

Accessibility and enzymatic degradation of native and model cellulose substrates

Miro Suchy

Accessibility and enzymatic degradation of native and model cellulose substrates

Miro Suchy

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This thesis investigated two issues relevant to the bioconversion of lignocellulosic biomass. The first part relates to the accessibility of cellulose in wood, more specifically, the impact of initial drying on wood ultrastructure. The second part of this thesis pertains to the enzymatic hydrolysis of cellulose, including a characterization of a model film of amorphous cellulose and its subsequent utilization in a fundamental evaluation of the mechanism of cellulase action. In addition, the impact of cellulase treatment on the properties and the morphology of wood pulp fibers was evaluated.

The impact of drying on the ultrastructure of fresh wood was studied by deuterium exchange coupled with FT-IR analysis. Initially, the experimental concept and its reliability were evaluated using fresh wood samples. The same experimental concept was then applied to study the impact of initial drying on wood pulps of different characteristics and composition. The objective was to correlate the trends in drying-induced alterations observed for wood and for pulps, bleached chemical pulp in particular. Drying-induced alterations of a native wood sample exhibited a remarkable similarity to those observed for wood pulp samples. The results suggest that the supramolecular rearrangements in the native wood matrix upon dehydration are qualitatively identical to the well-known changes occurring in pulp fibers after drying, although the changes are considerably different in quantity.

Quantitative studies of cellulose degradation by cellulase enzymes were carried out using an amorphous cellulose model film with well-defined characteristics. First, the films were extensively characterized, particularly their crystalline nature and swelling behavior. The film swelled excessively in water, doubling its thickness, but returned to the original thickness upon water removal, while retaining its amorphous nature. This film was then used as a substrate in studying the action of monocomponent endoglucanase (EG) that acts on amorphous cellulose. The study was carried out using a quartz crystal microbalance with dissipation monitoring (QCMD). The main objectives were to study the impact of film thickness on the rate of hydrolysis and to directly quantify the extent of hydrolysis (decrease in thickness of films after hydrolysis). It was demonstrated that the amount of substrate available for hydrolysis did not have an impact on the rate of hydrolysis. The investigation also demonstrated impacts of various factors on the kinetic evaluation of the cellulase action. The investigation of the action of a commercial celobiohydrolase on wood pulp fibers showed that the treatment had no significant impact on the strength properties of the pulp.

Keywords Wood, ultrastructure, deuteration, FT-IR, drying, accessibility, cellulase, degradation, model film**ISBN (printed)** 978-952-60-4131-5**ISBN (pdf)** 978-952-60-4132-2**ISSN-L** 1799-4934**ISSN (printed)** 1799-4934**ISSN (pdf)** 1799-4942**Location of publisher** Espoo**Location of printing** Helsinki**Year** 2011**Pages** 162**The dissertation can be read at** <http://lib.tkk.fi/Diss/>

PREFACE

This study was carried out at the Department of Forest Products Technology at Helsinki University of Technology, now Aalto University, School of Chemical Technology, during 2007-2010. Parts of this research were supported by National Agency for Technology and Innovation (TEKES), Multidisciplinary Institute of Digitalization and Energy (MIDE) and UPM-Kymmene Corporation.

I am indebted to Professor Tapani Vuorinen for giving me the opportunity to join and work in his group under his supervision, on very interesting research topics. I am also extremely grateful for the opportunity to work with Dr. Eero Kontturi, instructor of this thesis. He had a huge influence on the research comprising this thesis and particularly on the final outcome - the publishing of the results in respectable journals. In addition, I am privileged to consider him a really good friend. The evenings spent with him and his lovely wife, my friend and colleague Katri, and their two little children, is something I will always remember and greatly miss. The enjoyment of gourmet meals, exquisite wines and stimulating conversations that covered a wide gamut of topics from science to the everlasting hits of musical acts of the 80's and 90's, which usually ended in a struggle to catch the last bus connection to Espoo - this will forever be a highlight of my time in Finland.

My sincere thanks go to Dr. Anna-Stiina Jääskeläinen, who was “responsible” for my coming to Finland by letting me know about the opportunity and helping with my acceptance at the department, for her support, encouragement and for providing a positive atmosphere throughout my studies.

None of this would perhaps have happened without Jyrki Juntunen (alumni of our Department) and his wife Sanna, my first Finnish friends, who initially introduced me to Finland. Thank you so much for your friendship and all your help.

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Co-authors of the papers, Jenni Virtanen, Dr. Terhi Hakala, Dr. Tekla Tammelin, Professor Markus Linder, Dr. Joe Campbell are all thanked for their contribution to the research and resulting publications. Joe is also thanked for proof-reading the thesis and papers.

Despite some frustrating times with experiments and writing, it was always enjoyable working at our department. This was because of the people that created a friendly, relaxed and often entertaining working atmosphere. Ritva, Anu, Marja, Aila and Rita are thanked for all of their assistance with the laboratory work. Kati Mäenpää, our former librarian, is thanked for her assistance with literature acquisitions along with former laboratory engineer Riitta Hynynen for helping with all of the practical issues related to my stay and work.

I would like to thank my colleagues and friends that were there over the course of my studies. The first group that was here when I started, who are now all doctors, Juha, Petri, Tuula, Tekla and Kyösti and, in particular, Susanna who always encouraged me when things did not go as well as they should have. Then, there is the fresher blood represented by Tiina, Elli, Laura, Tuomas, Anna, Anne and Delphine. They always made me forget my “high number”.

Special thanks and gratitude go to the M club. Mao, Michael, Michi, Marcelo and Marina. You guys know what you mean to me and I will never forget your support, the times we spent together and all that you did for me. In addition, Mao, it is hard to express how much I appreciate your help with all the arrangements and sorting out my personal issues. And Michael, sharing the office and birthdays, all the doctor’s orders and all your advice will be impossible to replace.

And finally, I want to thank my parents and brothers for their support. Peter and Andrea, thank you for convincing me to go back to school.

Espoo, April 18th, 2011

Miro Suchy

LIST OF PUBLICATIONS

This thesis is mainly based on the results presented in five publications which are referred as Roman numerals in the text. Some additional published and unpublished data is also related to the work.

- Paper I** Suchy, M., Virtanen, J., Kontturi, E., Vuorinen, T. (2010) Impact of drying on wood ultrastructure observed by deuterium exchange and photoacoustic FT-IR spectroscopy. *Biomacromolecules* 11(2), 515-520.
- Paper II** Suchy, M., Kontturi, E., Vuorinen, T. (2010) Impact of drying on wood ultrastructure: Similarities in cell wall alteration between native wood and isolated wood-based fibers. *Biomacromolecules* 11(8), 2161-2168.
- Paper III** Kontturi, E., Suchy, M., Penttilä, P., Jean, B., Pirkkalainen, K., Torkkeli, M., Serimaa, R. (2011) Amorphous characteristics of an ultrathin cellulose film. *Biomacromolecules* 12(3), 770-777.
- Paper IV** Suchy, M., Linder, M., Tammelin, T., Campbell, J., Vuorinen, T., Kontturi, E. (2011). Quantitative assessment of the enzymatic degradation of amorphous cellulose by using Quartz crystal microbalance with dissipation monitoring. *Langmuir* 27(14), 8819-8828.
- Paper V** Suchy, M., Hakala, T., Kangas, H., Kontturi, E., Tammelin, T., Pursula, T., Vuorinen, T. (2009) Effects of commercial cellobiohydrolase treatment on fiber strength and morphology of bleached hardwood pulp. *Holzforschung* 63(6), 731-736.

Author's contribution to the appended joint publications:

- I, II, IV,V** Miro Suchy was responsible for the experimental design, performed the main part of the experimental work, analysed the corresponding results, and wrote the manuscript.
- III** Miro Suchy was responsible for the sample preparation, carried out a part of the experiments and their analysis, wrote the corresponding part of the manuscript.

LIST OF ABBREVIATIONS

AFM	atomic force microscopy
AGU	anhydroglucose unit
CBH	cellobiohydrolase
DP	degree of polymerization
EG	endoglucanase
FT-IR	Fourier transform infra-red
GIXRD	grazing incidence X-ray diffraction
LB	Langmuir-Blodgett
LS	Langmuir-Schaefer
MFC	microfibrillated cellulose
NFC	nanofibrillated cellulose
NR	neutron reflectivity
PAS	photoacoustic spectroscopy
QCM-D	quartz crystal microbalance with dissipation
RH	relative humidity
SEM	scanning electron microscopy
SLD	scattering length density
SPM	scanning probe microscopy
TMSC	trimethylsilyl cellulose
TEM	transmission electron microscopy
WRV	water retention value
XPS	X-ray photoelectron spectroscopy

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1 INTRODUCTION AND OUTLINE OF THE STUDY

Global economic developments that have arisen during the first decade of the 21st century have had a pronounced impact on the pulp and paper industry. Moving production to countries with more favorable economic conditions, with more readily available and affordable resources and closer to markets with a potential for increase in paper products consumption, has markedly affected the traditional pulp and paper producing countries. In a not so long term perspective, it will be imperative for pulp and paper companies to find additional sources of revenue, for example, by producing value-added specialty products based on wood, cellulose, and by-products of conventional wood pulping processes. These developments provided incentives to major pulp and paper producers to increase their involvement in wood, pulp and cellulose research with a focus on seeking alternative utilization. Parallel to these trends in industry, the focus of predominately paper production related research, on both commercial and academic levels, has been gradually moving towards studying other prospective wood and cellulose products. The research pertinent to this PhD thesis was carried out during this period, and the scope of the thesis reflects these trends by focusing on two important aspects of lignocellulosic bioconversion - substrate accessibility and enzymatic hydrolysis. More specifically, the overall objective was to study the following: (i) impact of drying on fresh wood ultrastructure; (ii) enzymatic hydrolysis of cellulose model substrates; and (iii) cellulose in wood fibers.

The impact of initial drying on wood ultrastructure can have implications in the accessibility of cellulose in wood towards enzymatic treatment. The cellulose accessibility is one of the most important factors in wood or biomass conversion to biofuels. Fundamental studies of enzymatic hydrolysis combined with evaluation of the impact of enzymatic treatment on fiber properties can lead to a better understanding of the mechanism of enzymatic action on the actual wood fiber, and to an evaluation of environmentally benign alternatives to the current technology implemented in fiber production and modifications.

The first part of this thesis describes the investigation of the impact of initial drying on wood ultrastructure. Drying of harvested wood is unavoidable, even a requirement

for some processes, and these drying-induced alterations may have a pronounced impact on the accessibility of wood, the cellulose component in particular. The accessibility of cellulose plays an important role in alternative wood processing, for example, in cellulose degradation for biofuels production, or in the isolation of cellulose nanofibrils. The study, described in **Papers I and II**, utilised deuteration of cellulose in fresh wood and never-dried pulp samples and subsequent detection of the exchanged OH groups by FT-IR spectroscopy. The rationale of the experimental plan was to convert the accessible hydroxyl groups (OH) of the wood cell wall polysaccharides to OD groups with D₂O. The deuterated wood and pulp samples were then dried under controlled conditions, followed by reprotonation with an excess of water. The detection of the OD group in the samples after reprotonation would indicate conversion of the originally accessible OH groups that were readily exchanged to OD to inaccessible, reprotonation resistant OD groups during drying.

The objective of the initial work was to evaluate the experimental concept and its reliability (**Paper I**). In the following investigation described in **Paper II**, the same experimental concept was applied to study the impact of initial drying on wood pulps of different characteristics and composition. The importance of the inclusion of the pulp samples in this investigation was to correlate the trends in drying-induced alterations observed for wood and for pulps, bleached chemical pulp in particular. The impact of drying on the swelling properties of low yield pulps is well documented. Therefore, correlating the similarities between the behaviour of the pulp and the original wood sample during initial drying could indicate that the changes taking place in fresh wood ultrastructure to be of a similar nature as those occurring in the pulp fibers. The alterations in pulp fibres were shown to have a pronounced impact on their properties. The methods used for demonstrating and quantifying of these changes, however, are not, in most cases, applicable for the measurement of solid wood samples.

The second part of this thesis pertains to the enzymatic hydrolysis of cellulose. The efficient hydrolysis of cellulose in lignocellulosic biomass is essential for the production of cellulose based biofuels. In contrast, controlled partial hydrolysis of wood pulp fibers can be utilized in modification of some pulp properties. In general, better knowledge of cellulase action on cellulosic fibers is needed both for fiber modification and total hydrolysis of lignocellulosic materials. The complexity of the

native cellulose substrates often makes its utilization in fundamental studies challenging. Various cellulose model substrates have been utilised to gain more information on the mechanism of the enzymatic hydrolysis of cellulose. More recently, ultrathin cellulose model films have become popular tools for these studies. Prepared films can have different characteristics, including crystalline form and degree of ordering. Although the focus of the enzymatic hydrolysis investigation is mostly aimed at degradation of the crystalline part of cellulose, hydrolysis of amorphous cellulose plays an important role when, for example, in increasing reactivity of dissolving pulp or in production of microfibrilated cellulose. In our investigation, we intended to explore the interaction of the cellulases with amorphous cellulose. **Paper III** describes evaluation of the characteristics of amorphous model films using several pertinent analytical techniques. This characterization of the film properties and evaluation of their stability was essential for the studies of the cellulase action, particularly using, for example, the quartz crystal microbalance with dissipation (QCM-D) technique, where swelling and stability of the films can affect the measurement, and consequently interpretation of the results. **Paper IV** describes fundamental studies of the hydrolytic action of monocomponent cellulases on amorphous cellulose model films. In addition, we also focused on quantification of the extent of hydrolysis by a complementary method – atomic force microscopy (AFM). The decrease in thickness of hydrolyzed films measured by AFM was then correlated with the results obtained from the QCM-D measurements. In addition, we tried to demonstrate the impact of film thickness on the rate of hydrolysis.

Finally, **Paper V** describes the study of the impact of enzymatic treatment on the properties and morphology of bleached hardwood kraft pulp fibers. This research, carried out in collaboration with industrial partners, has particular relevance as more affordable enzymes have recently become commercially available. The study describes utilization of the cellulase action in modification of native cellulose fiber properties and evaluates its impact on fiber morphology. The study complements the investigation carried out using model cellulose substrates.

2 BACKGROUND

2.1 Wood and wood pulp fibers structure and composition

Whether used as a source of heat and energy, construction material, or for production of wood pulp, paper and cellulose, wood has always had a special place in the history of humankind. In addition to favourable mechanical properties and composition, it is a natural, biodegradable and renewable material - aspects highly regarded in the contemporary environmentally-cautious society. As a raw material, it is the most important source of industrial cellulose. The hierarchical structure of wood is shown in Figure 1. Included in the figure is the range of length scales from tree trunk down to individual cellulose molecules.

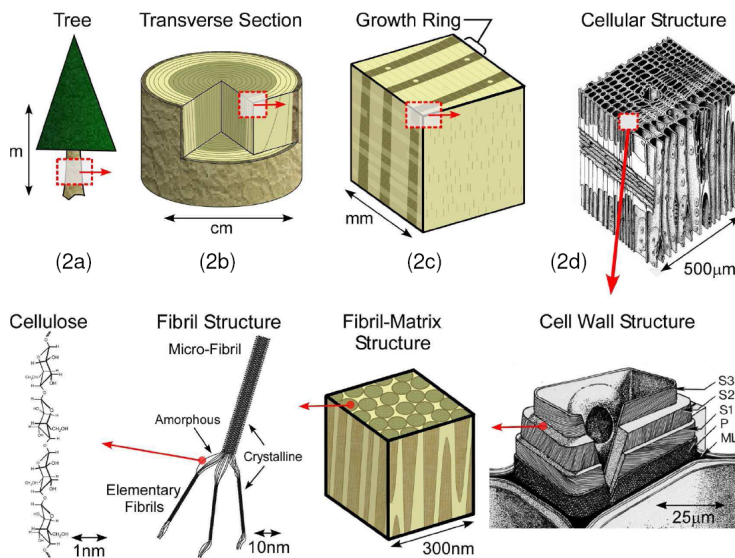


Figure 1. Hierarchical structure of wood (Moon 2008).

The smallest cellulosic strand, termed an elementary fibril, is in the mature wood cell wall and is between 2.5 and 3.5 nm wide (Heyn 1969; Jacob et al. 1995). The elementary fibrils are further arranged to form so called microfibrils. Depending on the source of cellulose and the position within the cell wall, these microfibrils are 5-30 nm in diameter, and are already visible in an electron microscope (Alén 2000).

The terminology of the cellulose arrangement is not clearly defined. In some recent publications, the elementary fibrils (4 nm) are termed microfibrils, and the microfibrils are described as cellulose aggregates, or bundles, 15-25 nm in diameter (Fahlén and Salmén 2003; Salmén 2004). The microfibrils, or cellulose bundles, are combined to form greater fibrils and lamellae and are embedded in the hemicellulose-lignin matrix. Layers of these embedded microfibrils form multilayered walls of cells. Neighboring cells are held together by the middle lamella containing lignin and pectin that surrounds the cells. This organisation makes wood an exceptionally strong material.

2.1.1 Ultrastructure and composition of wood and pulp fiber

Perhaps the most fitting and common description of wood as a material is that of a natural biocomposite made up of semi-crystalline cellulose microfibrils embedded in an amorphous hemicellulose-lignin matrix. The actual structure and organization of wood is rather complex, considering that wood comes from living trees in which the cells serve a variety of specific purposes, i.e. transport, storage and support. Wood consists of different types of fibers. Tracheids and ray cells are the main constituents of softwoods, while hardwoods consist of vessel elements, fiber tracheids or libriform cells and ray cells (Sjöström 1993). The majority of fiber cell wall models describing the structure of wood fiber are based on softwood tracheids. A model of wood cell organisation and composition is illustrated in Figure 2.

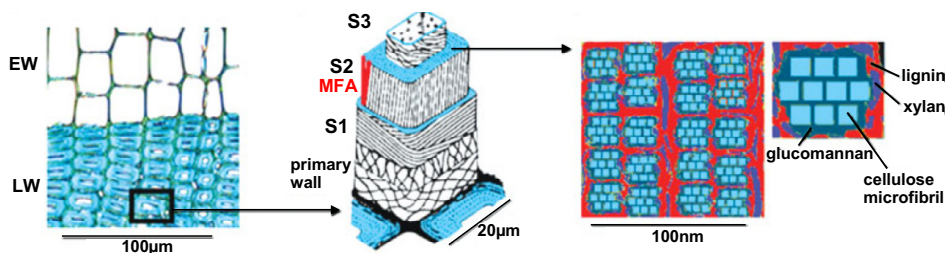


Figure 2. Structure of wood cells: different wall thickness of earlywood (EW) and latewood (LW); layered structure of the cell wall with different microfibril angle (MFA) and composite structure within the S2 layer of the secondary wall (Schmidt et al. 2006).

The thickness of the cell wall depends on the growth time of the year and the function of the cell. The cell wall of earlywood (EW) is usually thinner and the role is mostly water transport within the tree, while the thicker latewood (LW) cell wall fulfils the role of mechanical support of the tree. A more detailed model of the cell wall structure with the thicknesses of the individual layers is shown in Figure 3.

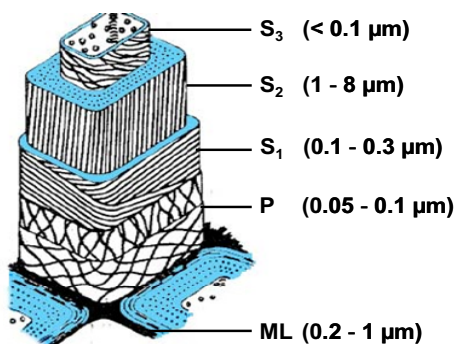


Figure 3. Model of the cell wall structure (Adapted from Fengel and Wegener 1984).

The cell wall is essentially composed of two layers, consisting of the relatively thin primary wall (P) and the thick secondary wall (S). The primary wall is rich in hemicelluloses, pectin, protein and lignin. The secondary cell wall layer is divided into three sublayers - the outer layer (S1), the middle layer (S2), and the inner layer (S3), as illustrated in Figure 3. In addition to thickness, the layers differ in the orientation of the microfibrils along the fibre axis, often described as microfibril angle (MFA). The MFA refers to the angle between the direction of the helical windings of cellulose microfibrils in the secondary wall of fibres and the long axis of cell, and it is mostly applied to the orientation of cellulose microfibrils in the S2 layer. The S1 layer, although not investigated in as much detail as the thick middle S2 layer, has a very important role for pulp fibres since it is in direct contact with chemicals or enzymes. The S1 layer is composed of two striations of cellulose microfibrils with alternate helical orientations, winding around the cell axis at flat angle to the left (S-helix) and to the right (Z-helix) (Daniel 2009). Some studies, however, suggested that the S1 layer is composed of microfibrils oriented in a single helix (Brändström et al. 2003).

