying glasses: With observations and inquiries thereupon**. London: J. Martyn and J. Allestry; 1665.
31. White AR, Brown RM: **Enzymatic hydrolysis of cellulose: visual characterization of the process**. *Proc Natl Acad Sci USA* 1981, 78(2):1047-1051.
32. Wilkie J: **Carl Nägeli and the fine structure of living matter**. *Nature* 1961, 190:1145-1150.
33. VanderHart DL, Atalla R: **Studies of microstructure in native celluloses using solid-state carbon-13 NMR**. *Macromolecules* 1984, 17(8):1465-1472.
34. Kunze J, Scheler G, Schröter B, Philipp B: **^{13}C High resolution solid state NMR studies on cellulose samples of different physical structure**. *Polymer Bulletin* 1983, 10(1):56-62.
35. Moon RJ, Martini A, Nairn J, Simonsen J, Youngblood J: **Cellulose nanomaterials review: structure, properties and nanocomposites**. *Chem Soc Rev* 2011, 40(7):3941-3994.
36. Credou J, Berthelot T: **Cellulose: From biocompatible to bioactive material**. *J Mater Chem B* 2014, 2(30):4767-4788.
37. You C, Chen H, Myung S, Sathitsuksanoh N, Ma H, Zhang X-Z, Li J, Zhang Y-HP: **Enzymatic transformation of nonfood biomass to starch**. *Proc Natl Acad Sci USA* 2013, 110(18):7182-7187.
38. Reese ET: **Enzymatic hydrolysis of cellulose**. *Appl Microbiol* 1956, 4(1):39.
39. Brown RM: **Cellulose structure and biosynthesis: what is in store for the 21st century?** *J Polym Sci Pol Chem* 2004, 42(3):487-495.
40. Römling U: **Molecular biology of cellulose production in bacteria**. *Res Microbiol* 2002, 153(4):205-212.
41. Nobles DR, Romanovicz DK, Brown RM: **Cellulose in cyanobacteria. Origin of vascular plant cellulose synthase?** *Plant Physiol* 2001, 127(2):529-542.
42. Kimura S, Laosinchai W, Itoh T, Cui X, Linder CR, Brown RM: **Immunogold labeling of rosette terminal cellulose-synthesizing complexes in the vascular plant *Vigna angularis***. *Plant Cell* 1999, 11(11):2075-2085.

43. Keshk S: **Bacterial Cellulose Production and its Industrial Applications**. *J Bioprocess Biotech* 2014, 4(150):2.
44. Bidlack J, Malone M, Benson R: **Molecular structure and component integration of secondary cell walls in plants**. In: *Proc Okla Acad Sci: 1992*. 51-56.
45. Eibinger M, Ganner T, Bubner P, Rošker S, Kracher D, Haltrich D, Ludwig R, Plank H, Nidetzky B: **Cellulose surface degradation by a lytic polysaccharide monooxygenase and its effect on cellulase hydrolytic efficiency**. *J Biol Chem* 2014, 289(52):35929-35938.
46. Bubner P, Dohr J, Plank H, Mayrhofer C, Nidetzky B: **Cellulases dig deep *in situ* observation of the mesoscopic structural dynamics of enzymatic cellulose degradation**. *J Biol Chem* 2012, 287(4):2759-2765.
47. Glas D, Paesen R, Depuydt D, Binnemans K, Ameloot M, De Vos DE, Ameloot R: **Cellulose amorphization by swelling in ionic liquid/water mixtures: A combined macroscopic and second-harmonic microscopy study**. *ChemSusChem* 2015, 8(1):82-86.
48. Zhang Y-HP, Lynd LR: **Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulase systems**. *Biotechnol Bioeng* 2004, 88(7):797-824.
49. Sipos B, Benkő Z, Dienes D, Réczey K, Viikari L, Siika-aho M: **Characterisation of specific activities and hydrolytic properties of cell-wall-degrading enzymes produced by *Trichoderma reesei* Rut C30 on different carbon sources**. *Applied Biochem Biotech* 2010, 161(1):347-364.
50. Ahola S, Turon X, Österberg M, Laine J, Rojas OJ: **Enzymatic hydrolysis of native cellulose nanofibrils and other cellulose model films: Effect of surface structure**. *ACS* 2008, 24(20):11592-11599.
51. Sjöström E: **Wood chemistry: Fundamentals and applications, 2nd ed.**: Academic Press, San Diego, CA; 1993.
52. Söderholm K, Bergquist A-K: **Growing green and competitive—A case study of a Swedish pulp mill**. *Sustainability* 2013, 5(5):1789-1805.
53. Rydholm SA: **Pulping processes**. *New York, US: Interscience Pub* 1965.
54. Sixta H: **Multistage kraft pulping**. In: *Handbook of Pulp.* Edited by Sixta H. Weinheim: Wiley-VCH; 2006.
55. Karlström K, Sjögren B, Vorwerg W, Volkert B: **Sulphur-free cooking for value added cellulose** *Cellulose Chem Technol* 2014, 48(9-10):781-786.
