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Enzymatic hydrolysis of lignocellulose

substrate interactions and high solids loadings

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FACULTY OF LIFE SCIENCES
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PREFACE

This thesis describes the most important results achieved during my PhD study. The work was carried out at Forest & Landscape, Faculty of Life Sciences, University of Copenhagen in the period from November 2005 to November 2008. From June to September 2007, I spent three months with Novozymes Inc. in Davis, CA, USA. The thesis was submitted to Faculty of Life Sciences, University of Copenhagen on November 14, 2008 as part of the requirements of the academic degree of Doctor of Philosophy (PhD). The project was funded by the Danish Agency for Science, Technology and Innovation, contract 2104-05-0008.

Working in the exciting and quickly changing field of biofuels has been a thrill. I would like to thank my supervisors, associate professor Henning Jørgensen and professor Claus Felby for valuable guidance, support and enthusiasm. Their trust in me and freedom to pursue ideas in untraditional ways has been great.

The open and friendly atmosphere at Department 4 of Forest & Landscape has made for an excellent time. I would like to thank all colleagues and fellow students for three very good years. In particular my good friends Niels Peter K. Nielsen and Kiki L. Larsen; thank you for all the help, discussions, fun and beer - sharing office with you has been great. I also wish to thank Lisbeth G. Thygesen for invaluable help with spectroscopy and reading manuscripts; lab technicians Joanna M. Nielsen and Britta Skov for always helping me out with my samples whenever I needed it; and Mette Maj N. Kirsch for proof-reading.

Novozymes A/S and DONG Energy A/S (Inbicon A/S) have been industrial partners on the project and I thank them for their contributions and collaborations. In particular, I would like to thank Mai Østergaard and Jan Larsen at Inbicon, and senior researcher KC McFarland at Novozymes, Davis, for his contagious enthusiasm and many great discussions, making my stay in Davis very enjoyable.

Thomas Elder from the US Forest Service stayed with us several times during my PhD study. I would very much like to thank Tom for all his help, Christmas parties and crawfish in Louisiana - and for teaching me to do AFM microscopy!

To all my friends in flyfishing; thank you for keeping me relatively sane over these three years.

Finally, I would like to thank Majse for her love, support and patience.

Jan B. Kristensen
Copenhagen, November 14, 2008

LIST OF PUBLICATIONS

This thesis is based on the work presented in the following publications. They are referred to in the text by their roman numerals.

I Jørgensen, H., Kristensen, J.B., Felby, C.:
Enzymatic conversion of lignocellulose into fermentable sugars: Challenges and opportunities
Biofuels, Bioproducts and Biorefining 2007, 1(2):119-134.

II Kristensen J.B., Thygesen, L.G., Felby, C., Jørgensen, H., Elder, T.:
Cell-wall structural changes in wheat straw pretreated for bioethanol production
Biotechnology for Biofuels 2008, 1(5).

III Kristensen, J.B., Börjesson, J., Bruun, M.H., Tjerneld, F., Jørgensen, H.:
Use of surface active additives in enzymatic hydrolysis of wheat straw lignocellulose
Enzyme and Microbial Technology 2007, 40(4):888-895.

IV Kristensen, J.B., Felby, C., Jørgensen, H.:
Determining yields in high solids enzymatic hydrolysis of biomass
Applied Biochemistry and Biotechnology, DOI: 10.1007/s12010-008-8375-0.

V Kristensen, J.B., Felby, C., Jørgensen, H.:
Yield determining factors in high solids enzymatic hydrolysis of lignocellulose
Submitted for publication.

Publications contributed to, but not part of thesis (found in Appendix):

VI Felby, C., Thygesen, L.G., Kristensen, J.B., Jørgensen, H., Elder, T.:
Cellulose-water interactions during enzymatic hydrolysis as studied by time domain NMR
Cellulose 2008, 15(5):703-710.

VII Elder. T., Kristensen, J.B., Thygesen, L.G., Jørgensen, H.:
Hydrothermal pretreatment of wheat straw. Temperature effects
Submitted for publication.