The bulk of cellulose, and also lignin, is contained in the thick S2 layer, which represents about 80% of the total cell wall thickness (Fengel and Stoll 1973) and

contains between 80 to 85% of the cellulose matter of the wood fiber (Krässig 1993). The S2 layer is considered as the main load-bearing element in wood fibres and both the thickness and the microfibrillar angle of the S2 layer affect the mechanical strength of a single fiber (Page et al. 1977). The S3 layer is sometimes also referred to as the tertiary wall. The microfibrils are arranged in a parallel, tightly wound helical order forming a gentle angle with the cell axis (Fengel and Wegener 1984). The cavity in the center of the hollow fiber is termed the lumen. The middle lamella (ML), composed mostly of lignin and pectin, is located between the P walls of adjacent cells and its role is to bind the cells together (Alén 2000).

Pulping is a process in which the native wood matrix is disintegrated and the wood is converted into a fibrous pulp. Depending on the pulping process, the extent of the fiber cell wall alteration varies. The processes range from mechanical pulping, where the fibers are separated from each other by means of elevated temperature and mechanical forces (i.e. grinding), to chemical pulping (delignification), in which chemicals in combination with elevated temperatures and high pressure are utilized to separate the pulp fibers. Mechanical pulps, such as stone groundwood (SGW) pulp that is produced by grinding the wood logs (Blechschildt and Heinemann 2006), are referred to as high yield pulp, and their chemical composition is comparable to that of fibers in native wood. In contrast, chemical pulping processes, such as the most common kraft process, disintegrate the wood matrix and separate the fibers by degrading and extracting a substantial part of the lignin (Gierer 1980; Sjöström 1993). In addition to lignin, a portion of carbohydrates, mainly hemicelluloses, is partially removed, and the chemical pulps are thus referred to as low yield pulps. The removal of the lignin and exposure of the fibers to high temperatures results in alterations of the cell wall. The impact of chemical pulping on fiber cell wall structure has been studied previously using several methods, including atomic force microscopy coupled with image analysis (Fahlén and Salmén 2003; 2005). Based on these investigations, a structural model of the arrangement of the cell wall polymers and its alterations as a result of chemical pulping was proposed (Fahlén and Salmén 2005). The schematic of these changes taking place during chemical pulping is illustrated in Figure 4.

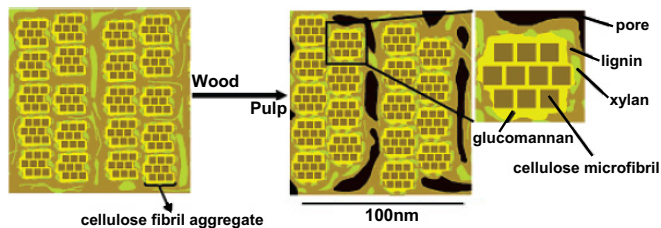


Figure 4. Schematic illustration of a cross-section of part of the S2 layer in spruce wood (left) and the corresponding chemical pulp (right) with a detailed image of cellulose fibril aggregate in the lignin-hemicellulose matrix (Fahlén and Salmén 2005).

It has been demonstrated that water-swollen pores are formed in place of removed lignin (Maloney and Paulupuro 1999). In addition to increased porosity, aggregation of cellulose fibrils during the pulping has been observed (Hult et al. 2001; Fahlén and Salmén 2003). Although these cell wall alterations may not considerably impact the properties of produced paper, they may be of a significant importance when considering the pulp as an intermediate product for alternative cellulose utilization, in which accessibility of cellulose may be very important.

2.1.2 Supramolecular organization of cellulose and accessibility

Cellulose is produced by plants, trees, bacteria and some animals (tunicates) via the condensation polymerisation of glucose. It is a linear homopolymer made of anhydroglucose units (AGU) linked together by β -1,4-glycosidic linkages formed between the carbon atoms of C(1) and C(4) of adjacent glucose units. The β -link requires that the pyranose ring of every second unit along the chain is turned around the axis C(1)-C(4) by 180° (Krässig 1993). Therefore, the actual repeating unit of the cellulose chain is cellobiose. The macromolecular structure of cellulose chain is illustrated in Figure 5.

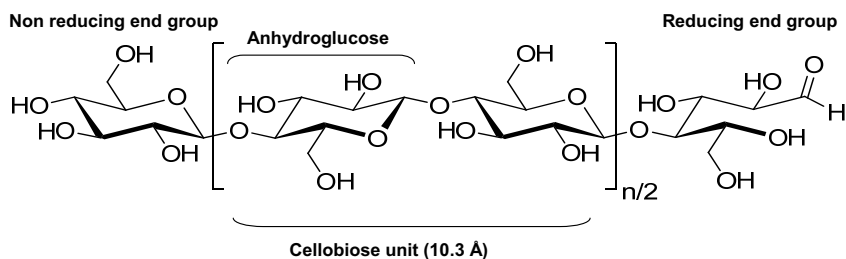


Figure 5. Structure of cellulose. The n in the formula designates the amount of anhydroglucose units linked together.

The cellulose chain molecule has two different termini, often referred to as the reducing and the non-reducing ends. The C(1) hydroxyl group at one end is an aldehyde hydrate group (in equilibrium with its cyclic hemiacetal form) with reducing activity, while the C(4) hydroxyl groups on the other end of the chain are alcoholic and as such non-reducing (Krässig 1993). The importance of this distinction is realised later in the enzymatic hydrolysis section of this thesis.

Depending on its origin, the amount of repeating AGU in the naturally occurring cellulose, commonly known as degree of polymerization (DP), can be approximately 10,000 in wood and up to 15,000 in native cotton cellulose (Goring and Timell 1962). In wood pulp fibers used in papermaking, the DP of cellulose is between 500-2000, depending on the wood source and the pulping and bleaching processes (Alén 2000). Native cellulose, in the cell walls of plants and trees, favors organization of the parallel individual chains into crystalline structures (crystallites or crystalline strands). The three hydroxyl groups of the AGU interact with those of neighbouring AGUs in the same or an adjacent cellulose chain through hydrogen bonding. The linear structure and large number of hydroxyl groups on the cellulose chain enables formation of an extensive intra- and intermolecular network of hydrogen bonding (Marchessaults and Liang 1960). This extensive hydrogen bonding renders native cellulose insoluble in common solvents. The intramolecular hydrogen bonds, O3-H \rightarrow O5 in particular, are partly responsible for cellulose chain stiffness. The intermolecular hydrogen bonds, such as O6-H \rightarrow O3 are basis for the crystalline organisation of native cellulose (Gardner and Blackwell 1974; Nishiyama et al. 2002 and 2003). Cellulose in its native state, in the cell walls of plants and woods occurs in two known crystalline forms - cellulose I α and I β (Atalla and Van der Hart 1984;

Sugiyama et al. 1991). In addition to these forms, cellulose can also adopt other polymorphic crystal structures (Krässig 1993). For example, the cellulose crystal structure is modified after mercerization (a chemical treatment) or when the cellulose is precipitated from suitable solutions. The crystalline allomorphs of cellulose and their formation are described in detail elsewhere (O'Sullivan 1997).

Cellulose is a semicrystalline polymer. The crystalline regions of cellulose are interspersed with less ordered paracrystalline or amorphous areas. These regions were shown to be distributed both on the surface (Larsson et al. 1997), and along the cellulose microfibrils (Nishiyama et al. 2003b). One of the earlier models (Hess et al. 1957) depicting the elementary fibrils and microfibril organization with ordered crystalline and amorphous areas is shown in Figure 6.

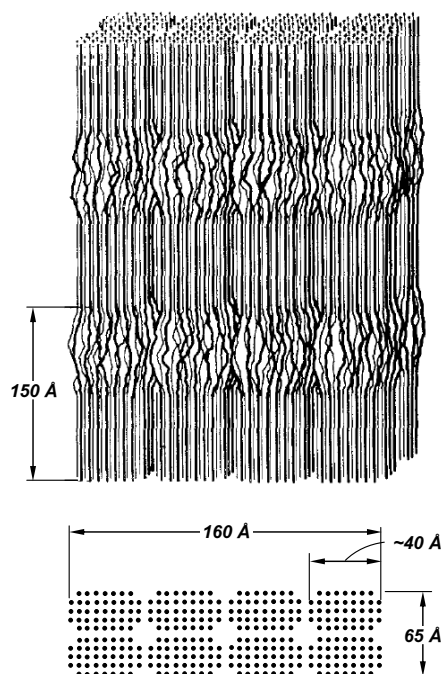


Figure 6. Structure of elementary fibrils and microfibrils according to Hess et al. 1957 (Adopted from Krässig 1993).

Depending on the source, the degree of crystallinity in native cellulose is typically around 50-70% (Hermans and Weidinger 1949) but can be over 90% (Hon 1994). Due to crystallinity, cellulose is very resistant to complete hydrolysis (Gross and Chu 2010). However, the amorphous parts of the cellulose structure are susceptible to acid hydrolysis. This selective susceptibility has been utilised in breaking down the

cellulose structure by strong acids and obtaining individual cellulose crystallites (Rånby 1951; Battista and Smith 1962; Araki et al. 1998). The cellulose crystallites (or cellulose nanocrystals) obtained by sulphuric acid hydrolysis have been studied extensively, in particular their aqueous suspensions that were shown to form chiral nematic liquid crystal phases above a critical concentration (Revol et al. 1992 and 1994; Fleming et al. 2001). The research on CNC preparation, properties, and possible applications has recently been reviewed by several authors (De Souza Lima and Borsali 2004; Samir et al. 2005; Habibi et al. 2010).

In addition to the amorphous parts of the cellulose substrate, surfaces of crystalline areas are also accessible to chemical interactions. Several methods have been utilised to determine the overall cellulose accessibility including water and gas sorption, weight gain by swelling in water or organic solvent, and deuterium exchange (Krässig 1993; Zhang and Lynd 2004). One of the methods commonly used to determine accessibility of cellulose is based on deuteration of cellulose in combination with a pertinent detection technique. Accessible OH groups of cellulose are readily exchanged to OD upon exposure to D₂O, as shown in Figure 7.

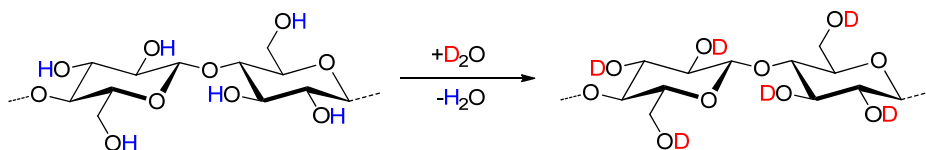


Figure 7. Schematic of deuteration of OH groups on cellulose chain.

The reaction of the hydroxyl groups of cellulose with D₂O was first observed by Bonhoeffer (1934). Utilization of IR spectroscopy for detecting the exchanged OH groups was later described by Rowen and Plyler (1950), followed by a more systematic study by Mann and Merinam (1956). These and additional studies of cellulose deuteration have demonstrated that the rate and extent of OH→OD exchange depend on several factors, including the type and crystallinity of the cellulose sample (Jeffries 1963), temperature, and relative D₂O vapour pressure (Rowen and Plyler 1950; Jeffries 1963), as well as mode (liquid or vapour phase) of deuteration (Mann and Marrinan 1956). Depending on the cellulose type and conditions, the deuterium exchange in accessible regions has been shown to be complete from less than or close to 1 h (Frilette et al. 1948; Jeffries 1964) up to

several hours (Hishikawa et al. 1999). Deuteration coupled with FT-IR spectroscopy has recently been used to measure accessibility and the size of microfibrils of *Valonia* cellulose (Horikawa and Sugiyama 2008). Deuteration at high temperatures (260 °C) led to complete exchange of all OH groups to OD. A similar concept was used to localize I_{α} and I_{β} domains within cellulose microfibrils of *Valonia* and *Cladophora* cellulose (Horikawa and Sugiyama 2009).

The D_2O and other deuterium labelled compounds in combination with near IR spectroscopy were utilized to study the diffusion and accessibility of wood (Tsuchikawa and Siesler 2003a; 2003b). Based on those results, the authors proposed models of the arrangement of elementary fibrils for softwood and hardwood, with the dimensions of microfibrillar areas. An adaptation of the model for softwood, including the areas of D_2O accessibility is shown in Figure 8.

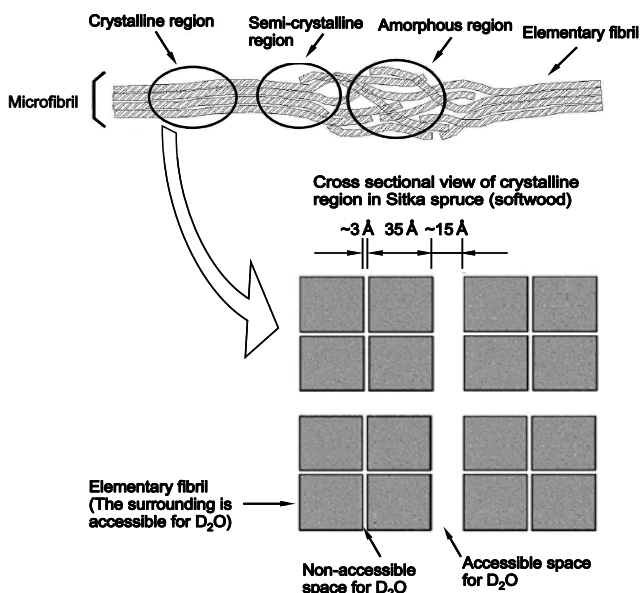


Figure 8. Elementary fibril arrangement in softwood and D_2O accessibility (Adapted from Tsuchikawa and Siesler 2003b).

Although the deuteration of cellulose and cellulose derivatives has been studied extensively over a period of over 80 years, the utilization of deuteration of cellulose within fresh never-dried wood has not been exploited. This was made possible by utilizing FT-IR spectroscopy with photoacoustic detection. The main advantage of this detection technique is that it does not require sample preparation, therefore a fresh

sample can be measured and information on the alteration from its native state is possible. This concept was utilised in our studies of native wood ultrastructure alteration induced by drying described in Paper I and II.

2.2 Drying of wood and wood pulp fibers

The water content of fresh wood and never dried pulp is comprised of free and bound water (Berthold et al. 1994). Free water includes water in cell voids and pores between fibers and in the lumens of fibers. Bound water, i.e. cell wall water, is further divided into freezing bound water in the pores of fiber wall and non-freezing bound water that is chemically bonded to the hydroxylic and carboxylic acid groups in fiber. Two structural components of wood fiber, cellulose and hemicellulose, are mainly responsible for absorption of water. Crystalline regions of cellulose are not accessible to water; however, surfaces of these cellulose crystallites provide additional sites available for water absorption. In contrast, mainly hydrophobic lignin decreases the permeation of water in the fiber wall and restricts swelling of the fiber. In addition, swelling of the fibers is also restricted due to structural constraints imposed by the cell wall arrangement.

Trees grow in water-swollen conditions, and once a tree is felled, the wood quickly undergoes clearly perceptible changes in its texture. These changes are mostly associated with water escape from wood structure. A number of studies have previously demonstrated that this loss of water has a direct impact on the mechanical properties of wood (Gerhards 1982; Hillis 1984; Kelley et al. 1987; Kifetew et al. 1998).

When considered as a raw material for production of other materials, alterations of wood structure associated with drying may have unfavourable consequences, for instance in wood or wood pulp bioconversion processes. It has been shown that in addition to chemical components and their interactions, physical features of wood and cellulosic biomass ultrastructure can affect the efficiency of enzymatic hydrolysis of the carbohydrates (Nazhad et al. 1995; Mooney et al. 1998; Mansfield et al. 1999; Zhu et al. 2008).

2.2.1 Ultrastructural changes of wood

It has been suggested that the drying of wood can lead to irreversible damage of the cell wall on the ultrastructural level. Analysis of the fracture surface by SEM after applying a tensile load showed differences in the fracture mechanisms between green and rewetted dried samples (Kifetew et al. 1998). A significant difference in the distribution of silver particles in the cell walls of green and dried wood was observed by TEM (Wallström and Lindberg 2000). Authors suggested that the inhomogeneous distribution in the dried wood sample was a result of damage in the cell wall during drying. A reduction of tensile strength of a wood sample impregnated with a bulking compound (glycerol) after drying compared to the wood impregnated in the green state was also observed (Thuvander et al. 2001). In a similar study, while studying penetration of bulking chemicals (for dimensional stability) into the cell wall of wood fibers, formation of microcracks as a result of the initial drying of wood was reported (Wallström and Lindberg 1999). Recently, *in situ* formation of microcracks occurring during the drying of wood was visualized by confocal laser scanning microscopy (Sakagami et al. 2009).

Assessing the actual changes in native wood ultrastructure during the initial drying is a challenging task. These alterations have mostly been studied by microscopic methods. The main difficulty associated with these methods is the requirement of sample preparation. During preparation, water in the sample is often removed or replaced with a suitable organic solvent. This removal or exchange can alter the original structure of the cell wall, which therefore may not be entirely representative of the wood in its native swollen hydrated state. In addition, artefacts may be introduced during the sample preparation by microtoming. For studying changes of wood ultrastructure of green wood, it would be beneficial to utilize methods that do not alter the structure of native wood. Deuterium exchange coupled with FT-IR spectroscopy, utilised in our investigation described in Paper I and II, fulfils this requirement.

2.2.2 Changes in ultrastructure and properties of pulp fiber

The properties of pulp fibers are significantly altered after the fibers are dried. A decrease in reactivity and diminished bonding properties of recycled fibers are the most notable alterations. In general, once dried, the pulp fiber cannot regain its original swelling characteristic, and that consequently impacts other fiber properties and the strength of paper produced from recycled (dried) fibers (Higgins and McKenzie 1963; Nazhad and Paszner 1994). The decrease in strength of the dried fiber has been attributed to structural changes in the cell wall due to irreversible bonding of cellulose surfaces. On dehydration, the molecules in the less ordered and highly swollen surface areas of the fibrils form new hydrogen bonds, removing the space between the fibrils. This aggregation of fibrils reduces the internal surface that is accessible to water (Krässig 1993). The phenomenon encompassing these irreversible structural changes in pulp fibers occurring after drying has been termed hornification, and was initially demonstrated as a decrease of water retention value (WRV) of dried pulp (Jayme 1944). Since then, hornification has been studied extensively using a variety of methods and these efforts were summarized and reviewed in several papers (Laivins and Scallan 1993; Minor 1994; Young 1994; Kato and Cameron 1999; Fernandes Diniz 2004). The proposed causes and mechanisms of hornification include closure of capillaries, collapse of pores between cellulose microfibrils and an increased degree of crosslinking within the fiber cell wall, tighter packing of cellulose chains and fibril aggregation. It has been demonstrated that hornification occurs once the water content in the fibres decreases below the fiber saturation point (FSP – water is contained in the cell wall of fiber only), and that the actual water removal, not the temperature of drying, is the determining factor in hornification (Laivins and Scallan 1993; Kato and Cameron 1999).

Hornification has been mainly described as a feature of low yield pulps. The properties of dried mechanical (high yield) pulps are affected to a much lesser extent. In contrast to low yield pulps, the presence of lignin and hemicellulose between the microfibrils is likely to prevent the internal cross-linking and aggregation of cellulose fibrils. Recently, the role of hemicellulose in hornification and the adsorption of hemicelluloses on cellulose to inhibit hornification has been studied (Oksanen et al. 1997; Duchesne et al. 2001; Köhnke et al. 2010).

These extensive studies of hornification have helped to better understand the mechanism, its implications on fiber properties and consequently to find means of preventing, limiting or compensating for its impacts. Recent interest in hornification is associated with the current extensive research on the enzymatic degradation of cellulose and on the preparation and characterization of nanocellulosic materials, including microfibrillated cellulose (MFC), nanofibrillated cellulose (NFC) and cellulose nanocrystals (CNC). It has been demonstrated that drying of cellulose fibers has a significant impact on adsorption of cellulases (Gerber et al. 1997). Drying of fibers also resulted in an increase in the fibril aggregate dimensions within chemical pulps (Hult et al. 2001). Although the aggregation of cellulose fibrils can be controlled or prevented by surface modification of isolated fibrils (Araki et al. 2001; Gousse et al. 2004; Andresen et al. 2006; Saito et al. 2006; Stenstad et al. 2008), little can be done if irreversible aggregation occurs prior to fibrillation.