56. Saadatmand S, Edlund U, Albertsson A-C, Danielsson S, Dahlman O, Karlström K: **Turning hardwood dissolving pulp polysaccharide residual material into barrier packaging**. *Biomacromolecules* 2013, 14(8):2929-2936.
57. Ragauskas AJ, Beckham GT, Biddy MJ, Chandra R, Chen F, Davis MF, Davison BH, Dixon RA, Gilna P, Keller M: **Lignin valorization: improving lignin processing in the biorefinery**. *Science* 2014, 344(6185):1246843.
58. Olsson L, Saddler J: **Biorefineries, using lignocellulosic feedstocks, will have a key role in the future bioeconomy**. *Biofuel Bioprod Bior* 2013, 7(5):475-477.
59. van den Brink J, de Vries RP: **Fungal enzyme sets for plant polysaccharide degradation**. *Appl Microbiol Biot* 2011, 91(6):1477-1492.
60. Sánchez C: **Lignocellulosic residues: biodegradation and bioconversion by fungi**. *Biotechnol Adv* 2009, 27(2):185-194.
61. Doi RH, Kosugi A: **Cellulosomes: plant-cell-wall-degrading enzyme complexes**. *Nat Rev Microbiol* 2004, 2(7):541-551.
62. Rabinovich M, Melnick M, Bolobova A: **The structure and mechanism of action of cellulolytic enzymes**. *Biochemistry (Moscow)* 2002, 67(8):850-871.
63. Tomme P, Tilbeurgh H, Pettersson G, Damme J, Vandekerckhove J, Knowles J, Teeri T, Clayssens M: **Studies of the cellulolytic system of *Trichoderma reesei* QM 9414**. *Eur J Biochem* 1988, 170(3):575-581.

64. Boraston A, Bolam D, Gilbert H, Davies G: **Carbohydrate-binding modules: fine-tuning polysaccharide recognition**. *Biochem J* 2004, 382:769-781.
65. Carrard G, Koivula A, Soderlund H, Beguin P: **Cellulose-binding domains promote hydrolysis of different sites on crystalline cellulose**. *Proc Natl Acad Sci USA* 2000, 97:10342-10347.
66. Sørensen TH, Cruys-Bagger N, Windahl MS, Badino SF, Borch K, Westh P: **Temperature effects on kinetic parameters and substrate affinity of Cel7A cellobiohydrolases**. *J Biol Chem* 2015, 290(36):22193-22202.
67. Várnai A, Siika-aho M, Viikari L: **Carbohydrate-binding modules (CBMs) revisited: reduced amount of water counterbalances the need for CBMs**. *Biotechnol Biofuels* 2013, 6(1):1-12.
68. Payne CM, Resch MG, Chen L, Crowley MF, Himmel ME, Taylor LE, Sandgren M, Ståhlberg J, Stals I, Tan Z: **Glycosylated linkers in multimodular lignocellulose-degrading enzymes dynamically bind to cellulose**. *Proc Natl Acad Sci USA* 2013, 110(36):14646-14651.
69. Davies G, Henrissat B: **Structures and mechanisms of glycosyl hydrolases**. *Structure* 1995, 3(9):853-859.
70. Teeri TT: **Crystalline cellulose degradation: new insight into the function of cellobiohydrolases**. *Trends Biotechnol* 1997, 15(5):160-167.
71. Rouvinen J, Bergfors T, Teeri T, Knowles J, Jones T: **Three-dimensional structure of cellobiohydrolase II from *Trichoderma reesei***. *Science* 1990, 249(4967):380-386.
72. Divne C, Stahlberg J, Reinikainen T, Ruohonen L, Pettersson G, Knowles J, Teeri TT, Jones T: **The three-dimensional crystal structure of the catalytic core of cellobiohydrolase I from *Trichoderma reesei.*** *Science* 1994, 265(5171):524-528.
73. Kleywegt GJ, Zou J-Y, Divne C, Davies GJ, Sinning I, Ståhlberg J, Reinikainen T, Srisodsuk M, Teeri TT, Jones TA: **The crystal structure of the catalytic core domain of endoglucanase I from Trichoderma reesei at 3.6 Å resolution, and a comparison with related enzymes**. *J Mol Biol* 1997, 272(3):383-397.
74. Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS: **Microbial cellulose utilization: fundamentals and biotechnology**. *Microbiol Mol Biol Rev* 2002, 66(3):506-577, table of contents.
75. Wilson DB, Irwin DC: **Genetics and properties of cellulases**. In: *Recent Progress in Bioconversion of Lignocellulosics.* Springer; 1999: 1-21.
76. Sørensen A, Lübeck M, Lübeck PS, Ahring BK: **Fungal beta-glucosidases: a bottleneck in industrial use of lignocellulosic materials**. *Biomolecules* 2013, 3(3):612-631.
77. Horn SJ, Vaaje-Kolstad G, Westereng B, Eijsink VG: **Novel enzymes for the degradation of cellulose**. *Biotechnol Biofuels* 2012, **5**:45.