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INTRODUCTION

It can no longer come as a surprise that the ecological impact of human beings is having a significantly adverse effect on the global environment. Depletion of fossil fuels has increased the carbon dioxide content of the atmosphere, causing a steady increase in global temperatures [1]. Combined with a dependency on increasingly scarce fossil fuels, this has sparked a tremendous interest in alternative renewable fuel sources. Consequently, there has been an emphasis on plant biomass as a source of fermentable sugars. Often being touted as the world's most abundant polymers, cellulose and other plant carbohydrates are believed to be the renewable energy source that can provide liquid fuels and chemicals on a sustainable basis, in turn displacing fossil fuels and decreasing carbon dioxide emissions [2].

One of the liquid biofuels receiving the most interest is bioethanol. Although attractive due to its simplicity, so-called first-generation bioethanol made from starch and sugar is now considered less desirable due to its alleged influence on food prices. Cellulosic bioethanol, also known as second-generation bioethanol, is seen as a more attractive alternative. It can be produced from all kinds of plant materials, ranging from corn stover and wheat straw to forest residues. Furthermore, cellulosic ethanol has the potential to produce large quantities of fuel with more significant reductions in greenhouse gas emissions [3]. The carbohydrates from plant biomass can also be utilised for a range of chemicals, thus providing a platform for biorefineries [4]. The main process steps involved in producing cellulosic bioethanol are illustrated in Fig. 1.

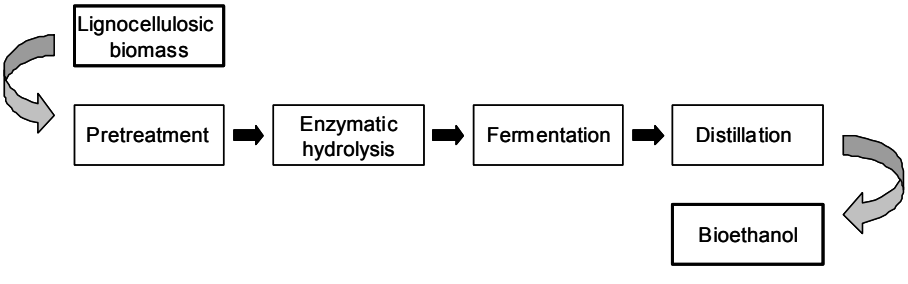

Figure 1*: Simplified flow sheet for ethanol production from lignocellulosic biomass. The pretreatment step prepares the biomass for the second step; enzymatic hydrolysis. After hydrolysis, sugars are fermented into ethanol. The two steps of enzymatic hydrolysis and fermentation may be combined into a single processing step known as simultaneous saccharification and fermentation (SSF). Lastly, the generated ethanol must be isolated through distillation.*

Despite intensive research over the last decades and investments in bioenergy-related biotechnology having sky-rocketed in recent years [5], no full-scale, commercial cellulosic ethanol plants exist today. Although several plants are planned or are under construction in a number of countries, several technical challenges remain. Plant cell walls are highly recalcitrant to degradation, both microbially and mechanically, and one of the main challenges is concerned with enzymatic conversion of cellulosic plant biomass into fermentable sugars [6,7].

To address this challenge of efficiently hydrolysing cell walls into fermentable sugars - also known as saccharification - two key aspects of cellulosic bioethanol production have been improved upon: Pretreatment and enzyme optimisation. Pretreatment is a balancing act that involves unlocking the cell wall structure without forming inhibitors, which affect hydrolysis and/or fermentation [8]. Being an insoluble and highly heterogeneous substrate, cellulosic materials pose several challenges in enzymatic conversion. However, recent years have shown important advances in understanding, improving and producing synergistic cellulases [9,10]. Although both enzyme systems and the understanding of cell walls are still subject to improvement [11], it is believed that an improved understanding of the mechanisms where the two areas come together is needed in order to further increase the viability of bioethanol production. This view was recently reinforced in a paper on focus areas vital to advancing cellulosic bioethanol where the author stated: “A better understanding of the factors that control the interactions of substrates and enzymes would be invaluable in identifying pathways to better systems” [12].