Fibrillated wood pulps from dried pulps showed coalescence of microfibrils compared to fibrils produced from never-dried pulps (Iwamoto et al. 2008). Length distribution analysis of a cellulose nanocrystal suspension prepared from never-dried pulp showed a higher number of longer crystals compared to CNC isolated from dried pulp (Kontturi and Vuorinen 2009). Reportedly, cellulose microfibrils form lamellar structures in the cell wall already during biosynthesis and it can be expected that the microfibrils within the lamellae are so close to each other that they can aggregate upon drying. This hypothesis correlates with the findings of Elazzouzi-Hafraoui et al. (2008) who observed aggregated cellulose crystallites even after severe acid hydrolysis and sonication treatments. The authors suggested that the biological origin promotes lateral adhesion of the cellulose microfibrils. Aggregation could also be the reason on why mechanically separated nanofibrillar cellulose has larger dimensions than the native microfibril even in the case when wood, not pulp, is used as a starting material.

2.3 Enzymatic degradation of cellulose

The concept of cellulose degradation by cellulolytic enzymes was first proposed by Reese in 1950 (Reese et al. 1950). Since then, the enzymatic degradation of cellulose has been studied extensively. The initial studies were mainly aimed to better understand the fundamental mechanism of degradation. Later, the objective of investigations focused on the actual transformation of cellulose into soluble and fermentable sugars and thus a source of renewable energy and chemicals. Mild and selective modification of cellulosic fibers was another area of cellulase application that was investigated, and to some extent implemented in textile as well as in the paper manufacturing industry.

At the turn of the century the interest in the enzymatic hydrolysis of cellulose increased dramatically. This renewed attention was a result of increased awareness of the critically declining resources of fossil fuels and an urgent need for finding alternative and sustainable sources of energy. Lignocellulosic biomass, an abundant, renewable, natural source of cellulose, has been identified as the only foreseeable sustainable source of fuels and materials (Lynd et al. 1999). The appeal of cellulase-assisted hydrolysis of cellulose was further enhanced by recent advances in the mechanistic understanding of cellulase action as well as by progress in the production of the enzymes. The cost of cellulase, or cost of the actual hydrolysis, has been identified as one of the main determinants in the overall economics of the process; therefore, more efficient cellulase production at lower cost is essential for commercial feasibility of the process. In general, a better understanding of the cellulose hydrolysis is crucial for designing efficient and cost competitive bioconversion of biomass, justifying the ongoing research in the area.

2.3.1 Cellulases: classes, structure and mechanisms of hydrolysis

Cellulases belong to a glycoside hydrolase family of enzymes which hydrolyse the β -1,4-glycosidic linkages in cellulose. The name cellulase refers to a system of several different enzymes that, based on their mode of action, are divided into three main classes. These include (i) cellobiohydrolases (CBH, 1,4- β -D-glucan cellobiohydrolase, EC 3.2.1.91) that cleave cellobiose from the cellulose chain

termini; (ii) endoglucanases (EG, 1,4- β -D-glucan glucohydrolase, EC 3.2.1.4) that randomly cleave internal bonds within the chain and (iii) β -glucosidase (EC 3.2.1.21), which hydrolyzes cellobiose and short cello-oligosaccharides. The majority of the investigations involved the cellulase system of filamentous fungus *Trichoderma reesei*, which is a very efficient producer of a potent set of cellulolytic enzymes. Its cellulase system has been found to be one of most effective in hydrolysis of cellulosic materials. The *T. reesei* fungus produces two distinct cellobiohydrolases (CBH I and II), at least five different endoglucanases (EG I-V) and one β -glucosidase (Knowles et al. 1987; Rabinovich et al. 2002). The individual components work synergistically and a cellulase system containing all three enzymes can efficiently degrade cellulose. It is generally accepted that cellulose degradation by cellulases is initiated by endoglucanase randomly cleaving internal glucosidic bonds along the length of the cellulose chain in the amorphous regions, decreasing the DP of the substrate in the process. The newly created chain ends then become available sites for cellobiohydrolase action. Cellobiohydrolases are processive enzymes that initiate their action from the ends of the cellulose chains, producing primarily cellobiose that is then released to solution (Teeri 1997). They attack the crystalline parts of the substrate and decrease the DP of the substrate, albeit slowly. The discrimination between CBH I and II is necessary due to the preference in cellulose chain end on which they act. The CBH I acts on the reducing end of the cellulose chain while CBH II operates on the non-reducing end of the chain (Vrsanska and Biely 1992). The hydrolysis of cellulose into the glucose is completed by β -glucosidase, which splits cellobiose into glucose monomers. A schematic of cellulase action is shown in Figure 9.

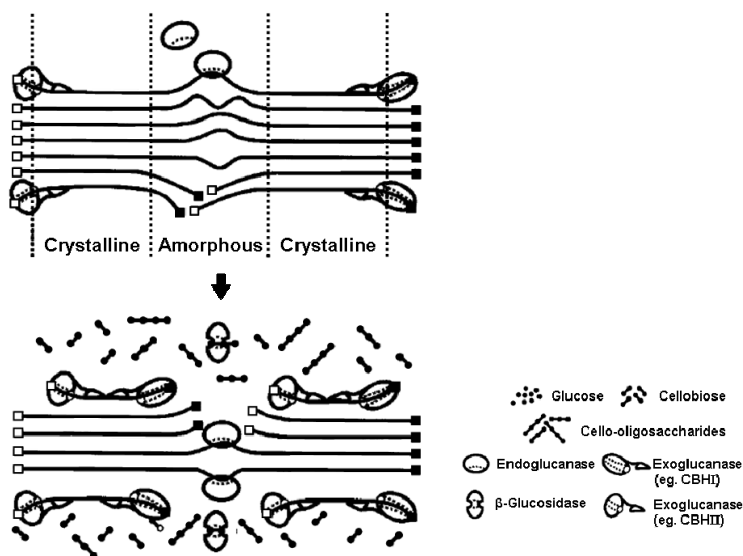


Figure 9. Schematic of cellulose hydrolysis by a cellulase system. The solid and open squares represent the reducing and non-reducing ends of cellulose chain. Cellulose, enzymes, and hydrolytic products are not shown to scale (Lynd et al. 2002).

Cellobiohydrolase I (CBH I) is the most abundant enzyme produced by *T. reesei*, making up 60% of the total cellulolytic protein and thus accounting for majority of its cellulolytic activity (Teeri 1997). The structure of isolated CBH I and its mechanism of action have been thoroughly investigated. The CBH I and other *T. reesei* cellulases consist of two distinct domains – a cellulose binding modulus (CBM) and an active catalytic core. These two domains are linked together by a flexible linker peptide (Tomme et al. 1988). The CBM does not possess hydrolytic activity and has no effect on the hydrolysis of soluble substrates. Its role is mainly the adsorption of the active catalytic core onto an insoluble substrate and it is essential for enzymatic activity on crystalline cellulose. The structure, studied by multidimensional NMR, was shown to be wedge shaped with one of the two sides being very flat (Kraulis et al. 1989). The flat side (face) is composed of three aromatic residues (tyrosines) that are approximately 1.04 nm apart from each other, which coincides with the size of glucose unit and suggests that the flat size would bind to cellulose through these residues (Henrissat 1994). The catalytic core is responsible for the actual hydrolysis of the substrate, and its structure has been shown to be in the shape of a tunnel,

approximately 50 Å long (Divne et al. 1994). A model of the CBH catalytic core and comparison with the core of EG is shown in Figure 10.

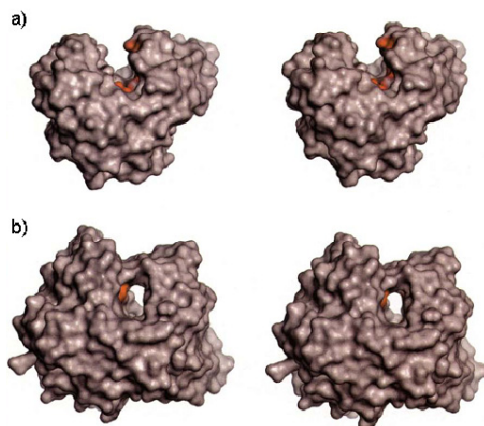


Figure 10. Active sites found in glycosyl hydrolases. (a) The cleft (endoglucanase E2 from *T. fusca*) and (b) the tunnel (cellobiohydrolase II from *T. reesei*). The proposed catalytic residues are shaded in red (Lynd et al. 2002).

This tunnel (Figure 10b) can accommodate a single glucan chain binding up to 10 glucose units. The chain enters the tunnel from one end, then threads through the entire length of the tunnel. During this time the chain bonds are cleaved and subsequently cellobiose is released from the other end of the tunnel. This topology allows the CBH to release cellobiose while remaining firmly bound to the polysaccharide chain, thus creating the conditions for processivity, which is probably a key factor for the efficient enzymatic degradation of insoluble crystalline cellulose (Davies and Henrissat 1995). The schematic of CBH I structure and action is shown in Figure 11.

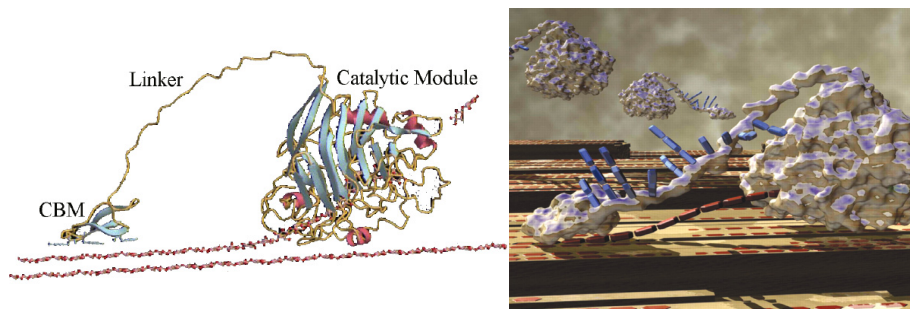


Figure 11. Depiction of the mechanism of exoglucanase (*T. reesei* CBH I) acting on crystalline cellulose. The carbohydrate-binding module (CBM) binds to the cellulose surface, isolating a single chain of cellulose that is then directed into the active-site tunnel of the catalytic module (Teeri and Henriksson 2009). To the right is an artistic portrayal of the CBH action (Himmel et al. 2007).

The structure of the endoglucanase catalytic core is slightly different. The more open active site structure, in form of a cleft (see Fig 10), allows random binding of several sugar units in polymeric substrates (Rouvinen et al. 1990; Divne et al. 1994). Because of this open active site, the endoglucanases can access internal glycosidic bonds along the cellulose chains.

2.3.2 Modification of pulp fiber properties

Early attempts to utilize enzymes in the pulp and paper industry date back to the mid 1980s. Interest in the use of enzymes at that time was stimulated by the need of the industry to adopt environmentally benign technologies in pulp production and processing. Later, a better understanding of the mechanism of action, the environmentally agreeable nature of the technology and the promise of more efficient production of commercial enzymes at lower cost were the main drivers of the extensive investigative efforts in the final decade of last century. The status of enzyme applications in the pulp and paper industry at that time was reviewed by several authors (Buchert et al. 1998; Bajpai 1999; Wong and Mansfield 1999). The research on the applicability of cellulase systems specifically was summarized by Viikari and co-workers (2000). The potential benefits of cellulase treatment for pulp fibers were identified in the reduction of refining energy demands for mechanical pulps, in

modifications of chemical fiber properties, in enhanced de-inking of recycled fibers, and in process water treatment. Although many of these enzymatic applications that have been proposed showed potential, only a few found their way to a successful commercial implementation.

Acting on the pulp fiber cellulose, cellulases have traditionally been considered detrimental to pulp and paper properties, particularly yield and strength properties. However, limited hydrolysis of fiber carbohydrates can also have positive effects. The extent of the hydrolytic action can be controlled by adjusting the treatment conditions (e.g. dosage, treatment time), or by eliminating the synergism of the cellulase components. As described earlier, cellulase components act synergistically and complete hydrolysis of cellulose requires the presence of both endo- and exocellulases (Henrissat 1994; Medve et al. 1994). The use of monocomponent cellulases or defined cellulase mixtures has been shown to have potential in pulp and paper applications. The concept of using individual components of cellulase was initially evaluated by Pere et al. (1995). The individual components exhibited significant differences in their mode of action and the ensuing effects on pulp properties. The cellobiohydrolases (CBH) had only little impact on pulp viscosity and a limited impact on strength properties. In contrast, the endoglucanases (EG), and particularly EG II dramatically decreased pulp viscosity and consequently the strength properties after refining. It has been proposed that CBH treatment did not cause any structural damage to the fibres, while EG, and EG II in particular, acted on the amorphous parts of the fibers where even a low level of hydrolysis resulted in a significant reduction of the strength properties of treated fibers.

In mechanical pulping, treatment with *T. reesei* CBH I was found to reduce the energy demand in secondary refining without affecting pulp quality (Pere et al. 1996). For chemical pulps, cellulases and cellulase-hemicellulase mixtures were reported to enhance the beatability of the coarse fibres and thus to improve paper properties (Mansfield et al. 1996). The treatment of bleached kraft pulp with *T. reesei* endoglucanases EG I and EG II considerably enhanced the beatability of the pulps (Oksanen et al. 1997b). The benefits obtained by the cellulase treatment however, were compromised by impaired strength properties of the treated pulps, attributed to EG attack on the amorphous part of cellulose in the defects and irregular zones of the fibres. Similar findings were observed in the treatment of recycled fibers with isolated cellulases and their mixtures (Stork et al. 1995). It was shown that the action of EG

was essential for the improvement of drainage characteristics of the pulps; however, a negative impact on strength properties was observed for recycled kraft pulp fibers. Other efforts were concisely summarized by Buchert et al. (1998). In general, treatments of pulp fibers with individual cellulases resulted in different effects on the fiber properties. The CBH treatment was shown to have only a moderate or slight effect on technical properties and viscosity. In contrast, EG treatment improved some properties of the pulps; however a noticeable decrease in viscosity and loss in pulp strength often accompanied these improvements.

The majority of previous efforts on cellulase treatment of pulp fibers have been conducted using pulp fibers of softwood species. When compared to softwoods, the structure of hardwoods is more heterogeneous due to the diversity of cell types. This complexity has limited the utilization of hardwoods fibers in fundamental studies. Although information on cellulase treatment of hardwood pulp fiber is available (Kamaya 1996; Rahkamo et al. 1996 and 1998), the overall extent of the research is somewhat limited. The growing demand for new raw materials coupled with extensive resources of hardwoods available for production of pulp resulted in an increased interest in hardwood fibers, and initiated further research on their modification with cellulases (Köpcke et al. 2008; Ibarra et al. 2010). The objective of the study described in Paper V was to evaluate the effect of a monocomponent commercial cellobiohydrolase treatment on fiber strength and the accompanying morphological changes of bleached hardwood kraft pulp.

2.3.3 Cellulose model films and enzymatic hydrolysis studies

A cellulose model film can be described as an ultrathin layer of cellulose deposited on a smooth flat surface. Recent intensive efforts in the preparation of cellulose thin films coincide with technological advances and increased utilization of surface sensitive analytical techniques. Even coverage, smoothness, defined composition and morphology are the main attributes that allow utilization of model films in fundamental studies of the interactions of cellulosic material with dissolved or dispersed matter. Obtaining this kind of information using native cellulosic fibers is often challenging due to their complex surface morphology and heterogeneous

composition. There are two established techniques that have been implemented in preparation of model films. They are: Langmuir–Blodgett deposition (Schaub et al. 1993) and spin-coating of cellulose or cellulose derivative solutions (Gunnars et al. 2002; Kontturi et al. 2003; 2003b). Both techniques require solubilisation of cellulose either using direct cellulose solvents, preparation of cellulose derivatives that can be dissolved in non-polar solvent and subsequently regenerated to cellulose, or alternatively, using fine dispersions of cellulose nanoparticles. Direct dissolution of cellulose can be achieved using solvents such as dimethylacetamide with lithium chloride (DMAc-LiCl) or N-methylmorpholine-N-oxide (NMMO). Trimethylsilyl cellulose (TMSC) is an example of the derivatization of cellulose, rendering it soluble in common non-polar solvents, such as chloroform or toluene. Prepared films of TMSC can be readily regenerated to cellulose by acid hydrolysis (HCl) in vapour phase. Model films prepared by both dissolution and by derivatization produce films of regenerated cellulose II with different degrees of ordering (Kontturi et al. 2003b; Fält et al. 2004; Aulin et al. 2009). Smooth model films from suspensions of cellulose nanocrystals (CNC) can also be prepared using either the LB deposition (Habibi et al. 2007) or the spin-coating techniques (Edgar and Gray 2003). Although these films have the same crystallinity (cellulose I) as that of the native cellulose fibers, they do not consist of pure cellulose; they have sulphate groups on the crystal surface. Recently, other approaches for the preparation of cellulose model films have been described. Cellulose dissolved in NMMO was derivatized to cellulose-thiosemicarbazone, which was used to construct a cellulose I nanolayer with a parallel chain alignment via self-assembly on a gold substrate (Yokota et al. 2007). Model films of cellulose nanofibrils deposited on a silica substrate were also introduced recently (Ahola et al. 2008). The deposited cellulose nanofibrils contain both crystalline and amorphous regions as well as hemicellulose, making the characteristics of this model substrate closest to a native cellulosic fiber. More detailed information on the development, characterization and application of cellulose model films is included in reviews by Kontturi et al. (2006), Kontturi and Österberg (2009) and Roman (2009).

Cellulose films have been widely utilised in fundamental studies of adsorption on cellulose (Kontturi et al. 2006). They are also ideal model substrates for studying the enzymatic degradation of cellulose. For instance, degradation of model cellulose films by cellulase has been investigated by ellipsometry (Eriksson et al. 2005). More

recently, the quartz crystal microbalance with dissipation monitoring (QCM-D) technique has been applied to the study of the enzymatic degradation of cellulose films. QCM-D is a relatively new method that allows in situ monitoring of adsorption, swelling, degradation, and viscoelastic changes in thin model surfaces. A more detailed description of the method is included in the experimental section of this thesis. In general, the method utilises the piezoelectric properties of the quartz crystal on which the substrate is deposited in an ultrathin layer (such as cellulose model films). The change in oscillation frequency and dissipation of energy of the crystal indicate changes in the film characteristics, such as swelling of the films (Fält et al. 2003; Tammelin et al. 2006).

The action of cellulase enzymes consists of two main stages: adsorption (binding) and degradation (hydrolysis). Since the QCM-D allows monitoring of both of these steps, its utilization can provide further information on mechanism and kinetics of the cellulase action. The action of individual cellulases (Josefsson et al. 2008) as well as a commercial cellulase mixture (Turon et al. 2008) on cellulose films prepared from NMMO solution showed different behaviours endo- and exo-glucanases, as well as rapid hydrolysis by the cellulase mixture. The impact of the commercial cellulase mixture on a film of native cellulose nanofibrils containing both crystalline (cellulose I) and amorphous domains was compared with that observed for other model films of cellulose from regenerated cellulose as well as cellulose nanocrystal cast films (Ahola et al. 2008b). The method was also used in evaluations of the treatment conditions, including enzyme concentration, temperature and pH (Jeong et al. 2005) and crystallinity (Ahola et al. 2008b). In our investigation, we used a cellulose model film of different characteristics to that used in a majority of previous studies and focused on the action of pure individual cellulases. The main objective was to assess the impact of the initial amount of substrate on the rate of hydrolysis, and attempted a direct, complementary quantification of the hydrolysis.

3 EXPERIMENTAL

This chapter describes the materials and the methods used in the experiments pertinent to this thesis, including the general background on the main methods and an overview of the experimental design. More detailed experimental descriptions can be found in Papers I-V.

3.1 Materials

3.1.1 Wood and pulps

Wood samples for experiments in Paper I were freshly felled pine (*Pinus sylvestris*) and spruce (*Picea abies*). The experiments described in Paper II were carried out using fresh pine (*Pinus sylvestris*) only. All wood samples were from Eastern Finland. The samples were supplied in the form of discs 7-10 cm thick and 20-40 cm in diameter. In the initial testing (Paper I), the discs were cut with a saw to form a rectangle from which slivers of ~1 mm thickness were sliced with a chisel. Using a sharp knife, small squares of approximately 5 × 5 mm were cut from the slivers. In the subsequent testing (Paper II), the specimens were sampled from the fresh wood discs using a Suunto increment borer (Suunto, Finland). The cores (5.15 mm in diameter) were then sliced into 0.5-1 mm thick discs by using a sharp knife. The sampling of the wood specimen is depicted in Figure 12.