78. Quinlan R, Sweeney M, Lo Leggio L, Otten H, Poulsen J, Johansen K, Krogh K, Jorgensen C, Tovborg M, Anthonsen A: **Insights into the oxidative degradation of cellulose by a copper metalloenzyme that exploits biomass components**. *Proc Natl Acad Sci USA* 2011, 108(37):15079-15084.
79. Karkehabadi S, Hansson H, Kim S, Piens K, Mitchinson C, Sandgren M: **The first structure of a glycoside hydrolase family 61 member, Cel61B from *Hypocrea jecorina*, at 1.6 angstrom resolution**. *J Mol Biol* 2008, 383(1):144-154.
80. Isaksen T, Westereng B, Aachmann FL, Agger JW, Kracher D, Kittl R, Ludwig R, Haltrich D, Eijsink VG, Horn SJ: **A C4-oxidizing lytic polysaccharide monooxygenase cleaving both cellulose and cello-oligosaccharides**. *J Biol Chem* 2014, 289(5):2632-2642.
81. Beeson W, Phillips C, Cate J, Marletta M: **Oxidative cleavage of cellulose by fungal copper-dependent polysaccharide monooxygenases**. *J Am Chem Soc* 2012, 134(2):890 - 892.

82. Vu VV, Beeson WT, Phillips CM, Cate JH, Marletta MA: **Determinants of regioselective hydroxylation in the fungal polysaccharide monooxygenases**. *J Am Chem Soc* 2013, 136(2):562-565.
83. Lombard V, Ramulu HG, Drula E, Coutinho PM, Henrissat B: **The carbohydrate-active enzymes database (CAZy) in 2013**. *Nucleic Acids Res* 2014, 42(D1):D490-D495.
84. Henrissat B: **A classification of glycosyl hydrolases based on amino acid sequence similarities**. *Biochem J* 1991, 280:309 - 316.
85. Levasseur A, Drula E, Lombard V, Coutinho PM, Henrissat B: **Expansion of the enzymatic repertoire of the CAZy database to integrate auxiliary redox enzymes**. *Biotechnol Biofuels* 2013, 6(1):41.
86. Hemsworth GR, Henrissat B, Davies GJ, Walton PH: **Discovery and characterization of a new family of lytic polysaccharide monooxygenases**. *Nat Chem Biol* 2014, **10**:122-126.
87. Leggio LL, Simmons TJ, Poulsen J-CN, Frandsen KE, Hemsworth GR, Stringer MA, von Freiesleben P, Tovborg M, Johansen KS, De Maria L: **Structure and boosting activity of a starch-degrading lytic polysaccharide monooxygenase**. *Nat Commun* 2015, **6**. DOI:10.1038/ncomms6961
88. Karlsson J, Saloheimo M, Siika-aho M, Tenkanen M, Penttilä M, Tjerneld F: **Homologous expression and characterization of Cel61A (EG IV) of *Trichoderma reesei***. *Eur J Biochem* 2001, 268(24):6498-6507.
89. Gourlay K, Hu J, Arantes V, Penttilä M, Saddler JN: **The use of carbohydrate binding modules (CBMs) to monitor changes in fragmentation and cellulose fiber surface morphology during cellulase- and swollenin-induced deconstruction of lignocellulosic substrates**. *J Biol Chem* 2015, 290(5):2938-2945.
90. Quiroz-Castañeda RE, Martínez-Anaya C, Cuervo-Soto LI, Segovia L, Folch-Mallol JL: **Loosenin, a novel protein with cellulose-disrupting activity from *Bjerkandera adusta***. *Microb Cell Fact* 2011, 10(8).
91. Lehmann L, Petersen N, Jørgensen CI, Olsson L, Stocks SM, Jørgensen HS, Hobley T: **Linking hydrolysis performance to *Trichoderma reesei* cellulolytic enzyme profile**. *Biotechnol Bioeng* 2015. DOI:10.1002/bit.25871
92. Zhao X, Rignall TR, McCabe C, Adney WS, Himmel ME: **Molecular simulation evidence for processive motion of *Trichoderma reesei* Cel7A during cellulose depolymerization**. *Chem Phys Lett* 2008, 460(1):284-288.
93. Jalak J, Väljamäe P: **Mechanism of initial rapid rate retardation in cellobiohydrolase catalyzed cellulose hydrolysis**. *Biotechnol Bioeng* 2010, 106(6):871-883.
94. Chia-wen CH, Cannella D, Jørgensen H, Felby C, Thygesen LG: **Cellobiohydrolase and endoglucanase respond differently to surfactants during the hydrolysis of cellulose**. *Biotechnol Biofuels* 2015, 8(1):52.
95. Aro N, Pakula T, Penttila M: **Transcriptional regulation of plant cell wall degradation by filamentous fungi**. *FEMS Microbiol Rev* 2005, **29**(4):719-739.
96. Merino S, Cherry J: **Progress and challenges in enzyme development for biomass utilization**. *Biofuels* 2007:95-120.
97. Artzi L, Morag E, Barak Y, Lamed R, Bayer EA: ***Clostridium clariflavum*: Key cellulosome players are revealed by proteomic analysis**. *mBio* 2015, 6(3):e00411-00415.