Objective and outline of thesis

The main objective of the work presented in this thesis has been to gain a better understanding of enzyme-substrate interactions in the enzymatic hydrolysis of pretreated biomass. An efficient conversion of lignocellulose into fermentable sugars is a key step in producing bioethanol in a cost-effective and environmentally friendly way.

Microscopic and spectroscopic work was performed on hydrothermally pretreated wheat straw in order to understand how pretreatments increase enzyme digestibility. The results of these investigations are presented in **Paper II**. The work has also included investigating the effect of surface active additives, or surfactants, in enzymatic hydrolysis, as seen in **Paper III**. The ability to work at high solids concentrations has been thoroughly investigated and is a vital component in the viability of ethanol production. In **Paper IV**, the challenges of determining yields in high-solids enzymatic hydrolysis are presented. **Paper V** deals with the factors responsible for

decreasing yields when hydrolysing lignocellulose at increasing solids concentrations.

Together with the review of **Paper I**, the first four chapters of this thesis provide background information and put the work in perspective. These chapters include an introduction to cellulosic biomass and how its composition and structure influence the recalcitrance to hydrolysis, pretreatment methods, enzymatic hydrolysis and surfactants. Some of the results presented in the published papers are included and discussed. The final chapter provides a more detailed discussion on enzyme-substrate interactions in high solids enzymatic hydrolysis. There is some overlap between **Paper V** and the final chapter of this thesis. However, the results are more extensively discussed and elaborated on in the thesis.

LIGNOCELLULOSIC BIOMASS

A defining feature of terrestrial plants is the highly developed cell walls. It is indeed these cellulosic cell walls that are the target of our bioconversion, whether for the purpose of production of liquid fuels or chemicals. The plant cell wall is described as a dynamic structure and is a complex composite of polysaccharides, aromatic compounds and proteins. In general, the three major constituents of secondary cell walls are cellulose, hemicelluloses and lignin; from which the generic term *lignocellulose* is coined. In order to efficiently convert the carbohydrate polymers into the desired products, it is important to understand the composition, nature, structure and interactions of these cell wall components and how they affect the degradability of lignocellulosic biomass

Composition, structure and recalcitrance

The resistance to deconstruction of plant biomass is often referred to as "biomass recalcitrance", a term that has become well-known in the field of liquid biofuels [7]. This recalcitrance is a result of the way terrestrial plants have evolved over time, in part turning the plant cell wall into an efficient barrier against intrusion and degradation. Not only does the structural arrangement prevent decomposition but some components of the cell wall are able to retard enzymatic hydrolysis themselves.

The load-bearing framework of the cell wall is the cellulose fibrils. Being the most abundant polymer and entirely made up of fermentable glucose units, cellulose is the main target for bioconversion. The D-glucose units of cellulose are linked by β-1,4-glucosidic bonds into a linear structure with a high degree of polymerisation. This structure results in the formation of intra- and intermolecular hydrogen bonds, creating para-crystalline cellulose fibrils [13], see Fig. 2. The 3-5 nm wide elementary fibrils adhere together to form micro- and macrofibrils up to 200 nm wide, that spool around the cell, often in a parallel manner [14]. In addition to being chemically stable and resistant to microbial degradation, the cellulose fibrils are responsible for the great tensile strength of the cell wall [15]. This structural and inherent integrity of cellulose is believed to play an important role in the recalcitrance of lignocellulosic biomass [16]. It is generally accepted that cellulose can be crystalline, para-crystalline and even amorphous. However, the detailed crystal and allomorphic structure of native cellulose and how it influences degradability is still a matter of investigation and debate [14,17,18].