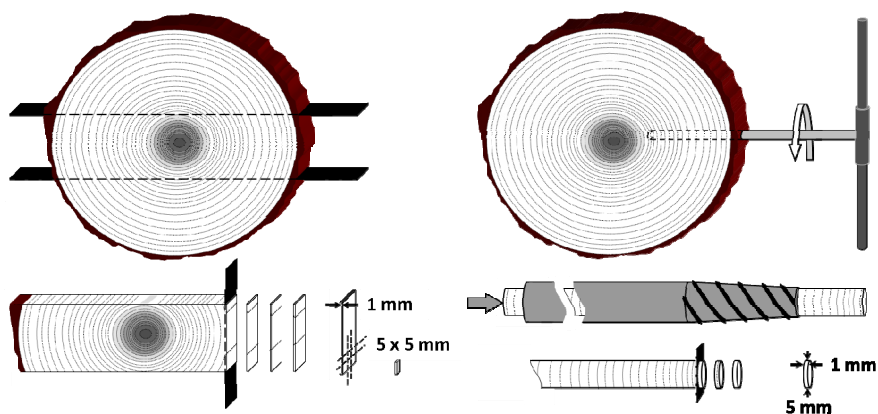


Figure 12. Schematic of wood sampling. Dimensions are not to scale.

The initial experiments (Paper I) were carried out using a commercially produced never-dried bleached (lignin-free) kraft pulp sample. The pulp was made from spruce and pine wood (approximately 60% spruce and 40% pine, according to the manufacturer). Experiments described in Paper II were carried out using commercially produced softwood groundwood and chemical (kraft) pulp samples. The kraft pulps were made from spruce and pine wood (approximately 60% spruce and 40% pine). The collected samples were washed and centrifuged to approximately 30% solid content before shipping. The kappa numbers (an approximation of lignin content) of the kraft pulps, as determined by ISO standard 302:2004, were 23.1 for unbleached sample and 0.7 for fully bleached sample.

The evaluation of enzymatic treatment on fiber properties described in Paper V was carried out using a commercially produced, never-dried ECF bleached birch (*Betula pendula*) kraft pulp sample.

3.1.2 Chemicals and enzymes

Deuterium oxide (99.9 atom % D, Sigma-Aldrich) was used for deuteration and relative humidity control in experiments described in Papers I and II. The salts used for humidity control were NaCl (99.5%, J.T. Baker) and NaOH (p.a. Merck).

The conductivity and pH of the pulp slurry prior to enzymatic treatments (Paper III) were adjusted with 0.1 M MgSO₄ (p.a. Merck) and 0.1 M NaOH (Oy FF chemicals, CAS 1310-73-2), respectively. The pulp was treated with a commercial cellobiohydrolase Ecopulp®Energy (AB Enzymes, Finland). The enzyme is a family Cel7A cellobiohydrolase from a thermophilic ascomycetous fungus *Thermoascus aurantiacus* (Viikari et al. 2007).

The enzymes used in enzymatic degradation of amorphous cellulose films were EG I and CBH I from *Trichoderma reesei*. EG I was purified from culture filtrates lacking CBH I and CBH II as described in by Suurnäkki et al. (2000). Briefly, culture supernatant was desalted on a Sephadex G-25 coarse column (GE) equilibrated with 6 mM sodium phosphate buffer, pH 7.0 and then loaded on a DEAE Sepharose FF (GE). EG I was eluted using a NaCl gradient and then loaded on a Phenyl Sepharose FF (GE) column equilibrated with 25 mM sodium phosphate at pH 6.0 with 0.4 M (NH₄)₂SO₄. EG I was eluted by a decreasing gradient of 20 mM pH 6.0. EG I containing fractions were further concentrated by ultrafiltration with 0.025 sodium acetate, pH 4.5, and applied to a DEAE Sepharose FF column, and eluted by a NaCl gradient. Finally the protein was desalted and concentrated by ultrafiltration.

CBH I was purified from culture filtrate devoid of EG I and EG II as described by Suurnäkki et al. (2000). Briefly, concentrated culture was desalted on Sephadex G-25 using 10 mM sodium phosphate buffer, pH 7.0 and applied on DEAE Sepharose FF. Elution was with a NaCl gradient. The CBH I pool was then applied on Phenyl Sepharose FF equilibrated with 20 mM sodium acetate pH 5.0 containing 0.4 M (NH₄)₂SO₄. Elution was performed with a decreasing gradient of (NH₄)₂SO₄ 20 mM sodium acetate. The protein was finally desalted and concentrated by ultrafiltration.

3.2 Methods

3.2.1 Deuteration and controlled drying experiments

The deuteration of wood samples was carried out in 10 mL glass vials by immersing the specimens in an excess of deuterium oxide (D₂O) for 60 min. The pulp samples were deuterated in plastic bags for a period of 2 × 20 min. Excess D₂O was added to

the pulp and then the slurry was mixed by kneading. After 20 min, the free D₂O was squeezed out of the bag before fresh D₂O was added and the slurry was mixed again. After deuteration, the samples were dried under different conditions and then flushed with an excess of water for an identical period of time as the deuteration process (2 × 20 and 60 min for pulp and wood, respectively). A schematic of the experiments is shown in Figure 13.

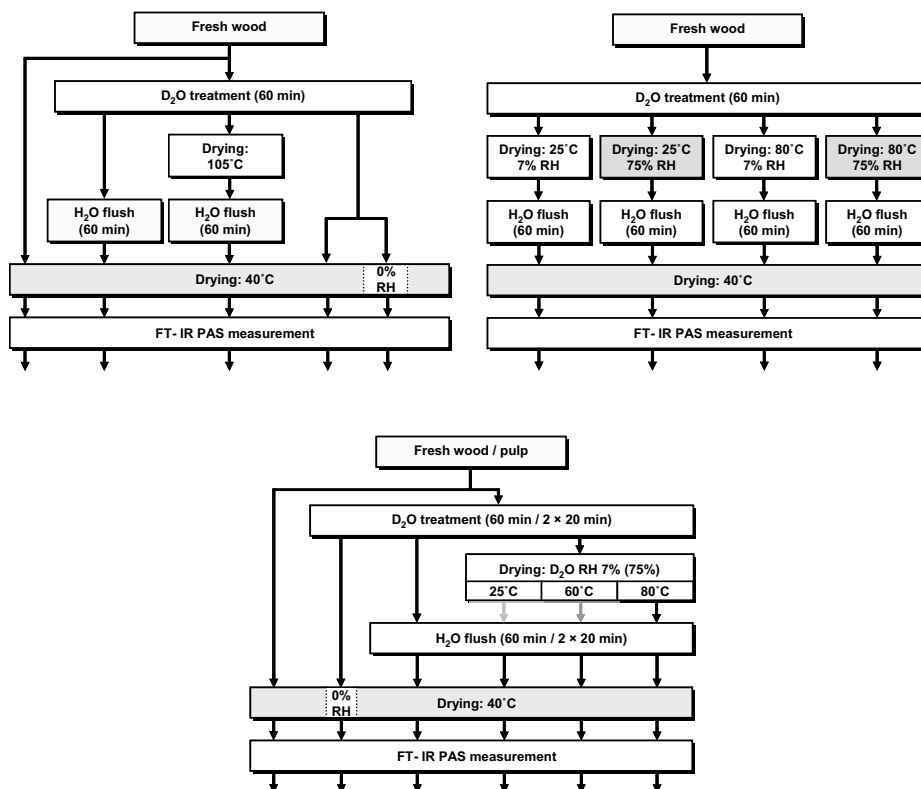


Figure 13. Schematic of deuteration (top left) and controlled drying (top right) of wood; wood and pulp evaluation in Paper II (bottom).

Both deuteration and flushing were carried out at room temperature. All samples were then dried in a convection oven at 40 °C prior to measurement with the FT-IR spectrometer. Drying at controlled D₂O relative humidity was carried out in vacuum desiccators. The samples were held in perforated aluminum containers and placed on the porcelain plate of desiccators containing D₂O saturated solutions at the bottom. The desiccators were then evacuated and placed into an oven (25, 60, and 80 °C) for conditioning (7 days).

3.2.2 FT-IR spectroscopy

Infrared (IR) radiation refers to the part of electromagnetic spectrum that extends from the red end of the visible light to the microwave range. The region covers wavelengths between 0.78 μm and 1 mm, however the most useful for organic chemistry is the mid-IR region between 2.5 - 25 μm , or when expressed in wavenumbers ($1 / \text{wavelength in cm}$), between 4000 - 400 cm^{-1} . Practically all common functional groups in organic compounds absorb IR radiation in this region.

IR spectroscopy is a vibrational spectroscopy in which an organic molecule is exposed to incident IR radiation. The radiation is absorbed by the molecule and converted into the energy of molecular vibration. The absorption occurs when the radiant energy matches the energy of a specific molecular vibration. A molecule gives a signal in IR spectroscopy only if there is a change in a dipole moment during a vibration. There are two types of molecular vibrations, stretching (stretching and compressing) and bending (oscillation of bond angles). Stretching vibrations can be symmetrical or asymmetrical, and bending can be in plane or out of plane. Complex molecules, such as the polymers in wood, have a large number of vibrational modes. A schematic of the vibrational modes of the cellulose hydrogen bond network with the wavenumber bands assigned in the IR spectrum is shown in Figure 14.

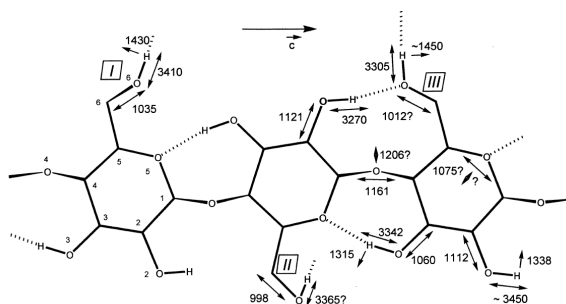


Figure 14. Vibrations and assignment of corresponding IR bands for the hydrogen bond network of I_{β} cellulose in Valonia. Stretching bands are depicted as two headed arrows and bending bands of alcoholic groups as single headed arrows (Marechal and Chanzy 2000).

An IR spectrum describes the amount of absorbed energy at a given wavenumber for the whole range of IR radiation applied (usually $4000\text{-}400\text{ cm}^{-1}$). The absorbed amount (band intensity) is expressed either as transmittance or absorbance. The wavenumber region between $4000\text{-}1500\text{ cm}^{-1}$ in the IR spectrum is referred to as a functional group area. Bands in this region can be assigned to individual bonds or functional groups of the molecules. The spectral region below 1500 cm^{-1} is referred to as fingerprint region ($1500\text{-}400\text{ cm}^{-1}$) and the IR bands in this region are often utilised to confirm the identity of the compound. An example of an IR spectrum measured for spruce holocellulose with an overview of the band assignments and characteristic absorption maxima from the different wood polysaccharides is shown in Figure 15.

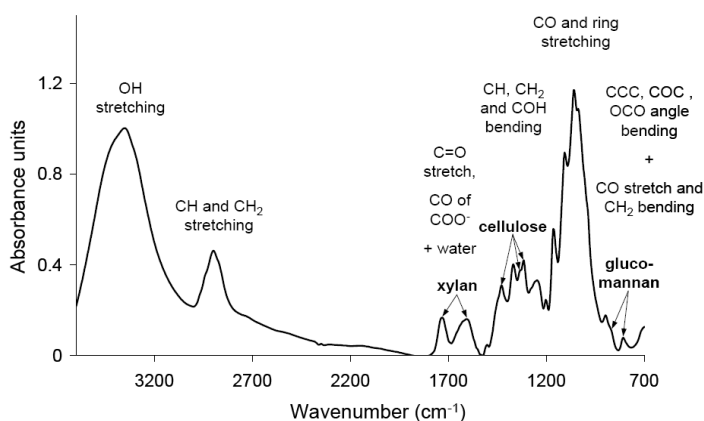


Figure 15. FT-IR spectrum of spruce holocellulose including band assignments and characteristic absorption maxima for the polysaccharides in wood pulps (Åkerholm 2003).

Modern instruments for IR spectroscopy are fitted with Fourier transform (FT) spectrometers that allow rapid analysis of the samples. FT-IR spectroscopy is based on an interferometric measurement, making use of the entire source spectrum with all wavelengths recorded simultaneously. The advantages of FT in IR include a better signal-to-noise ratio, reduced scan time, higher energy throughout, superior spectral resolution, better wavelength accuracy, and ease of sample handling without sample destruction during the measurement.

Photoacoustic spectroscopy (PAS) has become a valuable analytical tool with the following developments in FT-IR: low noise electronics, high sensitivity

microphones, and computerized data handling. In PAS, a sample sealed in a cell is purged with helium and then illuminated with IR radiation with the intensity modulated at a frequency in the acoustic range. The absorbed radiation is converted to heat and the thermal diffusion from within the sample causing a pressure oscillation of helium in the cell. These pressure waves (sound) are then detected using a microphone of high sensitivity, transformed into a corresponding electrical signal that generates a spectrum. The main advantages of FT-IR PAS are the possibility to analyse different types of materials, simple sample preparation, nondestructive, noncontact measurement and depth profiling capability.

FT-IR PAS measurements. The spectra shown in Paper I and II were collected using a Bio-Rad FTS 6000 spectrometer (Cambridge, MA) with a MTEC 300 photoacoustic cell (Ames, IA) at a constant mirror velocity of 5 kHz, 1.2 kHz filter, and 8 cm⁻¹ resolution. A background spectrum with standard carbon black was measured at the beginning of each set of measurements. After collecting the background spectrum, the wood or pulp sample was put into the sample holder that was then placed into the PA detection cell which was purged with helium gas for 5 min. Then the cell was sealed and the actual spectrum of the sample was recorded. A minimum of 400 scans per spectrum were collected and processed using the Win-IR Pro 3.4 software (Digilab, Randolph, MA). Each spectrum was normalized to have the same value at 1200 cm⁻¹. Each spectrum is an average of at least four measurements.

3.2.3 Enzymatic treatment and analysis of released sugars

The enzymatic treatments were carried out with CBH dosages of 100, 300 and 900 g of protein per ton of pulp (oven dry basis) at 70 °C, pH 6.0, 4% consistency and 60 minutes retention time. The conductivity of the pulp slurry was adjusted to 70 mS m⁻¹ prior to the treatments. Released carbohydrates in the filtrates were analyzed after pH adjustment and acid hydrolysis with 72% sulphuric acid performed according to Hausalo (1995). The monosaccharides were separated using an anion exchange column (Dionex CarboPac PA10, width 4 mm, length 250 mm) and quantified by pulsed amperometric detection (HPAEC-PAD) with a Dionex DX-500 (Dionex Corporation, Sunnyvale, CA) liquid chromatograph.

3.2.4 Preparation of cellulose model surfaces and enzymatic hydrolysis

Trimethylsilyl cellulose (TMSC) was synthesized from cellulose from spruce for column chromatography (Fluka) as described in detail by Kontturi et al. (2003). Ultrathin films of different thickness were prepared by spin coating from TMSC solutions of different concentrations. TMSC was dissolved into toluene to obtain solutions in concentration range from 1 to 20 g dm⁻³. Silicon wafers (Okmetic, Espoo, Finland) cut to ca. 1×1 cm² squares were used as substrates. The cut pieces were cleaned in a UV/ozonator for 15 minutes and rinsed twice with toluene (4000 rpm for ca. 15 s) before spin coating. The spin coating was performed with spinning speed of 4000 rpm and acceleration of 2200 rpm s⁻¹, for about 45 s. The films for QCM-D studies were prepared in a similar manner using silica QCM-D crystals (Q-Sense AB, Gothenburg, Sweden) as substrates. The spin coater used was the WS-400B-6NPP/LITE (Laurell Technologies Corporation, North Wales, PA). The TMSC spin coated films were hydrolyzed in a 2 M HCl vapor environment for 2 minutes. During hydrolysis the spin coated TMSC film is converted back to cellulose.

3.2.5 Quartz crystal microbalance with dissipation (QCM-D)

The QCM-D technique is based on measuring frequency changes of an oscillating quartz crystal, which indicates a change in mass deposited on the crystal. Simultaneously, a change in dissipation is measured, providing information on changes in viscoelastic / softness properties of the deposited substrate (Rodahl et al. 1995). The model substrate, such as an ultrathin layer of cellulose, is deposited on a sensor (quartz crystal). The sensor oscillates at a certain resonant frequency, and the frequency change detected is indicative of the change in mass on the sensor. The adsorption (mass increase), for example, is indicated by a decrease in frequency, while detachment or dissolution of a substrate results in an increase in frequency. If the adsorbed layer is uniform and rigid, the adsorbed mass per unit surface (Δm) is proportional to the change in frequency (Δf), according to the Sauerbrey equation (Höök et al. 1998; Sauerbrey 1959),

$$\Delta m = -\frac{C\Delta f}{n} \quad (1),$$

where n denotes a number of the overtone of the sensed frequency and C is a sensitivity constant for the device. The relation is valid only when the adsorbed mass is small compared to the mass of the sensor crystal.

The viscoelastic properties of the adsorbed layer can be monitored by following the dissipation of energy during one cycle of oscillation. When the voltage applied to the QCM-D is cut off, the amplitude decays due to frictional losses in the system, and rate of the decay depends on the viscoelastic properties of the substrate, adsorbed matter, surrounding solution and the crystal itself. The dissipation factor D is defined as

$$D = \frac{E_{diss}}{2\pi E_{stored}} \quad (2),$$

where E_{diss} is the energy dissipated in one cycle of oscillation and E_{stored} is the total energy of the oscillating system.

For a rigid adsorbed layer the change in dissipation is negligible. However, adsorption of loose and viscous layers is indicated by increased dissipation where the extent of increase is proportional to the adsorbed amount. The dissipation evaluation can be also utilised to study viscoelastic property alterations of the substrate by various treatments, including exposure to NaOH and enzymatic hydrolysis, as shown later in this thesis. A schematic of the QCM-D principle and an example of the data obtained during an adsorption experiment are shown in Figure 16.

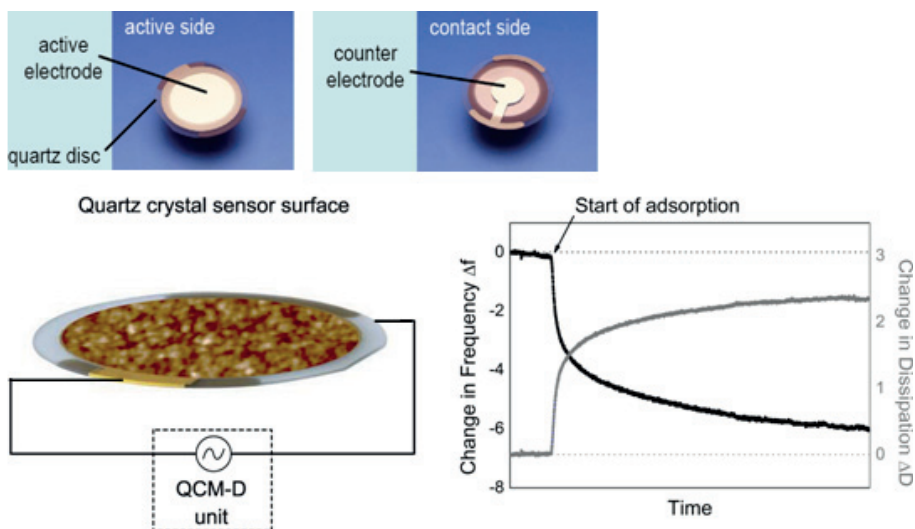


Figure 16. Working principle of QCM-D and an example of the data obtained from an adsorption study (Adapted from Myllytie 2009).

QCM-D measurements. The studies on the stability and enzymatic hydrolysis of the films described in Papers III and IV, respectively, were carried out using Q-Sense E4 QCM-D instrument (Q-Sense AB, Gothenburg, Sweden).

In stability testing (Paper III), the spin-coated and acid hydrolyzed crystals were placed in distilled (Milli-Q) water overnight. The crystals were then rinsed with water and thoroughly dried with nitrogen before being mounted in the measuring chamber. First, the pump was turned on at high speed (0.8 mL min^{-1}) until the measuring chamber was filled with water and then the water flow was lowered to 0.1 mL min^{-1} before the measurement commenced. Once the resonance response was stable, water was replaced with NaOH solutions of different concentration (0.0001, 0.001, and 0.01 M). The flow continued for 60 min and then the pump was turned off. The total time of measurement was 10 hours. All measurements were recorded at a 5 MHz fundamental resonance frequency with its overtones at 15, 25, 35, 55, and 75 MHz. The third overtone (15 MHz) was used in the evaluation of the data. The measurement was carried out at $25 \text{ }^{\circ}\text{C}$.