98. Suen G, Weimer PJ, Stevenson DM, Aylward FO, Boyum J, Deneke J, Drinkwater C, Ivanova NN, Mikhailova N, Chertkov O: **The complete genome sequence of *Fibrobacter succinogenes* S85 reveals a cellulolytic and metabolic specialist**. *PLoS One* 2011, 6(4):e18814.
99. Xie G, Bruce DC, Challacombe JF, Chertkov O, Detter JC, Gilna P, Han CS, Lucas S, Misra M, Myers GL: **Genome sequence of the cellulolytic gliding bacterium *Cytophaga hutchinsonii***. *Appl Environ Microb* 2007, 73(11):3536-3546.

100. Martinez D, Challacombe J, Morgenstern I, Hibbett D, Schmoll M, Kubicek CP, Ferreira P, Ruiz-Duenas FJ, Martinez AT, Kersten P: **Genome, transcriptome, and secretome analysis of wood decay fungus *Postia placenta* supports unique mechanisms of lignocellulose conversion**. *Proc Natl Acad Sci USA* 2009, 106(6):1954-1959.
101. Eastwood DC, Floudas D, Binder M, Majcherczyk A, Schneider P, Aerts A, Asiegbu FO, Baker SE, Barry K, Bendiksby M: **The plant cell wall–decomposing machinery underlies the functional diversity of forest fungi**. *Science* 2011, 333(6043):762-765.
102. Levasseur A, Saloheimo M, Navarro D, Andberg M, Pontarotti P, Kruus K, Record E: **Exploring laccase-like multicopper oxidase genes from the ascomycete *Trichoderma reesei*: a functional, phylogenetic and evolutionary study**. *BMC biochemistry* 2010, 11(1):32.
103. Riley R, Salamov AA, Brown DW, Nagy LG, Floudas D, Held BW, Levasseur A, Lombard V, Morin E, Otillar R: **Extensive sampling of basidiomycete genomes demonstrates inadequacy of the white-rot/brown-rot paradigm for wood decay fungi**. *Proc Natl Acad Sci USA* 2014, 111(27):9923-9928.
104. Martinez D, Larrondo LF, Putnam N, Gelpke MDS, Huang K, Chapman J, Helfenbein KG, Ramaiya P, Detter JC, Larimer F: **Genome sequence of the lignocellulose degrading fungus *Phanerochaete chrysosporium* strain RP78**. *Nat Biotechnol* 2004, 22(6):695-700.
105. Levasseur A, Lomascolo A, Chabrol O, Ruiz-Dueñas FJ, Boukhris-Uzan E, Piumi F, Kües U, Ram AF, Murat C, Haon M: **The genome of the white-rot fungus *Pycnoporus cinnabarinus*: a basidiomycete model with a versatile arsenal for lignocellulosic biomass breakdown**. *BMC genomics* 2014, 15(1):486.
106. Schwarze FW, Spycher M, Fink S: **Superior wood for violins–wood decay fungi as a substitute for cold climate**. *New Phytol* 2008, 179(4):1095-1104.
107. Grigoriev IV, Nikitin R, Haridas S, Kuo A, Ohm R, Otillar R, Riley R, Salamov A, Zhao X, Korzeniewski F: **MycoCosm portal: gearing up for 1000 fungal genomes**. *Nucleic Acids Res* 2014, 42:D699–D704.
108. Peterson R, Nevalainen H: ***Trichoderma reesei* RUT-C30 - thirty years of strain improvement**. *Microbiology* 2012, 158(1):58-68.
109. Mandels M, Reese ET: **Induction of cellulase in *Trichoderma viride* as influenced by carbon sources and metals**. *J Bacteriol* 1957, 73(2):263-278.
110. Montenecourt BS, Eveleigh DE: **Preparation of mutants of *Trichoderma reesei* with enhanced cellulase production**. *Appl Environ Microbiol* 1977, 34(6):777-782.
111. Montenecourt BS, Eveleigh DE: **Selective screening methods for the isolation of high yielding cellulase mutants of *Trichoderma reesei***. In.; 1979.
112. Le Crom S, Schackwitz W, Pennacchio L, Magnuson JK, Culley DE, Collett JR, Martin J, Druzhinina IS, Mathis H, Monot F: **Tracking the roots of cellulase hyperproduction by the fungus Trichoderma reesei using massively parallel DNA sequencing**. *Proc Natl Acad Sci USA* 2009, 106(38):16151-16156.
113. Martinez D, Berka RM, Henrissat B, Saloheimo M, Arvas M, Baker SE, Chapman J, Chertkov O, Coutinho PM, Cullen D *et al*: **Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. *Hypocrea jecorina*)**. *Nat Biotechnol* 2008, 26(5):553-560.
114. Häkkinen M, Arvas M, Oja M, Aro N, Penttilä M, Saloheimo M, Pakula TM: **Re-annotation of the CAZy genes of *Trichoderma reesei* and transcription in the presence of lignocellulosic substrates**. *Microb Cell Fact* 2012, 11(1):134.