In the enzymatic hydrolysis studies (Paper IV), the spin-coated and acid hydrolyzed crystals were left to swell in buffer solution overnight. The crystals were then rinsed with water and thoroughly dried with nitrogen before measurement. Prior to the measurement the cells were filled with buffer solution to allow the films to fully

swell, as indicated by stable frequency and dissipation values. The measurement commenced by running buffer solution for ca. 5 min (0.1 mL min^{-1}) to attain the measurement baseline, and then the buffer solution was replaced by enzyme. The enzyme was pumped for a total of 60 min, and then the eluent was switched back to buffer solution for another 60 min (both at the same 0.1 mL min^{-1} flow rate). The pump was then turned off, and the measurement continued for additional 2 hours. Total time of measurement was 4 hours. All measurements were recorded at the 5 MHz fundamental resonance frequency and its overtones, 15, 25, 35, 55, and 75 MHz. The third overtone (15 MHz) was used in the evaluation of the data. The temperature inside the measurement chamber was maintained at $25 \text{ }^{\circ}\text{C}$.

3.2.6 Atomic force microscopy (AFM)

AFM belongs to a group of scanning probe microscopic (SPM) methods. It is a very high resolution microscopic technique in which the scanning probe is a tiny sharp tip connected to a supporting thin and flexible cantilever. The near-field of the sample surface is scanned by the tip and the interactions between the surface and the tip are recorded. The vertical movement of the tip, which corresponds to the height profile of the scanned sample surface, is monitored using a laser beam (directed to the cantilever) which reflects off the backside of the cantilever. This reflected laser beam is detected with a multi-segment photodiode which can track the movement of the beam, and therefore the movement of the cantilever and tip.

AFM can operate in different operating modes, specifically, contact and dynamic modes. In the contact mode the AFM tip scans the surface in contact with the surface. In the dynamic mode, that can be further divided into intermittent contact (tapping) and non-contact modes, the cantilever is vibrating at or near its resonance frequency and the changes in amplitude or frequency when scanning near the sample surface are recorded.

AFM measurements. Film thickness measurements were carried out using a Nanoscope IIIa Multimode scanning probe microscope (Digital Instruments, Inc., Santa Barbara, CA). The images were scanned in tapping mode with a J-scanner and silicon cantilevers (NSC15/AIBS from Ultrasharp μmasch , Tallinn, Estonia). The

radius of curvature for the tip, according the manufacturer, was less than 10 nm and the typical resonance frequency of the cantilever was 325 kHz. Relatively soft tapping (50-70% of the free oscillating amplitude) was applied in the imaging. The films on silicon wafers, films swollen in buffer and films after enzymatic hydrolysis were dried in oven at 80 °C for 15 minutes. The films were then scratched using a sharp needle. The scratched area was scanned by the AFM (10 μm \times 10 μm). The height difference between the uncovered substrate and the intact areas of the film determined the film thickness. A minimum of three scans were imaged for each film sample and at least three points in the image were measured. All quantitative data were extracted from the height images. No image processing except flattening was performed.

3.2.7 Other methods

X-ray diffraction. The grazing incidence X-ray diffraction (GIXRD) measurements were carried out at the beamline W1.1 (ROEWI) at HASYLAB in Hamburg, Germany. More information on the measurement can be found in the experimental section of Paper III.

Neutron reflectivity. Specular neutron reflectivity (NR) experiments were carried out on the time-offlight reflectometer EROS at the Laboratoire Léon Brillouin, CEA Saclay, France. More information on the measurement can be found in the experimental section of Paper III.

Contact angle measurements. Contact angles of the cellulose films were measured to calculate the surface energies of the cellulose films. A CAM 200 (KSV Instruments Ltd, Helsinki, Finland) contact angle goniometer was used for the advancing contact angle measurements. The software delivered by the instrument manufacturer calculates the contact angles based on numerical solution of the full Young-Laplace equation. Measurements were performed at room temperature with four probe liquids: water, ethylene glycol (Fluka), formamide (Sigma-Aldrich) and diiodomethane (Fluka). The droplet volume varied between 3.5-10 μl . The contact angle was measured on three different spots on each sample. The surface energies were

calculated from the contact angle data at equilibrium by Fowkes' method (Fowkes 1964).

Scanning electron microscopy (SEM). The SEM analysis was carried out using Hitachi S-3400N SEM (Hitachinaka, Japan). The samples were gold-sputtered and then the images were taken in secondary electron imaging mode with an acceleration voltage of 5 kV. Wet zero-span samples were freeze-dried before coating.

X-ray photoelectron spectroscopy (XPS). A Kratos Analytical AXIS 165 electron spectrometer with a monochromatic Al_{Kα} X-ray source was used to analyze the elemental and chemical compositions of the sample surfaces in a manner reported previously (Joahnsson and Campbell 2004). All spectra were collected at an electron take-off angle of 90° from sample areas less than 1 mm in diameter. Survey Spectra were recorded at 1 eV intervals at a Pass Energy of 80 eV. Higher resolution regional spectra were recorded at 0.1 eV intervals at a Pass Energy of 20 eV. The spectra were recorded at three different spots on each sample and the analysis vacuum was monitored during the experiments with an in-situ reference sample. No X-ray induced degradation was detected during the experiments.

Refining of the pulps and strength testing. The pulp was refined with a Voith Sulzer LR1 (Ravensburg, Germany) industrial type laboratory refiner equipped with disc fillings (2/3-1.46-40D). The specific edge load was 0.5 J m⁻¹ and the pulp was refined to three specific energy consumption levels (SEC 0, 25, 50 kWh ton⁻¹). Handsheets were prepared according to EN ISO 5269-1 and tested according to EN ISO 5720 and EN ISO 1924-2 (tensile index), EN ISO 5270, ISO 1974 (tear index) and ISO 15361 (zero-span) standards.

4 RESULTS AND DISCUSSION

The most important findings during this thesis work are summarized within this chapter. The complete results are presented in the attached Papers I-V.

4.1 Drying of wood and pulps studied by deuteration and FT-IR

Investigations of the native wood ultrastructure by microscopy or by spectroscopy often require sample preparation to obtain a dry, thin, flat specimen. During preparation water in the sample is usually removed or replaced with a suitable organic solvent. This can lead to structural alterations of the cell wall, so the sample may not be entirely representative of the wood in its native swollen hydrated state. To investigate possible alterations in the ultrastructure of fresh wood during initial drying, it was important to select a methodology that would require minimal sample preparation to preserve the native structure of the wood sample. The concept of deuterium exchange in combination with FT-IR spectroscopy is one of methodologies meeting this requirement. Accessible OH groups of cellulose in wood or in fiber can be readily deuterated by simple exposure to deuterium oxide vapor or by immersion in liquid deuterium oxide. Drying of the deuterated wood could result in changes of the ultrastructure that may cause irreversible deuteration, i.e., conversion of accessible OD groups to inaccessible, reprotonation resistant ones. Detection of these OD groups would then be an indicator of change. The exchanged deuterium in the cellulose sample can be easily detected by IR spectroscopy, because the OD band signal is in an area of spectrum without interference from other signals. Photoacoustic detection is optimal for this testing because, apart from partial drying prior to the measurement, it does not require additional sample preparation.

4.1.1 Concept validation

A fundamental requirement for this experimental concept was to be able to achieve complete reprotonation of the deuterated never-dried samples. Ideally, complete

reversibility of the exchange would be desirable. The experimental conditions, more specifically, the time of deuteration, were selected based on previously published studies. The deuteration of cellulose in wood samples (Tsuchikawa and Siesler 2003a and 2003b) has shown the majority of OH / OD exchange was completed within 100 min in all accessible regions. Therefore, a 60 min deuteration time was deemed sufficient for the purpose of this investigation. More accessible pulp samples were deuterated in two stages (20 minutes), with an exchange of D₂O between the stages. Comparison of FT-IR spectra measured for fresh (never-dried) samples and deuterated never-dried samples subsequently flushed with water is shown in Figure 17.

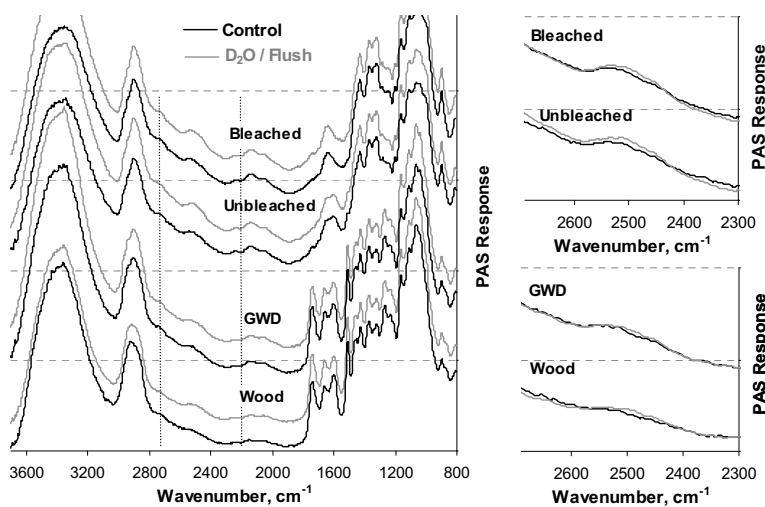


Figure 17. Reversibility of deuteration: Comparison of spectra measured for fresh samples with spectra of corresponding deuterated and subsequently flushed samples. Right: Enlargements of the OD band region of spectra.

The comparison showed that no residual OD groups were retained within the wood and pulp samples after exposure to an excess of water immediately following deuteration. This demonstrates a complete reversal of the OH / OD exchange for wood samples; therefore, the presence of OD groups in dried samples after flushing would indicate the occurrence of irreversible changes in wood structure taking place between the deuteration and the flushing (reprotonation) steps. The increase in the OD band of the spectra was not accompanied by a decrease in the OH region. Although previous studies on cellulose deuteration showed reduction in OH band area in

deuterated samples, those studies employed a different experimental setup – more specifically, closed cells in which the deuteration was carried out while collecting the IR spectra. However, despite using the closed cell system, only a modest decrease in OH absorption band in static FT-IR spectra reported by Hofstetter et al. (2006). In this investigation, as described in experimental part, the samples were first deuterated and then measured by FT-IR. As a part of methodology validation, uniformity of deuteration throughout the wood sample and reproducibility of the measurement were evaluated. The uniformity of deuteration was assessed by slicing the wood test specimen and comparing the spectra measured for the surface and for the inside of the specimen. Reproducibility of the measurement was verified by comparing the spectra of samples collected, treated and measured at different times. The spectra used in the reproducibility evaluation are from the wood drying experiments described later in the thesis. The comparisons are shown in Figure 18.

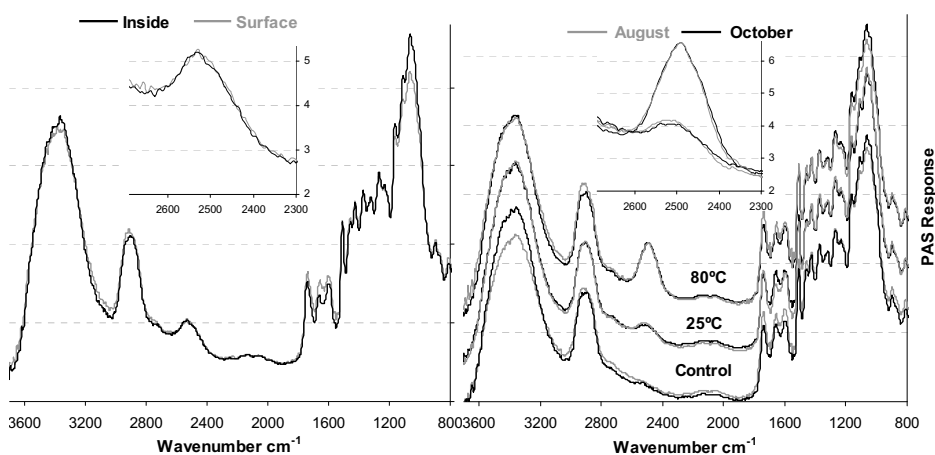


Figure 18. Uniformity of deuteration: comparison of spectra measured for the surface and for the inside of a wood sample (left) and comparison of spectra of treatments carried out at different times using samples collected from different locations (right).

The comparison of treatments and measurements carried out two months apart (August and October) and using fresh wood samples collected from different sources yielded very similar results. Similarly, the spectra of the inner part of the sample and from the original surface were identical, indicating that the alterations occurred uniformly throughout the whole thickness of the specimens. This demonstrates the exceptionally good reproducibility of this analytical technique.

4.1.2 Drying of deuterated wood and pulps

The deuterated fresh wood samples were dried for seven days under controlled D₂O relative humidity (7 and 75%) at 25 and 80 °C. The controlled D₂O environment during drying and different relative humidity levels were designed to prevent the undesirable impact of water on deuteration reversal occurring before any possible structural changes, and to evaluate the impact of RH on the exchange. After drying, the samples were flushed with an excess of water to reprotonate accessible OD groups in the sample. The measured FT-IR spectra for spruce and pine samples are shown in Figure 19. Detailed images of the spectral region of interest are included on the right.

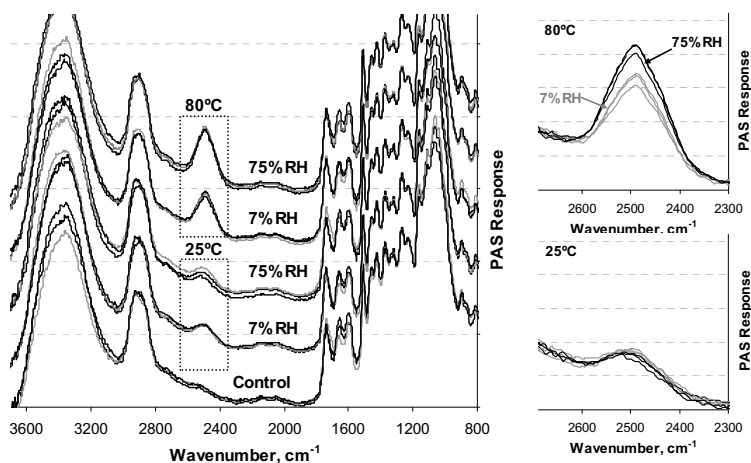


Figure 19. FT- IR spectra of wood samples dried at different temperatures and D₂O relative humidity levels. Comparison of spruce and two sets of pine wood samples.

In addition to the clearly visible OD stretch band in the spectra of all dried samples, a marked difference in the $\sim 2500\text{ cm}^{-1}$ band size was observed between samples dried at different temperatures. The band size of the samples dried at 25 °C was noticeably smaller than those observed for the samples dried at 80 °C. Also, the relative humidity of D₂O did not appear to have an effect on the amount of deuterium retained in the dried wood after reprotonation in samples dried at 25 °C.

In general, this investigation demonstrates that irreversible alterations in ultrastructure of fresh wood take place during the initial drying as indicated by conversion of the

formerly accessible OD groups (readily reprotonated by exposure to water) to inaccessible, reprotonation-resistant OD groups. It is evident that the accessibility of water is altered during drying of fresh wood samples, which could be indicative of supramolecular rearrangements between the wood polymers – something that is smaller in scale than the previously reported microcracks in the cell wall after drying (Kifetew et al. 1998; Thuvander et al. 2002). Based on this investigation, a hypothesis was proposed that the ultrastructural rearrangements are results of the irreversible aggregation of cellulose microfibrils in a similar manner (but to a smaller extent) as that which occurs during hornification of pulp fibers. A schematic of the proposed hypothesis, as investigated by deuterium exchange, is shown in Figure 20.

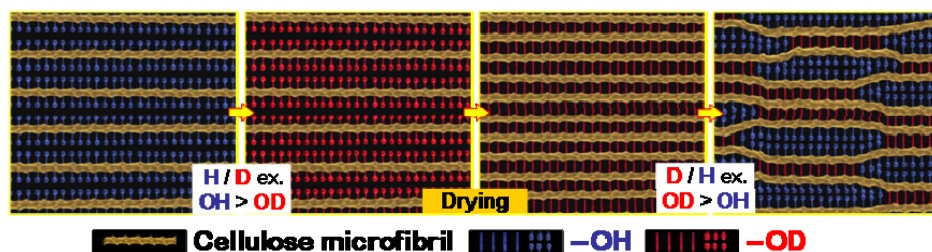


Figure 20. Schematic of the experimental concept and proposed mechanism of ultrastructural alterations in fresh wood during initial drying.

The initial study of the wood drying was followed by applying the same experimental concept in evaluation of the impact of drying on several industrially produced pulps of different characteristics and comparing their behavior to that of a native wood matrix. The trends observed for the pulp samples with known consequences to the fiber properties and mechanisms involved could be then correlated with the results observed for the drying of fresh native wood samples.

Prior to the drying measurement, it was important to assess the extent of deuteration for the pulp samples. A comparison of initial accessibility, expressed as the size of the OD band for wood, groundwood and chemical (bleached and unbleached) pulps is shown in Figure 21.

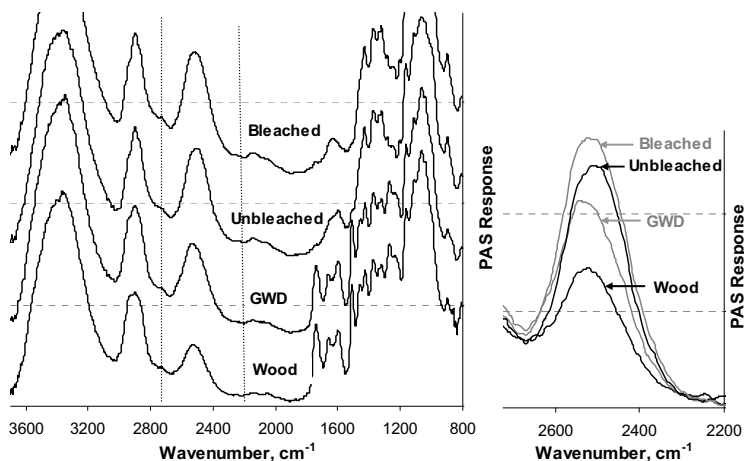


Figure 21. Comparison of maximum deuterium exchange measured for fresh wood and never dried pulp samples. The direct OD band size comparison is shown on the right.

It is clearly evident that, under the selected conditions, the amount of the deuterated accessible OH groups in pulps is considerably greater when compared to wood. Despite similar chemical composition of both wood and groundwood fibers, the measured amount of accessible OH groups in groundwood is almost twice that of the wood sample. The increase in OH group accessibility can be mainly attributed to the effects of wood matrix disintegration. Specifically, an increase in the surface area and the cell wall volume of the fibers liberated from the wood matrix due to the partial disruption of the fiber cell wall and the removal of the middle lamella. Mechanical pulping exposes significant amounts of fresh surface that was previously inaccessible to water in solid fresh wood. In addition, the lignin rich middle lamella that holds the wood fibers together is at least partially removed (Li et al. 2006). In addition to the wood matrix disintegration, the increase in accessibility can be a result of the elevated temperatures that are applied in the groundwood production (Blechsmidt and Heinemann 2006). The groundwood pulp usually contains a large amount of fines, a fraction of fibers of colloidal dimensions, which could have an impact on the measurement of the exchanged OD groups. The sample used in this investigation was thoroughly washed and centrifuged beforehand, and further washed during the deuteration/drying procedure. Therefore, it can be assumed that the small number of

residual fines should not contribute to the measured drying-induced alterations within the groundwood sample.

The greater amount of accessible OH groups in chemical pulps correlates with the difference in chemical composition (higher carbohydrate content) and structural alterations (increased porosity and swelling capacity) resulting from the chemical pulping process (Alén 2000; Fahlén and Salmén 2005; 2005b; Scallan and Tigerström 1992). In bleached pulps, a further increase in cell wall porosity, as well as a slightly higher relative cellulose content, are responsible for the greater amount of accessible OH groups when compared to unbleached pulp sample.

The drying experiments were conducted using the same experimental concept and conditions as the wood samples. To further examine the impact of drying temperature on the extent of alterations, an additional drying temperature level was evaluated. A comparison of the OD band region of spectra measured from samples dried at different temperatures and 7% D₂O relative humidity is shown in Figure 22.