115. Mandels M, Reese ET: **Induction of cellulase in fungi by cellobiose**. *J Bacteriol* 1960, 79(6):816-826.
116. Kubicek CP, Mikus M, Schuster A, Schmoll M, Seiboth B: **Metabolic engineering strategies for the improvement of cellulase production by *Hypocrea jecorina***. *Biotechnol Biofuels* 2009, 2(1):19.

117. Juhasz T, Szengyel Z, Reczey K, Siika-Aho M, Viikari L: **Characterization of cellulases and hemicellulases produced by *Trichoderma reesei* on various carbon sources**. *Process Biochem* 2005, 40(11):3519-3525.
118. Jørgensen H, Olsson L: **Production of cellulases by *Penicillium brasilianum* IBT 20888—Effect of substrate on hydrolytic performance**. *Enzyme Microb Tech* 2006, 38(3):381-390.
119. Bantscheff M, Lemeer S, Savitski MM, Kuster B: **Quantitative mass spectrometry in proteomics: critical review update from 2007 to the present**. *Anal Bioanal Chem* 2012, 404(4):939-965.
120. Bianco L, Perrotta G: **Methodologies and perspectives of proteomics applied to filamentous fungi: From sample preparation to secretome analysis**. *Int J Mol Sci* 2015, 16(3):5803-5829.
121. Lewis S, Currie I: **A novel experimental design for comparative twodimensional gel analysis: two-dimensional difference gel electrophoresis incorporating a pooled internal standard**. *Proteomics* 2003, 3:36-44.
122. Thompson A, Schafer J, Kuhn K, Kienle S, Schwarz J, Schmidt G, Neumann T, Johnstone R, Mohammed AK, Hamon C: **Tandem mass tags: a novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS**. *Anal Chem* 2003, 75(8):1895-1904.
123. Adav SS, Chao LT, Sze SK: **Protein abundance in multiplexed samples (PAMUS) for quantitation of *Trichoderma reesei* secretome**. *J Proteomics* 2013, 83:180-196.
124. Chundawat SP, Lipton MS, Purvine SO, Uppugundla N, Gao D, Balan V, Dale BE: **Proteomics-based compositional analysis of complex cellulase–hemicellulase mixtures**. *J Proteome Res* 2011, 10(10):4365-4372.
125. dos Santos Castro L, Pedersoli WR, Antoniêto ACC, Steindorff AS, Silva-Rocha R, Martinez-Rossi NM, Rossi A, Brown NA, Goldman GH, Faça VM: **Comparative metabolism of cellulose, sophorose and glucose in *Trichoderma reesei* using high-throughput genomic and proteomic analyses**. *Biotechnol Biofuels* 2014, 7(1):1-18.
126. Kubicek CP: **Systems biological approaches towards understanding cellulase production by *Trichoderma reesei***. *J Biotechnol* 2013, 163(2):133-142.
127. Adav SS, Ravindran A, Chao LT, Tan L, Singh S, Sze SK: **Proteomic analysis of pH and strains dependent protein secretion of *Trichoderma reesei***. *J Proteome Res* 2011, 10(10):4579-4596.
128. Jun H, Kieselbach T, Jönsson LJ: **Enzyme production by filamentous fungi: analysis of the secretome of *Trichoderma reesei* grown on unconventional carbon source**. *Microb Cell Facts* 2011, 10(1):68.
129. Herpoël-Gimbert I, Margeot A, Dolla A, Jan G, Mollé D, Lignon S, Mathis H, Sigoillot J-C, Monot F, Asther M: **Comparative secretome analyses of two *Trichoderma reesei* RUT-C30 and CL847 hypersecretory strains**. *Biotechnol Biofuels* 2008, 1(1):18.
130. Adav SS, Chao LT, Sze SK: **Quantitative secretomic analysis of *Trichoderma reesei* strains reveals enzymatic composition for lignocellulosic biomass degradation**. *Mol Cell Proteomics* 2012, 11(7):M111 012419.
131. Borin GP, Sanchez CC, de Souza AP, de Santana ES, de Souza AT, Leme AFP, Squina FM, Buckeridge M, Goldman GH, de Castro Oliveira JV: **Comparative secretome analysis of *Trichoderma reesei* and *Aspergillus niger* during growth on sugarcane biomass**. *PLoS One* 2015, 10(6):e0129275.
132. Zhang G, Zhu Y, Wei D, Wang W: **Enhanced production of heterologous proteins by the filamentous fungus *Trichoderma reesei* via disruption of the alkaline serine protease SPW combined with a pH control strategy**. *Plasmid* 2014, 71:16-22.
133. Rahikainen J, Mikander S, Marjamaa K, Tamminen T, Lappas A, Viikari L, Kruus K: **Inhibition of enzymatic hydrolysis by residual lignins from softwood - study of enzyme**

binding and inactivation on lignin-rich surface. *Biotechnol Bioeng* 2011, 108(12):2823-2834.
134. Paul GC, Thomas CR: **Characterisation of mycelial morphology using image analysis**. In: *Relation Between Morphology and Process Performances.* Springer; 1998: 1-59.
135. Papagianni M: **Fungal morphology and metabolite production in submerged mycelial processes**. *Biotech Ad* 2004, 22(3):189-259.