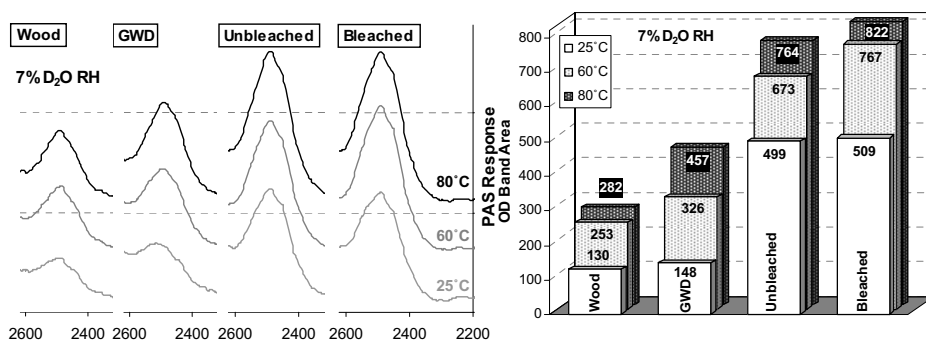


Figure 22. Comparison of the OD band segment of spectra (left) and OD band areas (right) measured for deuterated wood and pulp samples after drying under controlled D₂O relative humidity at different temperatures. Samples were flushed with water after drying.

The comparison clearly indicates the impact of drying temperature on the extent of ultrastructural alterations, particularly in the wood and groundwood samples. The overall extent of the alteration is visibly greater in the chemical pulps dried at all temperature levels. The differences in the extent of alterations between substrates and

the impact of temperature are more evident when the OD band areas are compared directly (Figure 22 right).

Because initial accessibility of the wood and pulps is different, the retained OD groups after drying and reprotonation were expressed as values relative to the maximum OD exchanged (accessible OH in original samples). A comparison of relative deuterium retention is shown in Figure 23.

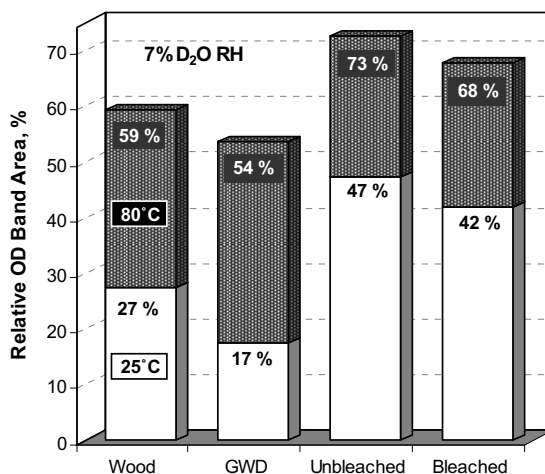


Figure 23. Comparison of the relative reduction of accessible OD band areas of deuterated wood and pulp samples dried under controlled conditions. Values calculated as the ratio between OD band area of dried and flushed sample vs the maximum OD exchanged in the never-dried sample (from Figure 19).

Although the mechanical disintegration of wood increased the accessibility of OH groups in never-dried groundwood (Figure 21) and subsequent retention of inaccessible OD groups after drying (Figure 22), the relative extent of the alterations is smaller compared to that of the wood sample at both temperature levels. Of all the exchanged accessible OH groups within the wood sample, 27% became converted to inaccessible during drying at 25 °C, while only 17% became inaccessible in the groundwood sample dried at this temperature. When dried at 80 °C, both wood and groundwood showed a similar relative amount of retained OD groups, 59 and 54%, respectively. The significantly greater surface area of the groundwood fibers compared to that of the solid wood sample and increase in the cell wall volume of the fibers liberated from the compact wood matrix are most likely responsible for the

increased initial accessibility. The impact of the greater surface area of the groundwood fibers in the drying experiments can be to some extent minimized because a similar area is then exposed to water during the flushing stage after drying. The lower relative amount of inaccessible OD in dried groundwood compared to dried wood indicates that the increased accessibility of the groundwood is not irreversibly altered by the drying and can for the most part be restored by rewetting. This finding correlates well with previous research showing that the high yield pulp swelling characteristics are not affected to a great extent by drying (Laivins and Scallan 1993). It is also possible that the elevated temperatures (up to 100 °C) that are common in mechanical pulping production might have an effect on the property of the fiber cell wall and be somewhat responsible for this difference in the relative extent and reversibility of the alterations.

The chemical pulps showed much greater retention of the OD groups compared to wood and groundwood. Almost half (47%) of the OD groups were retained in the unbleached sample dried at 25 °C, while a retention of 42% was measured for the bleached sample. At 80 °C, the retention of the OD groups was significantly higher, with the unbleached pulp showing greater relative OD retention than the bleached sample (73 vs 68%). The increase in the extent of irreversible alteration of the chemical pulp was expected due to greater cell wall porosity and minimal lignin content. The removal of water leads to irreversible closure of the pores and the formation of hydrogen bonds between adjacent microfibrils. These newly-formed bonds are resistant to hydration and do not break during rewetting, thus, the original swelling of the fiber cannot be restored. It was interesting to note, however, that, despite the greater porosity and negligible quantity of lignin in the bleached pulp sample, the relative amount of inaccessible OD groups formed during drying was slightly greater in the unbleached sample.

4.1.3 Comparison with WRV measurement

In the final part of the investigation, the results obtained by deuteration studies for pulps samples were compared with those obtained by the measurement of the water retention value (WRV). The WRV measurement is a standard empirical test that quantifies the total amount of water retained by a pulp sample after centrifugation

under well-defined and controlled conditions (Jayme 1958). The centrifugation removes the free water present between the fibers and inside the cell lumen, retaining only the water within the pore structure of the fiber, i.e., inside the fiber cell wall. The ratio of water to dry fiber after centrifugation is often used as a measure of fiber swelling capacity. Although deficiencies of the test have been presented in the past (Maloney et al. 1999b) and its explicit physical meaning is unclear, WRV is still accepted as a reliable and useful description of fiber swelling, particularly in cases where it is sufficient to assess the relative changes in fiber swelling.

It has been demonstrated that the properties of recycled (dried) chemical pulp fibers differ from those of fresh never-dried fibers. This phenomenon, termed hornification, has been investigated extensively (Laivins and Scallan 1993; Nazhad and Paszner 1994). Hornification has been described as a complex process and several mechanisms have been proposed, such as irreversible pore closure, microfibril aggregation, hydrogen bond formation and others (Kato and Cameron 1999; Fernandes Diniz et al. 2004).

Both WRV reduction and the OD retention measurement used in this investigation are used to indicate the changes in wood pulp fiber. One of the objectives was to establish a correlation between the pulp sample WRV reduction and the retained OD band area in the respective IR spectra. A comparison of the WRV measured for the pulp samples dried at different temperature is shown in Figure 24.

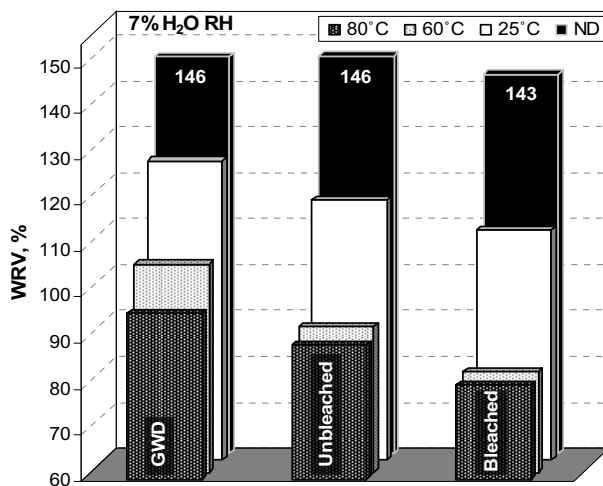


Figure 24. Comparison of WRV of pulp samples dried under controlled H₂O relative humidity (7%) at 25, 60 and 80 °C. ND indicates never-dried pulp samples.

Similarly, as observed in the deuteration studies, the WRV measurement demonstrates the impact of drying and drying temperature. The extent of hornification (WRV reduction) is most evident in the bleached pulp sample. The bleached pulp contains the largest segment of cellulose and practically no lignin, compared to the unbleached pulp, which contains a small amount of residual lignin and a slightly greater relative amount of hemicellulose. Both lignin and hemicelluloses were shown to inhibit hornification (Oksanen et al. 1997). In groundwood, the lignin is present in significantly greater amounts, yet a reduction of WRV was evident. Although the majority of previous research accounts have described limited impact of drying on the swelling characteristics of high yield pulps (Laivins and Scallan 1993), the reduction of mechanical pulps swelling ability, particularly after the first drying cycle, has also been previously described (Law et al. 1996).

All pulp samples dried at 25 °C already exhibited a significant reduction in WRV. Drying at higher temperatures resulted in even greater reductions. The reduction trend is quite steady with temperature for the groundwood sample. In contrast, the chemical pulps showed a significant drop in WRV between samples dried at 25 and 60 °C and only a marginal additional decrease between samples dried at 60 and 80 °C. This is in agreement with the previously reported investigation in which it was demonstrated that hornification is a direct result of the removal of water from the fiber, not the heat treatment associated with drying (Laivins and Scallan 1993). This is further confirmed by the moisture content of the samples after drying (Table 1).

Table 1. A comparison of moisture content (MC) and water retention values (WRV) of pulp samples dried at different temperatures at 7% relative humidity.

sample	groundwood		unbleached kraft pulp		bleached kraft pulp	
	MC (%)	WRV (%)	MC (%)	WRV (%)	MC (%)	WRV (%)
control	65.4	146	67.0	146	66.1	143
dried at 25°C	5.5	124	5.1	117	4.8	110
dried at 60°C	0.2	105	1.0	92	1.3	82
dried at 80°C	0.2	96	1.6	89	0.6	81

The water remaining in the pulp samples after drying at both temperatures is similar; no substantial further change in moisture content was observed by increasing the drying temperature above 60 °C under the selected conditions. The extent of WRV reduction in the 60 to 80 °C temperature range increased relative to the amount of lignin in the fibers, with the largest decrease measured for the groundwood and only a marginal decrease observed for the lignin-free bleached chemical pulp sample. It appears that drying at 80 °C possibly induces additional mechanisms of alteration, which are not directly related to the removal of water. These alterations may in some way be related to the presence of the hemicellulose/lignin matrix in the groundwood sample and its residues in the unbleached chemical pulp sample, because no significant change took place in the lignin-free sample.

Both WRV reduction and the OD retention measurement used in this investigation are used to indicate the changes in the wood pulp fiber. We attempted to establish a correlation between the pulp sample WRV reduction and the retained OD band area in the respective IR spectra. The correlations between WRV and OD band area measured for corresponding samples are shown in Figure 25.

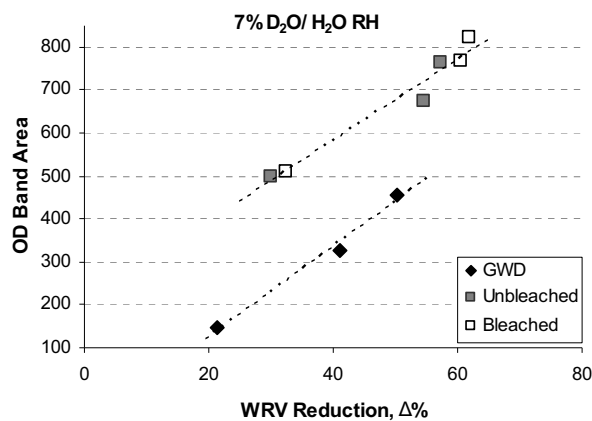


Figure 25. Correlations between WRV value reductions and OD band area for pulp samples dried under controlled relative humidity at different temperatures.

The correlation with the WRV of the pulps appears to be linear within the interval of the measured values. Although the amount of retained OD groups in groundwood is

lower, the incremental OD band area increase with WRV reduction is similar to that of chemical pulps.

The increase in the OD band area correlates well with the reduction in WRV, which is a widely applied standard for the extent of hornification in pulp. It can be safely assumed that the retained inaccessible OD groups within the pulp sample are also a reliable indicator for the extent of hornification. The wood samples exhibited the same trend in OD retention as those observed for pulps, although to a lesser extent. Therefore, the formation of inaccessible OD groups during drying clearly demonstrate that some kind of irreversible alteration, which, to a certain extent also takes place or proceeds through a similar mechanism in the cell wall of wood pulp fiber, occurs in the wood ultrastructure.

Initially, we hypothesized that a possible irreversible hardening of the lignin-hemicellulose matrix may also be responsible for accessibility changes during drying. However, the fact that the reduction in the water retention values of dried pulps appear to correlate with deuterium retention supports the hypothesis of microfibril aggregation in wood upon drying, because the drying-induced changes are ascribed to microfibril aggregation in the case of chemical pulps. Fundamentally, the nature and quantity of alterations in wood ultrastructure induced by drying is an important discovery because irreversible changes in swelling characteristics of wood are impossible to track down with conventional measures in swelling. Although the results of this investigation do not present unequivocal hard evidence, they provide a strong indication of the mechanism of changes occurring in the cell wall of wood and wood fibers during initial drying.

4.2 Enzymatic degradation of cellulose model films

The compositional and structural heterogeneity of native lignocellulosic biomass presents challenges for its utilization in the fundamental research of enzymatic hydrolysis. In addition, as demonstrated in the previous section, the ultrastructure of wood can be readily altered by initial drying, with implications on its accessibility and susceptibility towards enzymatic hydrolysis. Even when using pure cellulose substrates, the morphology of the substrate plays a significant role in the reactivity of cellulose. In model cellulose films, the impact of morphology is reduced (ultrathin

thickness). That makes model cellulose films an ideal substrate for the fundamental evaluation of the enzymatic degradation of cellulose, while enabling utilization of various surface analytical techniques which are not applicable for bulk samples. The prepared cellulose model films can have different characteristics (i.e. crystalline arrangement and degree of ordering), diverting from the native cellulose to various extents. In this investigation, an amorphous cellulose model film with well-defined characteristics has been applied in fundamental, quantitative studies of cellulose degradation by various cellulase enzymes. Amorphous cellulose differs significantly from its crystalline form - for example, susceptibility to degradation (e.g. acid hydrolysis), or accessibility to water. Amorphous cellulose cannot be readily isolated from the native microfibrils, therefore a need still exists to prepare amorphous cellulose substrates for utilization in fundamental studies.

The fundamental studies of enzymatic hydrolysis of amorphous cellulose model substrates, specifically by endoglucanase (EG), can provide valuable information, particularly when considering the increased interest in utilization of EG for application in, for example, increasing the solubility and reactivity of dissolving pulps, or in modification of delignified wood pulp fibers prior to preparation of MFC (Rahkamo et al. 1998; Engström et al. 2006; Pääkkö et al. 2007).

4.2.1 Characterization and stability evaluation of model cellulose film

The characterization of cellulose model film prepared from TMSC by Langmuir-Schaefer deposition revealed a relatively high degree of cellulose ordering, about 60% (Aulin et al. 2009). In that comparative study, however, the characteristics of the model films from TMSC prepared by spin coating were not evaluated.

The deposition technique has an impact on the ordering of the cellulose in the film. In our investigation, we intended to properly characterise the properties of the regenerated films prepared by spin coating of TMSC before using them in the enzymatic degradation study. The main objective was to evaluate the characteristics and behavior of amorphous cellulose within the ultrathin films. The characterization was carried out using grazing incidence x-ray diffraction (GIXRD), neutron reflectivity (NR), contact angle measurements (CA), atomic force microscopy (AFM)

and quartz crystal microbalance with dissipation monitoring (QCM-D). The results of GIXRD performed on the cellulose films are shown in Figure 26.

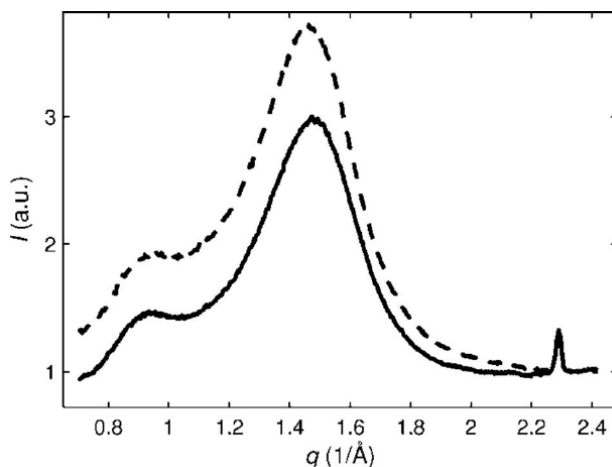


Figure 26. X-ray diffraction patterns for untreated, as-prepared cellulose film, regenerated from TMSC (solid line) and the same film after exposure to water and subsequent drying (dashed line).

The broad intensity distribution indicates a lack of long range order in the atomic arrangement of cellulose, therefore the films can be considered to consist mostly of amorphous cellulose without any larger crystallites. This result correlates with previous finding that, based on electron diffraction and reflection absorption infrared spectroscopy studies, suggested the amorphous nature of these films (Kaya et al. 2009). The comparison with the characteristics of LS deposited film demonstrated that despite the same starting material (TMSC) and regeneration procedure, the deposition method indeed makes a distinct difference. In contrast to the spin coated films, the film prepared by LS deposition exhibited a relatively high degree of crystallinity (Aulin et al. 2009). In spin coating, the dissolved substance is forced on the substrate by rapidly removing the solvent with high speed spinning. As a result, the films deposited by spin coating are far from thermodynamic equilibrium and it is possible that the cellulose chains are not favorably positioned or conformed for crystalline packing to occur. In contrast, the LS deposition is based on formation of a monolayer of a solid material on a liquid surface and subsequent adhesion of individual monolayers on top of each other on a solid substrate. Because the process is

based on self-organization, it seems reasonable to expect more advantageous conditions for crystallization to occur in LS deposition rather than in spin coating.

The characteristics of films prepared by spin coating obtained from the fits of the neutron reflectivity spectra, including scattering length density (SLD), thickness, density and roughness of the deposited cellulose film, and the roughness values of the films measured by AFM are summarized in Table 2.

Table 2. *Properties of amorphous cellulose film obtained from the NR curves and AFM (rms roughness).*

sample	SLD (10^{-6} \AA^{-2})	volume fract. (% cellulose)	thickness (nm)	roughness (nm)	rms roughness nm
as-prepared	1.42	79.9	19.5	0.5	0.67
swollen in D ₂ O	5.75	21.7	38.5	0.2	-
dried after swelling	1.57	88.2	20.0	0.5	0.85

The cellulose volume fraction has been derived from the SLD of the layer and from the bulk SLD of amorphous cellulose that is equal to $1.78 \times 10^{-6} \text{ \AA}^{-2}$. The results show that as prepared, the amorphous cellulose film is highly dense with a volume fraction close to 0.8, 19.5 nm thick and with a markedly low surface roughness. When exposed to liquid D₂O, the cellulose film thickness increased dramatically to reach about twice that of its original value (38.5 nm). At the same time, the cellulose volume fraction decreased to 0.22 (78% water uptake of the films). Upon drying, the layer thickness decreased from 38.5 nm to 20 nm, i.e., the cellulose layer practically recovered its original value, demonstrating that the swelling of the amorphous cellulose film was almost fully reversible. These results show that the spin-coated amorphous cellulose layer exhibited remarkable swelling properties while keeping a smooth outer interface. The extent of swelling correlates reasonably well with the reported value of 84-89% for thick amorphous cellulose films (Eckelt and Wolf 2007), yet it is still considerably smaller when compared with bulk hydrogels that are specifically designed for swelling. For example, more than 99% water uptake was reported for cross-linked carboxymethyl cellulose hydrogels (Barbucci et al. 2000). Still, this indicates that the supramolecular network in amorphous cellulose is substantial.

The roughness values extracted from the fits were very low regardless of the experimental conditions. Such a low value was expected for the as-prepared film but was somewhat unexpected in the case of the swollen sample, where a loose interface between the cellulose layer and the solvent rather than a sharp interface is expected. The AFM analysis of as-prepared films and films dried after swelling indicated a similar behavior in terms of morphology. Despite the slight increase in roughness – root mean square value increases from 0.67 nm to 0.85 nm – the morphology remained almost completely unchanged upon wetting and drying.

The next step in the characterization of the cellulose model film was the evaluation of the film stability in NaOH solutions. Native cellulose at least partially dissolves in concentrated alkali. For instance, amorphous cellulose prepared by ball milling has been shown to dissolve in 1 M NaOH solution at room temperature (Hermans and Weidinger 1946). The industrial appeal of NaOH as a cellulose solvent has initiated a number of fundamental research efforts in the dissolution mechanism (Isogai 1997; Isogai and Atalla 1998; Egal et al. 2007; Le Moigne and Navard 2010). Ultrathin films are attractive substrates for such research because the kinetics of dissolution can be followed, for instance, with the QCM-D technique. The QCM-D, as described in detail in the experimental section, is essentially a very sensitive balance where frequency changes are inversely proportional to mass changes in the film and alterations in dissipation correspond to its viscoelasticity. A decrease in frequency, indicating increased mass uptake, denotes swelling of or adsorption on the cellulose film whereas an increase in frequency, representing a mass loss, means that cellulose is detaching from the film, i.e., it dissolves. The frequency and dissipation curves for amorphous cellulose films exposed to 0.01M (pH 12), 0.001 M (pH 11), and 0.0001 M (pH 10) NaOH solutions at room temperature are shown in Figure 27. These concentrations were selected because stronger alkali solutions caused almost immediate dissolution of the whole cellulose film, rendering kinetic observations moot. The stability of these films in pure water has been evaluated previously (Nyfors et al. 2009), and those results are also included in the figure.

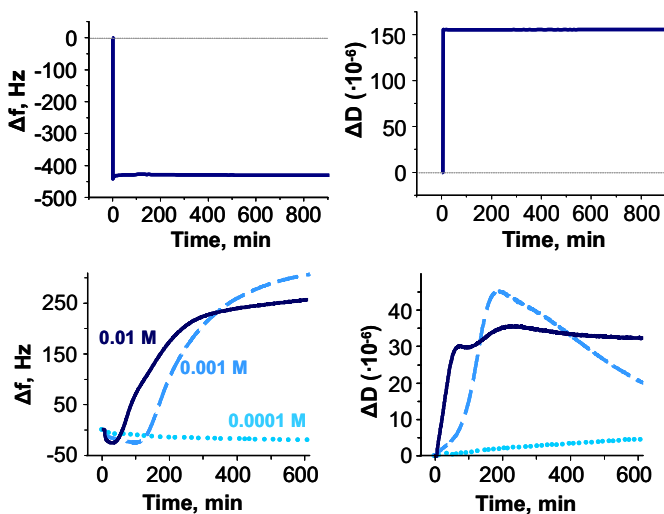


Figure 27. QCM-D plots of frequency change (Δf) and dissipation change (ΔD) as a function of time for amorphous cellulose films exposed to water (top) and different NaOH concentrations (bottom) In the top graph, water is added to the system at (t) = 0 min.

The frequency and dissipation changed immediately after the dry cellulose films were exposed to water, and remained stable over the period of testing (Figure 27 top). The swelling of the cellulose-containing films did not appear to significantly affect the softness of the films. When exposed to 0.0001 M NaOH solution, the swollen (in water) cellulose film underwent continuous swelling and increase in viscosity, as demonstrated by a slow, continuous decrease in frequency (-20 MHz over 600 min) and an increase in dissipation (4.5×10^{-6}). The swelling is probably due to the minor dissociation of the hydroxyl groups in cellulose causing an increased osmotic pressure. In general, the film remained relatively stable during the period of testing (600 min) with no apparent dissolution of cellulose.

The behavior of the films exposed to 0.001 M and to 0.01 M NaOH solutions was markedly different. The swelling of the cellulose films as indicated by a decrease in frequency was observed for both concentrations. However, the rate and extent of the film swelling was significantly greater compared to that observed at 0.0001 M NaOH concentration. The frequency of the films exposed to both 0.001 M and 0.01 M NaOH concentrations decreased by ~ 25 Hz. The minima were reached within 90 and 20

minutes, respectively, shortly after which a rapid increase in frequency was observed for both concentrations. This increase in frequency indicates dissolution of cellulose and started after ca. 105 min with 0.001 M NaOH solution and after ca. 35 min with 0.01 M NaOH solution. Overall, the initial swelling of cellulose with these higher concentrations was very different to the swelling in 0.0001 M NaOH, demonstrated by the abrupt and substantial rise in the dissipation values at the early stages of the NaOH exposure. The increase in dissipation, which denotes an increase in the viscoelasticity of the film, implies that intensive softening of the film precedes its dissolution. The softening coupled with the additional swelling may well indicate that the hydrogen bonding network of amorphous cellulose starts to disintegrate.

The dissolution rate appeared to slow significantly after ca. 250 min from the start of the dissolution for both higher NaOH concentrations. After that, the dissolution of cellulose in the film residues proceeds slowly, and is accompanied by a slow decrease in dissipation. Interestingly, there is a greater extent of dissolution for the 0.001 M NaOH solution, indicated by both changes in frequency and dissipation.

The extent of dissolution was quantified by using the Sauerbrey equation and according to these estimates, exposure of the films to dilute NaOH of 0.01 M and 0.001 M concentrations resulted in roughly a 50% removal of mass from the cellulose film. This also demonstrates that these dilute NaOH concentrations can facilitate only a partial dissolution of the film.

As the slopes of the frequency changes are fairly equal, the rate of dissolution is independent of concentration between 0.01 M and 0.001 M NaOH solutions. The difference, however, is in time after which the dissolution starts. The earlier start of dissolution of the film in 0.01 M NaOH solution is likely caused by faster ionization of the hydroxyl groups because of the close match with the pKa value of cellulose hydroxyl groups (13.7) (Neale 1930). The dissolution of cellulose by NaOH is facilitated by deprotonation of the hydroxyl groups on anhydroglucose units into soluble alkoxy ions (Isogai 1997). The dissolution of amorphous cellulose at room temperature in 1 M NaOH has been reported (Hermans and Weidinger 1946). The present results have revealed that even 0.001 M NaOH can partially dissolve an ultrathin film of amorphous cellulose at room temperature within a space of a few hours.

The ratio of ionized hydroxyl groups to protonated hydroxyl groups was estimated using Henderson-Hasselbalch equation, corrected by Manning's counter-ion condensation theory (Manning 1969). The obtained ratios were approximately 1/70 and 1/700 for cellulose in 0.01 M and 0.001 M NaOH, respectively. The extent of deprotonation for cellulose in 0.001 M NaOH in particular, is so minute that the ionization of hydroxyl groups cannot be the main reason for dissolution.

The limiting factor for dissolution of cellulose in aqueous NaOH is actually the morphology of the cellulosic fiber (Le Moigne and Navard 2010). The essentially featureless amorphous ultrathin film is the least structured morphology and, therefore, its dissolution in more dilute NaOH concentrations is logical.

4.2.2 Hydrolysis of amorphous cellulose model films by EG

For this investigation, an experimental system consisting of well-defined and noncomplex components was selected to allow a clear analysis of the enzyme action. The endoglucanase (EG) used in this investigation was a purified monocomponent EG I, which is known to act on amorphous cellulose (Lynd et al. 2002). QCM-D data of the action of EG I on amorphous cellulose and impact of the enzyme concentration is shown in Figure 28.

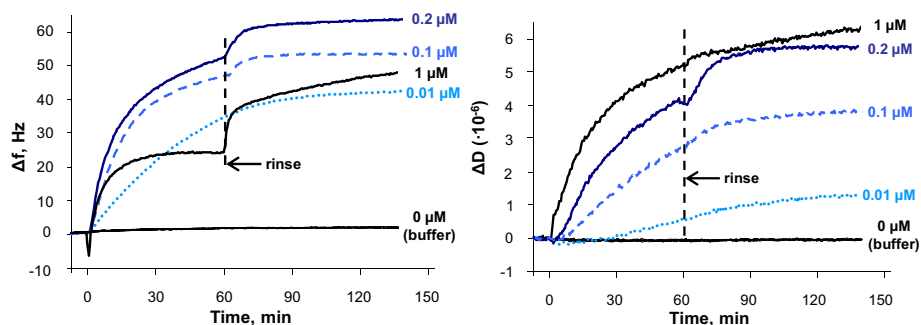


Figure 28. Change in frequency (left) and dissipation (right) measured by QCM-D for films of amorphous cellulose (18 nm thick) exposed to EG I of different concentrations. Enzyme is added at ($t = 0$ min).

The action of EG I on amorphous films leads to rapid degradation of the film. The hydrolysis commences almost immediately after contact of EG I with the substrate, indicated by a rapid increase in frequency. The hydrolytic action of EG I is clearly demonstrated, since no change in frequency was observed when the film was exposed to pure buffer. The rate of degradation starts to slow after ca. 30 min. Interestingly, after continuous exposure to the enzyme (60 min), the introduction of a buffer to the system (rinse stage) resulted in a rapid short burst of mass loss from the film. It appears that rinsing frees the activity of the enzyme, particularly at higher concentrations. In addition, it is possible that the hydrolyzed cellulose is more readily dissolved in pure buffer solution. When compared to the previously described investigation of EG I hydrolysis of semicrystalline cellulose model films studied with QCM-D (Josefsson et al. 2008), EG I exhibits markedly different action here. In that investigation, the interaction of the EG I with partially crystalline film and at higher enzyme loads resulted in adsorption of EG I on the substrate and subsequent swelling of the film. However, no detachment of the cellulose from the film was detected over extensive periods of exposure of the film to the enzyme.

The enzyme concentration was shown to be a factor in the hydrolysis as monitored by QCM-D. The comparison in Figure 28 showed that very low concentrations (0.01 μM), and particularly high (1 μM) concentrations have a different impact on the hydrolysis. At low concentration, a slower rate and a lower extent of hydrolysis were observed. The concentration in the middle range showed an increase in both the rate and the extent of hydrolysis. The high concentration, however, behaved differently. Only at this concentration (1 μM) was noticeable adsorption of the enzyme observed, indicated by a drop in frequency of ca. 10 Hz. The adsorption was rapidly surpassed by the hydrolytic action denoted by the rapid increase in frequency. At the initial stage, the rate of hydrolysis was among the fastest from the concentrations evaluated, when considering the initial adsorption. Shortly afterwards, however, the hydrolysis starts to rapidly level off, and appears to stop within 30 min of exposure. Introduction of the buffer to the system resulted in a burst of hydrolytic activity of the EG I. The frequency jumps almost 20 Hz within a minute after beginning the rinsing stage. Then the rate of hydrolysis starts to level off again, and gradually continues until the end of the measurement. Overall, the extent of cellulose film degradation is lower than that observed for lower concentrations, even that of the 100 times weaker enzyme

concentration. This indicates that once the film is saturated with the enzyme, overcrowding starts to impact the hydrolysis.

The impact of overcrowding on the efficiency and kinetics evaluation of cellulose hydrolysis by cellobiohydrolase (CBH) has recently been reported (Xu and Ding 2007; Igarashi et al. 2006). Although no data is available for EG I and an amorphous model film, it appears that overcrowding might be a phenomenon also associated with the endoglucanase hydrolysis of cellulose.

Dissipation data in Figure 28 (right) indicate that, despite no change in frequency after 30 min of exposure, the action of EG I increased the viscoelasticity of the films. The available surface for EG I binding appears to be fully saturated at that point; therefore this increase in dissipation is most likely due to continuous loosening of the swollen film. Therefore, while no significant amount of cellulose is released as indicated by the lack of frequency change, the hydrolytic activity of the enzyme continues. Previous findings on the action of monocomponent EG I on a semicrystalline cellulose model film investigated by QCM-D showed a similar, continuous increase of dissipation while a relatively stable frequency was maintained (Josefsson et al. 2008).

In summary, concentration of EG I was shown to have a pronounced effect on the hydrolysis of the cellulose films. Too high a concentration of the enzyme can lead to self-inhibition of the enzyme activity. Once the film is saturated with the enzyme, overcrowding starts to impact the hydrolysis probably because the dense binding of the enzyme molecules hinder each other from accessing the cellulose with their catalytic domains. This also shows that the mode of enzyme action is affected by the experimental conditions and that for a proper evaluation of hydrolysis, it is important to maintain the same enzyme load to obtain a system with steady state conditions.

The next step in evaluating the action of EG I on amorphous cellulose film was to assess the impact of the amount of substrate available for hydrolysis on the rate of hydrolysis as detected by QCM-D. Spin coating allows for the preparation of homogeneous amorphous films of different thickness. The thickness of these homogeneous films can be controlled by the concentration of the TMSC solution used in spin coating. Previous fundamental studies of the enzymatic degradation of cellulose model films (Eriksson et al. 2005; Josefsson et al. 2008; Turon et al. 2008)

have not addressed the issue of substrate available for hydrolysis, and as such, a direct comparison with previous work is not possible. The ability to control the film thickness, or volume of available substrate, however, can be valuable for obtaining additional information pertinent to the kinetic evaluation of cellulose degradation by cellulases.

The films swollen in buffer solution were exposed to the solution of EG I (0.2 μ M) in the QCM-D chamber for a period of 60 minutes. The development of frequency during the hydrolysis of the films with different thickness (2, 8, 18, 37 and 60 nm) is shown in Figure 29.

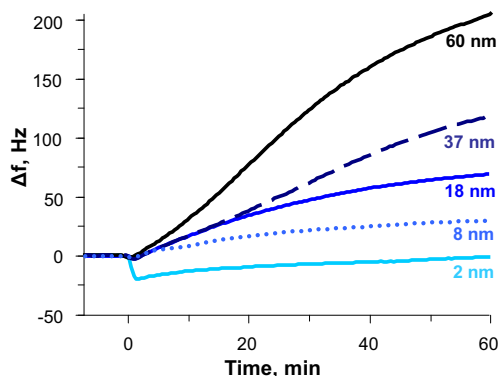


Figure 29. Frequency change development during hydrolysis of the films of different thickness. Time (t) = 0 min indicates the start of enzyme (EG I) addition.

Except for the thinnest film (2 nm), all the other films of different thicknesses showed a rapid degradation almost immediately following contact with the enzyme. The initial adsorption is rapidly surpassed by the concurrent hydrolytic action. The minimum frequency is reached for films of all thickness (except for the thinnest film) within ca. 2 min from the initial contact, followed by a rapid increase in frequency indicating the removal of the cellulose from the film. The thinnest film (2 nm), exhibited an anomaly in frequency development. A clearly visible and more pronounced adsorption stage and an instant increase in dissipation differ from the trends observed for the thicker films. The reason for this difference has not yet been identified, although it is possible that the cellulose in close contact with the silica substrate form some sort of arrangement that cannot be disrupted by the EG I treatment.

4.2.3 Quantitative assessment of hydrolysis

To attempt a direct quantification of the data obtained by QCM-D measurement, parallel to the experiments carried out by QCM-D, experiments using identical model films deposited on silicon wafers were conducted. This was necessary because the quartz crystal sensors used in the actual QCM-D measurement could not be used in thickness profiling by AFM. Identically prepared films deposited on silicon wafers were exposed to the flow of the enzyme. The enzyme, at the same rate as that in the QCM-D measurement (0.1 mL min^{-1}), was introduced on top of the film and the excess of the liquid (enzyme) was periodically removed from the top of the films by suctioning with a micropipette. To measure mass reduction, the thickness of the film was measured before and after the EG I treatment. The mass reduction was then determined from the difference between the initial thickness and that after the enzymatic treatment. This thickness difference can be considered a viable indicator of the mass reduction, based on the homogeneous characteristics of the films used in this investigation. The correlation between the change of frequency and the decrease in film thickness after treatment is shown in Figure 30.

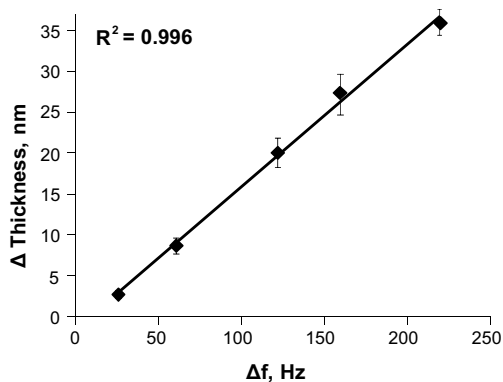


Figure 30. Correlation between measured QCM-D decrease and decrease of film thickness after 60 minute EG I treatment.

Both QCM-D frequency change and the thickness reduction measured by the AFM correlated very well with each other. Variation of the thickness measurements taken from different spots of the film were within an acceptable range, indicating that the degradation is even over the whole surface of the film. This shows that the QCM-D

frequency change reliably measures the mass loss of the cellulose model films for these films and conditions. These measured mass reduction data were used to determine the overall extent of hydrolysis for the film of different thicknesses.

Using the correlation of frequency and mass reduction (Figure 30) and the measured frequency change (Figure 29), the time dependent rate of hydrolysis was compared. The comparison of the rate of hydrolysis for films of different thicknesses (8, 18, 37 and 60 nm) is shown in Figure 31.

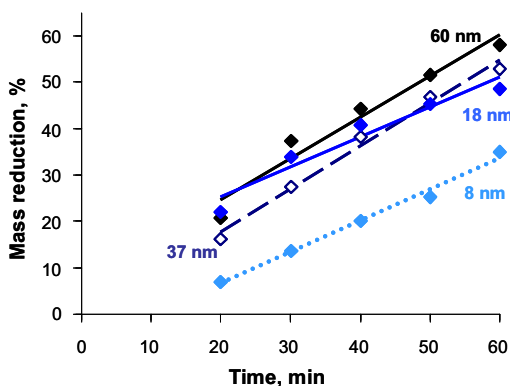


Figure 31. Correlation between relative decreases of the film thickness after 60 minutes of EG I treatment and initial film thickness.

The linear change in mass with time during hydrolysis demonstrated that steady state conditions were reached during the whole hydrolysis. The slopes of the correlation were similar for films of all measured thicknesses. This comparison indicates that the relative rate of hydrolysis for EG I hydrolysis of amorphous cellulose in the film is similar. In other words, our data implies that the film thickness does not ultimately affect the rate of endoglucanase hydrolysis of the amorphous cellulose film.

4.2.4 Comparison of CBH and EG action on amorphous cellulose model films

In the final part of this investigation, the action of monocomponent cellobiohydrolase (CBH I) was compared with the results obtained for EG I. The films, 18 and 60 nm thick, were exposed to EG I and CBH I under identical conditions. This comparison was carried out to obtain the initial information on action of CBH I on amorphous

cellulose film and to compare it to the action of EG I. The comparison is shown in Figure 32.

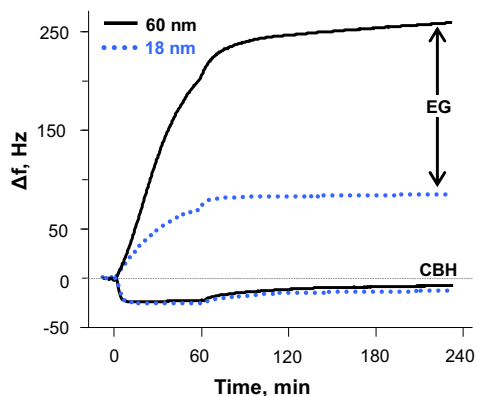


Figure 32. QCM-D evaluation of EG I and CBH I action on amorphous cellulose model films with different thickness. Enzyme introduced at $t = 0$, rinsing after 60 min of treatment.

The differences in action of CBH I and EG I were observed on two levels - extent of hydrolysis and the impact of film thickness. The films exposed to CBH I exhibited a rapid drop in frequency (ca. 25 Hz), that reached a minimum within ca. 10 min and remained constant over the period of enzyme exposure (60 min). This indicates that none or a limited decrease of mass occurred during that time. In addition, the film thickness did not appear to have an impact on the extent of frequency change. In contrast, the hydrolytic action of EG I, as shown previously, commenced almost immediately after the contact with the substrate, indicated by an instant rapid increase in frequency. Contrary to the action of CBH I, the impact of the film thickness is clearly evident; the change in frequency after 60 min of exposure markedly increases with the film thickness. Replacement of the enzyme with buffer solution (at 60 min) resulted in a gradual increase in frequency observed for CBH I treated film, while EG I treated sample exhibited a rapid increase in frequency followed by gradual leveling off. The fact that the thickness of the film (initial volume of cellulose available) does not affect the extent of the adsorption, nor the subsequent hydrolysis, could indicate that the adsorption of CBH I takes place on surface only. The gradual increase of the frequency after switching to buffer solution is a result of either desorption of the enzyme or limited hydrolysis of cellulose. It is possible that both, desorption and hydrolysis, take place simultaneously. The XPS analysis of the hydrolyzed films

(Table 3) indicated the presence of protein in the film. This would support the assumption that limited hydrolysis indeed takes place. The CBH I irreversibly adsorbs on the cellulose and proceeds with the limited hydrolysis. The bound CBH I is not removed even by extensive washing after the hydrolysis. In contrast, the protein was not detected in the film treated with EG I under identical conditions. This indicates that the adsorption of the EG I is reversible.