136. Kossen N: **The morphology of filamentous fungi**. In: *History of Modern Biotechnology II.* Springer; 2000: 1-33.
137. Domingues F, Queiroz J, Cabral J, Fonseca L: **The influence of culture conditions on mycelial structure and cellulase production by *Trichoderma reesei* Rut C-30**. *Enzyme Microb Tech* 2000, 26(5):394-401.
138. Haack MB, Olsson L, Hansen K, Lantz AE: **Change in hyphal morphology of *Aspergillus oryzae* during fed-batch cultivation**. *Appl Microbiol Biotechnol* 2006, 70(4):482-487.
139. Spohr A, Carlsen M, Nielsen J, Villadsen J: **Morphological characterization of recombinant strains of *Aspergillus oryzae* producing alpha-amylase during batch cultivations**. *Biotechnol Lett* 1997, 19(3):257-262.
140. Conesa A, Punt PJ, van Luijk N, van den Hondel CA: **The secretion pathway in filamentous fungi: a biotechnological view**. *Fungal Genet Biol* 2001, 33(3):155-171.
141. Nevalainen H, Peterson R: **Making recombinant proteins in filamentous fungi-are we expecting too much?** *Front Microbiol* 2014, 5:75.
142. Booker R, Sell J: **The nanostructure of the cell wall of softwoods and its functions in a living tree**. *Holz als Roh- und Werkstoff* 1998, 56(1):1-8.
143. King BC, Donnelly MK, Bergstrom GC, Walker LP, Gibson DM: **An optimized microplate assay system for quantitative evaluation of plant cell wall–degrading enzyme activity of fungal culture extracts**. *Biotechnology and bioengineering* 2009, 102(4):1033-1044.
144. Miller GL: **Use of dinitrosalicylic acid reagent for determination of reducing sugar**. *Anal Chem* 1959, 31(3):426-428.
145. Lindman B, Karlström G, Stigsson L: **On the mechanism of dissolution of cellulose**. *J Mol Liq* 2010, 156(1):76-81.
146. Glasser WG, Atalla RH, Blackwell J, Brown Jr RM, Burchard W, French AD, Klemm DO, Nishiyama Y: **About the structure of cellulose: debating the Lindman hypothesis**. *Cellulose* 2012, 19(3):589-598.
147. Testova L, Borrega M, Tolonen LK, Penttilä PA, Serimaa R, Larsson PT, Sixta H: **Dissolving-grade birch pulps produced under various prehydrolysis intensities: quality, structure and applications**. *Cellulose* 2014, 21(3):2007-2021.
148. Wickholm K, Larsson PT, Iversen T: **Assignment of non-crystalline forms in cellulose I by CP/MAS ^{13}C NMR spectroscopy**. *Carbohydr Res* 1998, 312(3):123-129.
149. Ding SY, Himmel ME: **The maize primary cell wall microfibril: a new model derived from direct visualization**. *J Agric Food Chem* 2006, 54(3):597-606.
150. Fernandes A, Thomas L, Altaner C, Callow P, Forsyth V, Apperley D, Kennedy C, Jarvis M: **Nanostructure of cellulose microfibrils in spruce wood**. *Proc Natl Acad Sci USA* 2011, **108**:E1195 - E1203.
151. Thomas LH, Forsyth VT, Šturcová A, Kennedy CJ, May RP, Altaner CM, Apperley DC, Wess TJ, Jarvis MC: **Structure of cellulose microfibrils in primary cell walls from collenchyma**. *Plant Physiol* 2013, 161(1):465-476.
152. Liu YS, Baker JO, Zeng Y, Himmel ME, Haas T, Ding SY: **Cellobiohydrolase hydrolyzes crystalline cellulose on hydrophobic faces**. *J Biol Chem* 2011, 286:11195-11201.
153. Lehtiö J, Sugiyama J, Gustavsson M, Fransson L, Linder M, Teeri TT: **The binding specificity and affinity determinants of family 1 and family 3 cellulose binding modules**. *Proc Natl Acad Sci USA* 2003, 100(2):484-489.

154. Heiner AP, Kuutti L, Teleman O: **Comparison of the interface between water and four surfaces of native crystalline cellulose by molecular dynamics simulations**. *Carbohyd Res* 1998, 306:205-220.
155. Felby C, Thygesen LG, Kristensen JB, Jørgensen H, Elder T: **Cellulose–water interactions during enzymatic hydrolysis as studied by time domain NMR**. *Cellulose* 2008, 15(5):703-710.
156. Selig MJ, Thygesen LG, Johnson DK, Himmel ME, Felby C, Mittal A: **Hydration and saccharification of cellulose Iβ, II and IIII at increasing dry solids loadings**. *Biotechnology Lett* 2013, 35(10):1599-1607.
157. Selig MJ, Thygesen LG, Felby C: **Correlating the ability of lignocellulosic polymers to constrain water with the potential to inhibit cellulose saccharification**. *Biotechnol Biofuels* 2014, 7(1):159.
158. Duchesne I, Daniel G: **The ultrastructure of wood fibre surfaces as shown by a variety of microscopical methods-A review**. *Nord Pulp Pap Res Jl* 1999, 14(2):129-139.