Table 3. XPS data for the amorphous films exposed to the EG I and CBH I treatment for 60 minutes and subsequent rinsing. Initial thickness the films was 60 nm.

sample	Atomic concentrations			
	O 1s	C 1s	Si 2p	N 1s
EG I	40.4 %	57.6 %	1.7 %	0.3 %
CBH I	38.1 %	57.8 %	0.1 %	4.1 %

Previously reported comparison of the action of monocomponent CBH and EG cellulases in the degradation of semicrystalline cellulose films showed different trends (Josefsson et al. 2008). The exposure of the film to EG resulted in a rapid decrease in frequency and continuous increase of dissipation, attributed to increased water uptake of the film due to film swelling. However no mass reduction was observed during the extended period of measurement. In contrast, the action of CBH resulted in rapid hydrolysis, indicated by an increase in frequency. In this investigation, the opposite trend was observed for both enzymes. A significant degradation of cellulose film was observed in the EG I treatment of the film, while only marginal hydrolysis was observed for CBH I. This agrees with the preference of EG I towards the amorphous parts of cellulose (Bailey et al. 1993; Kleman-Leyer et al. 1996; Lynd et al. 2002). The limited activity of CBH I is also different to that measured using semicrystalline films. Although CBH I preferentially acts on the crystalline part of cellulose, partial activity on amorphous cellulose has been reported (Nidetzky et al. 1993). It appears that the action of CBH I on swollen amorphous cellulose films is very limited.

With this comparison we intended to demonstrate the difference in action of CBH I and EG I in hydrolysis of amorphous substrates. Further investigation and more detailed study of the hydrolysis by CBH I is necessary to properly evaluate the mechanism of action of CBH I on the substrate.

In summary, this investigation demonstrated the action of EG I on amorphous cellulose film, and identified several important aspects relevant to the study the kinetics of cellulose degradation. These include the importance of steady state conditions for a proper evaluation of hydrolytic action, impact of enzyme load on hydrolysis efficiency and inability of EG I to completely degrade amorphous cellulose model substrate.

4.3 Cellulase treatment of bleached hardwood pulp fibers

In this final part of the thesis, the enzymatic treatment of wood pulp fibers was evaluated to complement the fundamental studies of enzymatic hydrolysis carried out using cellulose model films. This investigation was also aimed to provide additional information to the research already available on the action and effects of cellulase treatment on softwood pulp fibers. The objective of this study was to evaluate the effect of a monocomponent commercial cellobiohydrolase CBH treatment on fiber strength and possible morphological changes of bleached hardwood kraft pulp using standard strength testing methods and high resolution SEM analysis. Contrary to the model film investigation, this study focused on more technical measures of the enzymatic hydrolysis, such as strength properties. The intention was to provide a more realistic link to the fundamental model surface approach. Evaluation of the action of CBH on fibers of different characteristics helps to further understanding of the mechanism of modification and, together with the fundamental studies on cellulose model substrates (e.g. films), the action of cellulases in general.

4.3.1 Evaluation of pulp and fiber strength properties

The treatment of cellulose fibers with cellulose degrading enzymes can have a detrimental impact on the fiber strength, compromising possible benefits of the treatment. Therefore, it was important to evaluate the impact of CBH treatment on the strength properties of the treated pulp fibers. The evaluation was carried out using standard measurements of tensile and tear indices and of zero-span testing. The tensile index is an indicator of the overall sheet strength and encompasses contributions from

individual fiber strength and interfiber bonding (Page 1969). The tear index at a given tensile index value is an indicator of pulp or paper web strength and it is commonly expressed in the form of a tear-tensile indices correlation. The comparison of measured tensile indices of all samples and the tensile/tear indices correlation are shown in Figure 33 left and right, respectively.

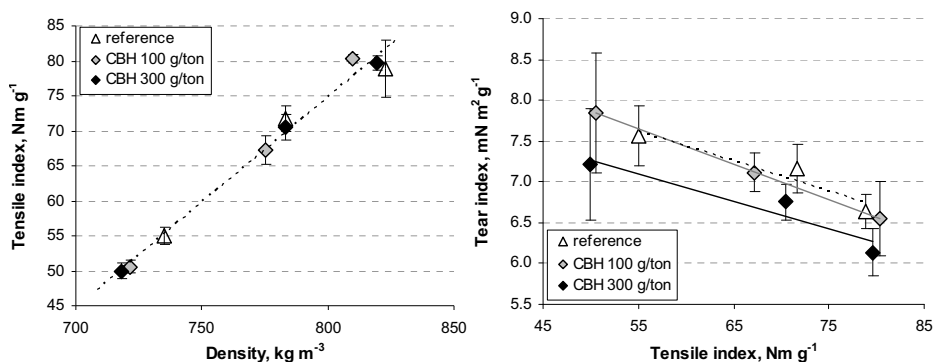


Figure 33. Comparison of for control and CBH treated pulp: Tensile index vs. apparent density relationship (left) and tensile and tear indices correlation (right)

Control and treated pulp samples were refined to two specific energy consumption levels (SEC 25 and 50 kWh ton⁻¹). Refining increased the density of the prepared paper sheets. The tensile indices at a given density were similar for both treated and control samples at both levels of refining. The values correlated well, in particular at lower density values, which correspond to levels of no refining and low refining for both CBH treated (at both dosages) and control samples. This indicates that the overall sheet strength of the treated pulp was not affected by the CBH treatment. The samples treated at low CBH dosage did not exhibit a marked difference in tensile-tear relation and correlated well with that of the control sample. The treatment at higher CBH dosage, however, showed a slight decrease of the tear index values at a given tensile index. Although the decrease was relatively small, the difference was consistent for both levels of refining. These observations agree with the development of tensile and tear indices previously reported for softwood pulps (Pere et al. 1995; Kibblewhite and Clark 1996; Oksanen et al. 1997b). In conclusion, the actual impact of the CBH treatment on the sheet strength properties of the pulp studied is very small.

The zero-span test is a direct measure of actual strength of individual fibers and can be measured for wet and dry samples. The summary of measured values for control and refined (reference and CBH treated) samples is shown in Figure 34.

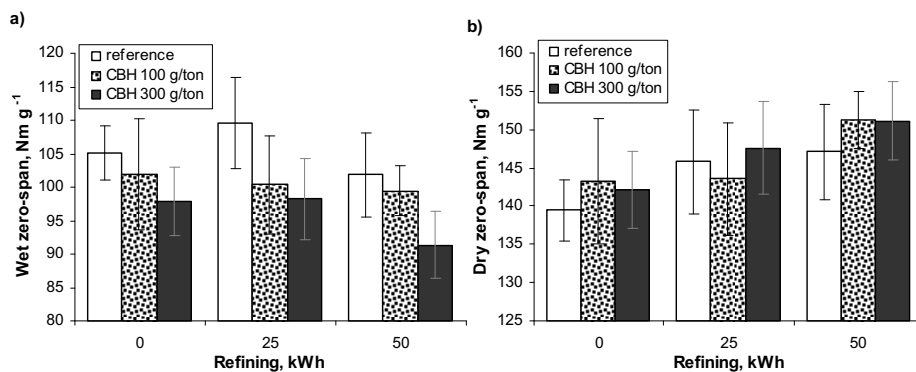


Figure 34. Effects of CBH treatment and refining on wet (a) and dry (b) zero-span tensile indices.

The wet zero-span testing (Figure 34a) showed a decrease in the measured values for the CBH treated samples at all refining levels. The value decreased with increasing enzyme dosage. A decrease in wet zero-span values as a result of extensive cellulase treatment of softwood pulp was reported previously (Mohlin and Pettersson 2002). It has been demonstrated that the zero-span tensile index of rewetted samples is more sensitive to localized cellulose degradation. The stress transfer between fibers that occurs in the dry state is eliminated, and thus the impact of fiber defects is greater in the wet state (Mohlin et al. 2003). The structurally irregular zones in the fiber wall are more susceptible towards enzymatic attack, which in turn affect the strength of the fiber (Gurnagul et al. 1992). It has been proposed that the irregular zones in the fiber wall are the preferential sites of cellulase action and dislocations also occur at these sites (Ander 2002; Nyholm et al. 2001).

The dry zero-span measurements (Figure 34b) showed an opposite tendency. The CBH treated samples measured slightly higher values than the reference (control) samples. Although relatively small, the trend is evident and consistent for both refined and non-refined samples. The dosage did not appear to have a pronounced effect on the increase in the measured values. An increase of the dry zero-span value after refining was predicted by Page (1985) and Seth and Chan (1999), and according to

Mohlin et al. (2003), the impact of fiber wall dislocation and localized cellulose degradations diminishes with drying. Nevertheless, the slight increase in the dry zero-span data after the CBH treatment was unexpected.

4.3.2 Evaluation of the Zero-span fiber fracture zone

To better understand the effect of CBH treatment on individual fiber strength, the tested wet zero-span strips were evaluated by SEM. The objective was to image the fracture zone of the tested strips at high magnification level (2000 \times) and visually evaluate possible differences in the fiber fracture mechanism between CBH treated and control samples. The measurement requires samples to be completely dry, therefore to prevent fiber alteration possibly caused by drying, the wet samples were freeze-dried prior to the measurement. The SEM images of the fracture zone of CBH treated (300 g prot. ton⁻¹) and control samples are shown in Figure 35.

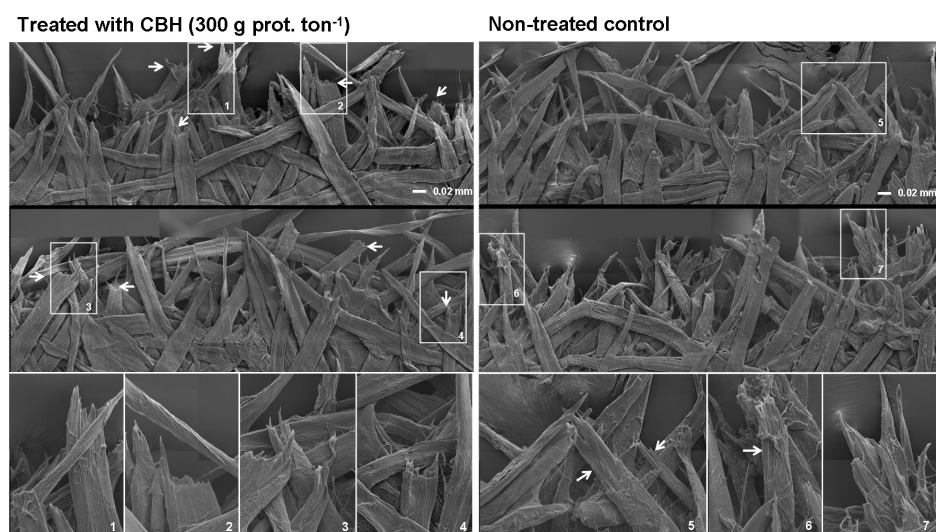


Figure 35. SEM images from the rupture zone of wet zero-span testing. Left: CBH (300 g prot. ton⁻¹) treated sample. Arrows indicate fibers with clean, straight fractures, hypothetically ascribed to the impact of CBH. Right: Non-treated control sample. Arrows indicate examples of fracture type predominant in control sample. Frames in the image represent magnified areas depicted in the lower part of the figure.

The comparison reveals differences in areas showing fiber fracture. On several occasions, the fractures of the CBH treated fibers appeared to be straighter in transverse direction. The arrows in Figure 35 indicate examples of such fractures. Although these types of fractures could also be found in the control sample and a statistical analysis of their distribution is not available, their increased presence in the CBH treated sample was evident. The major fracture type in the control sample was different. The fiber fractures did not appear to be straight, and on several occasions the outermost layer of the fibers was peeled or disrupted. The rupture zones of the individual fibers were much longer in axial direction, as shown in more detailed images in the lower part of Figure 35 (right).

It could be speculated that the stress applied to break the untreated control fiber is dissipated over the whole fiber, and thus the fiber can absorb a greater amount of energy. Consequently, more energy is required to rupture or break the fibers. In contrast, the enzyme treated samples are affected by the localized hydrolytic action of the CBH, which preferably targets the dislocation sites on fiber surfaces (Nyholm et al. 2001; Ander 2002). These localized points of cellulose degradation are the weak spots of the fiber. Once the fiber structure is disrupted at one point, the overall integrity and intrinsic strength of the fiber is compromised, leading to lower resistance toward the applied stress (zero-span value) and possibly resulting in a cleaner cut of the fibers.

4.3.3 Fiber surface morphology

The visual assessment of cellulose model substrates has clearly demonstrated the hydrolytic effect of cellulases (Chanzy et al. 1983; Hoshino et al. 1993, 1997; Samejima et al. 1998). The corresponding visual changes on surfaces of more complex wood pulp fibers were less evident after treatment with enzymes under industrially applicable conditions. SEM as a complementary method to the physical characterization of enzymatically modified softwood kraft fiber did not reveal any distinct alteration to the fiber surface (Mansfield et al. 1997). Similarly, no substantial effect on fiber morphology was observed on softwood kraft fibers treated by CBH to modify their papermaking properties (Dickson and Wong 1998).

In this evaluation, the CBH dosage and retention time were moderate in order to comply with the dosages intended for industrial application. To exaggerate the visibility of possible fiber surface alterations, an additional treatment at 900 g prot. ton⁻¹ CBH charge was carried out. Representative SEM images of each dosage trial are shown in Figure 36.

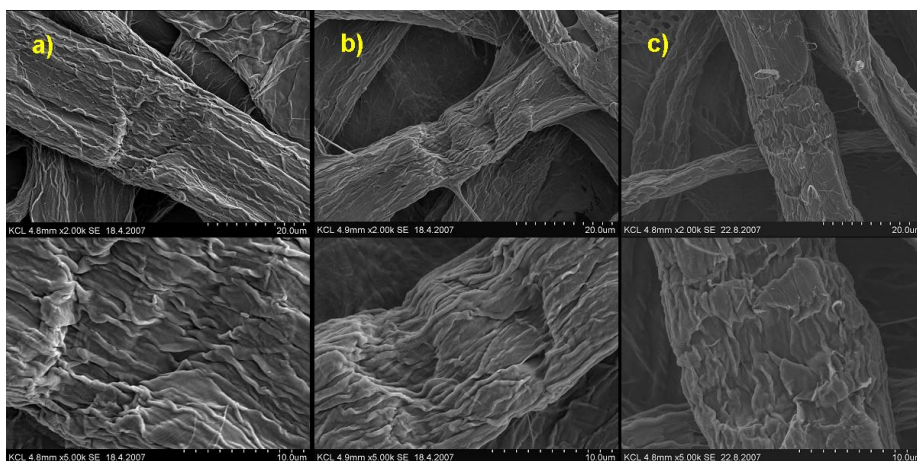


Figure 36. SEM images of CBH treated samples; dosage - 100 (a); 300 (b) and 900 g prot. ton⁻¹ (c); magnification: 2000× (top) and 5000× (bottom).

The initial observation of SEM images of CBH treated samples (100 and 300 g prot. ton⁻¹) did not reveal any distinctive surface alterations. However, compared to the reference sample, a noticeably greater amount of more apparent fiber wall dislocations was observed. Examples of these dislocations are shown in Figure 36a and b. The dislocations preferably occurred on fibers of smaller diameters, while the fibers of greater diameters and vessels did not appear to be affected. A closer examination of the images acquired at higher magnification showed that the outermost layer of the fiber wall was not interrupted and remained intact. This could indicate that the CBH possibly penetrates the outer layer of the fiber at the sites of original dislocations and proceeds with hydrolytic action to the inner layers of the fiber. The higher enzyme dosage treatment (300 g prot. ton⁻¹) resulted in somewhat increased occurrence of the fiber dislocations compared to the low enzyme dosage sample. The sample treated with the highest CBH dosage (900 g prot. ton⁻¹) showed, in addition to an increase in the number of dislocations, occasional disruptions in the form of cracks on the fiber surface (Figure 36c). It appears that the outer layer of the cell wall was

interrupted in several places, possibly exposing the S2 layer of the fiber wall. It is possible that these cracks are enlargements of structural irregularities which are originally present in the fibers prior to the enzymatic treatment. It has been previously suggested that the structurally irregular zones on the fiber surfaces are more susceptible towards enzymatic attack resulting in localized sites of degradation (Gurnagul et al. 1992). In addition, the localized action of cellulases preferentially acting on dislocations in softwood kraft pulp fibers was clearly visible by polarized light microscopy (Ander et al. 1996; Ander 2002).

It has to be noted that the SEM analysis of hardwood pulp fiber handsheets did not allow for a proper quantitative analysis of the dislocation occurrence on the fiber surface. However, their increased visibility and occurrence after high-dosage CBH treatment visually correlates well with the results of tear index and wet zero-span measurements. In both instances, the decrease in the measured values could be related to the increase in number of fiber wall dislocations.

5 CONCLUDING REMARKS

This thesis investigated two issues relevant to the bioconversion of lignocellulosic biomass. The first part relates to the accessibility of cellulose in wood, more specifically, the impact of initial drying on wood ultrastructure. Water accessibility of the substrate is a determining factor in degradation of cellulosic materials that occur in aqueous media, e.g., enzymatic hydrolysis. The second part of this thesis deals explicitly with the actual phenomenon of enzymatic hydrolysis of cellulose.

In the first part, it was demonstrated that the initial drying of the wood has an impact on wood ultrastructure. Although it has been long known that drying changes the mechanical properties of wood, the work of this thesis attempted to study these changes on an ultrastructural level. That was achieved by using a modified method widely used in the determination of cellulose accessibility - deuterium exchange coupled with FT-IR analysis. With this method, conversion of accessible deuterium exchanged OH groups in fresh wood and wood pulp fibers to inaccessible, reprotonation resistant OD groups during drying can be monitored. This conversion is then an indicator of the ultrastructure alterations. To study these alterations within a complex structure of native wood can be challenging, however, it was demonstrated that they can be reliably detected and quantified using this method.

The trends in the drying-induced alterations observed for wood and for pulps were similar; therefore the changes taking place during drying of pulp fibers and those occurring in the structure of native wood are similar processes. Based on the results of this investigation and previous studies on the drying of pulp fibers, a hypothesis was proposed that these ultrastructural alterations are results of the irreversible aggregation of cellulose microfibrils in a similar manner (but to a smaller extent) as those which occur during hornification of pulp fibers.

The behavior of cellulosic substrates with water was extended to a model surface in the subsequent part of the thesis. Thereafter, quantitative studies of cellulose degradation by cellulase enzymes were carried out using an amorphous cellulose model film with well-defined characteristics. First, the films were extensively

characterized, particularly their crystalline nature and swelling behavior. X-ray diffraction analysis demonstrated a lack of long range order in the atomic arrangement of cellulose, indicating that the film consists mostly of amorphous cellulose without any large crystallites.

This film was then used as a substrate in studying the action of monocomponent endoglucanase (EG I) that acts on amorphous cellulose. The study was carried out using a quartz crystal microbalance with dissipation monitoring (QCM-D). Quantification of the actual hydrolysis was done by measuring the film thickness reduction by Atomic Force Microscopy (AFM) after the enzymatic treatment. Thickness reduction of the film after the hydrolysis measured by AFM correlated well with the frequency change results obtained for the corresponding films in QCM-D.

The study showed that to properly evaluate the mechanism and kinetics of EG I action, steady state conditions in the experimental set up need to be reached. It was demonstrated that although the amount of substrate affects the absolute rate of hydrolysis, the relative rate of hydrolysis does not depend on initial amount of substrate. The enzyme was shown to work uniformly within the whole volume of swollen film; however, it was unable to fully degrade the amorphous film. In addition, a comparison between the action of monocomponent EG I and cellobiohydrolase (CBH I) on amorphous films was carried out. In contrast to EG I, CBH I exhibited only marginal hydrolytic action on films of amorphous cellulose.

Finally, the investigation of the action of a commercial cellobiohydrolase on wood pulp fibers was included to demonstrate the impact of cellulase on a complex structure of cellulose fiber. The study showed that the treatment had no significant impact on the strength properties of the pulp. Changes of fiber morphology and of fracture type in the treated fibers during zero span evaluation, however, were observed.

A better understanding of the mechanisms involved in ultrastructure alterations during initial drying of wood and cellulosic biomass and their subsequent impact on accessibility, as well as enzymatic hydrolysis of cellulose are vital in the light of current trends in alternative utilization of wood and biomass.

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