159. Duchesne I, Daniel G: **Changes in surface ultrastructure of Norway spruce fibres during kraft pulping - visualisation by field emission-SEM**. *Nord Pulp Pap Res J* 2000, 15:54-61.
160. Preston RD, Cronshaw J: **Constitution of the fibrillar and non-fibrillar components of the walls of *Valonia ventricosa***. *Nature* 1958, 181:248-250.
161. Park S, Baker JO, Himmel ME, Parilla PA, Johnson DK: **Cellulose crystallinity index: measurement techniques and their impact on interpreting cellulase performance**. *Biotechnol Biofuels* 2010, 3:10.
162. Larsson PT, Wickholm K, Iversen T: **A CP/MAS ^{13}C NMR investigation of molecular ordering in celluloses**. *Carbohydr Res* 1997, 302(1-2):19-25.
163. Larsson PT, Svensson A, Wagberg L: **A new, robust method for measuring average fibre wall pore sizes in cellulose I rich plant fibre walls**. *Cellulose* 2013, **20**:623-631.
164. Chunilall V, Bush T, Larsson PT, Iversen T, Kindness A: **A CP/MAS ^{13}C-NMR study of cellulose fibril aggregation in eucalyptus dissolving pulps during drying and the correlation between aggregate dimensions and chemical reactivity**. *Holzforschung* 2010, 64(6):693-698.
165. Hult E-L, Larsson P, Iversen T: **Cellulose fibril aggregation—an inherent property of kraft pulps**. *Polymer* 2001, 42(8):3309-3314.
166. Hubbe MA, Venditti RA, Rojas OJ: **What happens to cellulosic fibers during papermaking and recycling? A review**. *BioResources* 2007, 2(4):739-788.
167. Newman RH: **Carbon-13 NMR evidence for cocrystallization of cellulose as a mechanism for hornification of bleached kraft pulp**. *Cellulose* 2004, 11(1):45-52.
168. Idström A, Brelid H, Nydén M, Nordstierna L: **CP/MAS ^{13}C NMR study of pulp hornification using nanocrystalline cellulose as a model system**. *Carbohydr Polym* 2013, 92(1):881-884.
169. Aldaeus F, Larsson K, Srndovic JS, Kubat M, Karlström K, Peciulyte A, Olsson L, Larsson PT: **The supramolecular structure of cellulose-rich wood pulps can be a determinative factor for enzymatic hydrolysability**. *Cellulose* 2015, **22**(6):3991-4002.
170. Yu ZY, Jameel H, Chang HM, Philips R, Park S: **Evaluation of the factors affecting Avicel reactivity using multi-stage enzymatic hydrolysis**. *Biotechnol Bioeng* 2012, 109(5):1131-1139.
171. Grethlein HE: **The effect of pore size distribution on the rate of enzymatic hydrolysis of cellulosic substrates**. *Nature Biotechnology* 1985, **3**(2):155-160.
172. Boistelle R, Astier J: **Crystallization mechanisms in solution**. *J Cryst Growth* 1988, 90(1):14-30.
173. Stone J, Scallan A: **The effect of component removal upon the porous structure of the cell wall of wood. II. Swelling in water and the fiber saturation point**. *Tappi* 1967, 50:496-501.

174. Li T, Henriksson U, Ödberg L: **Determination of pore sizes in wood cellulose fibers by ^{2}H and ^{1}H NMR**. *Nord Pulp Pap Res J* 1993, 8(3):326-330.
175. Kohnke T, Ostlund A, Brelid H: **Adsorption of arabinoxylan on cellulosic surfaces: influence of degree of substitution and substitution pattern on adsorption characteristics**. *Biomacromolecules* 2011, 12(7):2633-2641.
176. Esteghlalian AR, Bilodeau M, Mansfield SD, Saddler JN: **Do enzymatic hydrolyzability and Simons' stain reflect the changes in the accessibility of lignocellulosic substrates to cellulase enzymes?** *Biotechnol Prog* 2001, 17(6):1049-1054.
177. Moura M, Ferreira P, Figueiredo M: **Mercury intrusion porosimetry in pulp and paper technology**. *Powder Technol* 2005, 160(2):61-66.
178. Park S, Venditti RA, Jameel H, Pawlak JJ: **Changes in pore size distribution during the drying of cellulose fibers as measured by differential scanning calorimetry**. *Carbohydr Polym* 2006, 66(1):97-103.
179. Goacher RE, Selig MJ, Master ER: **Advancing lignocellulose bioconversion through direct assessment of enzyme action on insoluble substrates**. *Curr Opin Biotech* 2014, 27:123-133.
180. Cataldi TRI, Campa C, De Benedetto GE: **Carbohydrate analysis by high-performance anion-exchange chromatography with pulsed amperometric detection: The potential is still growing**. *Fresenius J Anal Chem* 2000, 368(8):739-758.
181. Harris RK: **Nuclear magnetic resonance spectroscopy**. John Wiley & Sons: New York, 1987.
182. Andrew E, Bradbury A, Eades R: **Nuclear magnetic resonance spectra from a crystal rotated at high speed**. 1958, 182:1659.
183. Schaefer J, Stejskal E: **Carbon-13 nuclear magnetic resonance of polymers spinning at the magic angle**. *J Am Chem Soc* 1976, 98(4):1031-1032.
184. Larsson PT, Hult E-L, Wickholm K, Pettersson E, Iversen T: **CP/MAS ^{13}C-NMR spectroscopy applied to structure and interaction studies on cellulose I**. *Solid State Nucl Mag* 1999, 15(1):31-40.
185. Bergenstrahle M, Wohlert J, Larsson PT, Mazeau K, Berglund LA: **Dynamics of cellulose-water interfaces: NMR spin-lattice relaxation times calculated from atomistic computer simulations**. *J Phys Chem B* 2008, 112(9):2590-2595.
186. Zeng Y, Himmel ME, Ding SY: **Coherent Raman microscopy analysis of plant cell walls**. In: *Methods in Molecular Biology.* vol. 908; 2012: 49-60.
187. Moran-Mirabal JM: **Advanced-microscopy techniques for the characterization of cellulose structure and cellulose-cellulase interactions**. In: *Cellulose–Fundamental Aspects.* Dr. Theo G.M. Van De Ven (Ed.) In Tech; 2013:1-44.
188. Camp Jr CH, Cicerone MT: **Chemically sensitive bioimaging with coherent Raman scattering**. *Nat Photonics* 2015, 9(5):295-305.
189. Komis G, Šamajová O, Ovečka M, Šamaj J: **Super-resolution microscopy in plant cell imaging**. *Trends Plant Sci* 2015. DOI:10.1016/j.tplants.2015.08.013
190. Ding S-Y, Liu Y-S, Zeng Y, Himmel ME, Baker JO, Bayer EA: **How does plant cell wall nanoscale architecture correlate with enzymatic digestibility?** *Science* 2012, 338(6110):1055-1060.
191. Pedersen HL, Fangel JU, McCleary B, Ruzanski C, Rydahl MG, Ralet M-C, Farkas V, von Schantz L, Marcus SE, Andersen MC: **Versatile high resolution oligosaccharide microarrays for plant glycobiology and cell wall research**. *J Biol Chem* 2012, 287(47):39429-39438.
192. Chanzy H, Henrissat B, Vuong R: **Colloidal gold labelling of l, 4-β-D-glucan cellobiohydrolase adsorbed on cellulose substrates**. *FEBS letters* 1984, 172(2):193-197.
193. Chanzy H, Henrissat B: **Undirectional degradation of *Valonia* cellulose microcrystals subjected to cellulase action**. *FEBS letters* 1985, 184(2):285-288.

194. Imai T, Boisset C, Samejima M, Igarashi K, Sugiyama J: **Unidirectional processive action of cellobiohydrolase Cel7A on *Valonia* cellulose microcrystals**. *FEBS letters* 1998, 432(3):113-116.
195. Thygesen LG, Hidayat BJ, Johansen KS, Felby C: **Role of supramolecular cellulose structures in enzymatic hydrolysis of plant cell walls**. *J Ind Microbiol Biotechnol* 2011, 38(8):975-983.
196. Pohling C, Brackmann C, Duarte A, Buckup T, Enejder A, Motzkus M: **Chemical imaging of lignocellulosic biomass by CARS microscopy**. *J Biophotonics* 2014, 7(1-2):126-134.
197. Jeremic D, Goacher RE, Yan R, Karunakaran C, Master ER: **Direct and up-close views of plant cell walls show a leading role for lignin-modifying enzymes on ensuing xylanases**. *Biotechnol Biofuels* 2014, 7(1):496.
198. Liesche J, Ziomkiewicz I, Schulz A: **Super-resolution imaging with Pontamine Fast Scarlet 4BS enables direct visualization of cellulose orientation and cell connection architecture in onion epidermis cells**. *BMC Plant Biol* 2013, 13(1):226.
199. Slepkov AD, Ridsdale A, Pegoraro AF, Moffatt DJ, Stolow A: **Multimodal CARS microscopy of structured carbohydrate biopolymers**. *Biomed Opt Express* 2010, 1(5):1347-1357.
200. Zimmerley M, Younger R, Valenton T, Oertel DC, Ward JL, Potma EO: **Molecular orientation in dry and hydrated cellulose fibers: A coherent anti-stokes Raman scattering microscopy study**. *J Phys Chem B* 2010, 114(31):10200-10208.
201. Brackmann C, Bodin A, Åkeson M, Gatenholm P, Enejder A: **Visualization of the cellulose biosynthesis and cell integration into cellulose scaffolds**. *Biomacromolecules* 2010, 11(3):542-548.
202. Brown Jr RM, Millard AC, Campagnola PJ: **Macromolecular structure of cellulose studied by second-harmonic generation imaging microscopy**. *Optics letters* 2003, **28**(22):2207-2209.
203. Chimenez TA, Gehlen MH, Marabezi K, Curvelo AA: **Characterization of sugarcane bagasse by autofluorescence microscopy**. *Cellulose* 2014, 21(1):653-664.