Cellulose fibrils are often coated with complex, heterogeneous polymers collectively known as stereo-irregular polysaccharides or hemicelluloses. The specific structure and composition of hemicellulose largely depends on plant genus and cell type. For example, in grasses (*Poales*), the most

common hemicellulose is the branched polymer glucuronoarabinoxylan (GAX) [19]. The main function of hemicellulose as a constituent of the cell wall is two-sided. Through cross-linking it creates distance between cellulose fibrils, maintaining a certain degree of flexibility in the cell wall by avoiding cellulose fibrils to adhere to each other. However, at the same time the cross-linking helps anchor the cell wall matrix together [19]. Apart from hexose sugars (C-6 sugars; D-glucose, D-mannose, D-galactose and D-glucuronic acid) hemicelluloses also consist of pentose sugars (C-5 sugars; primarily D-xylose and L-arabinose), that are not readily fermentable by yeast. In general, agricultural products such as wheat straw and corn stover, as well as hardwoods, have higher contents of C-5 sugars than softwoods**.**

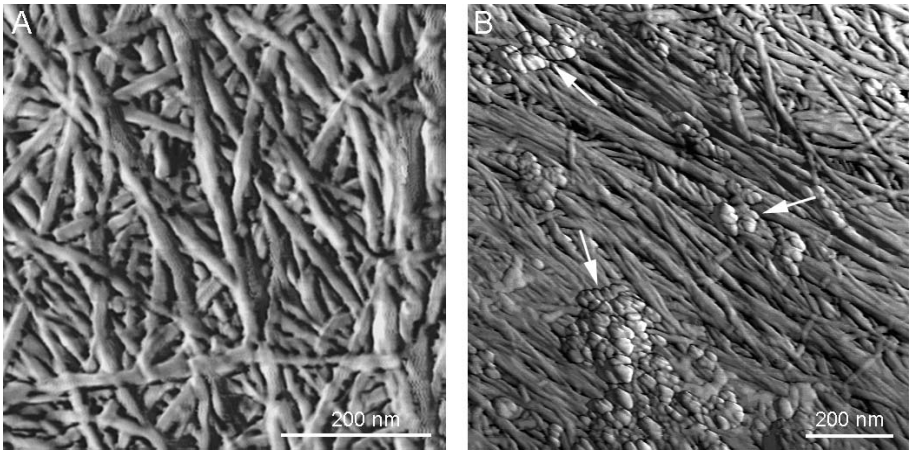

Figure 2*: Atomic force microscopy (AFM) tapping mode images of wheat straw parenchyma cell walls. A: The interwoven cellulose microfibrils that constitute the load-bearing structure of the primary cell wall are often cross-linked to hemicelluloses through covalent bonds. B: In secondary cell walls, the fibril orientation is highly parallel and fibrils coalesce into macrofibrils or cellulose aggregates that are encrusted in a matrix of hemicelluloses and lignin. For visualisation purposes the cell wall in B was partially de-lignified. Lignin deposits are seen as spherical shapes (white arrows) on top of the macrofibrils.*

After cell expansion, some cell walls are reinforced with thickenings to stabilise their structure. These secondary cell walls are deposited on the inside of the primary wall and on a weight basis, cells with secondary walls make up the majority of plant biomass. A significant difference between primary and secondary cell walls is that the polysaccharides of secondary walls are embedded in an aromatic polymer that is highly resistant to chemical and microbial attacks. This highly complex network of phenyl propane units is known as lignin. The addition of lignin to the cell wall

drastically increases mechanical strength, being responsible for terrestrial plants having evolved so successfully. However, from a bioconversion viewpoint lignin and low molecular phenolics are highly problematic; degree of lignification is often a limiting factor in degradability. This is not only due to lignin not being a carbohydrate and therefore not reducible to fermentable sugars, but also its ability to covalently bond to and mask carbohydrates, hindering enzyme accessibility and hydrolysis [20,21]. The phenolic groups of lignin may also be involved in inhibition [22]. Also, lignin may prevent the cell wall from swelling significantly, thus restricting enzyme accessibility [23]. As will be discussed in subsequent chapters, lignin has been shown to negatively influence enzymatic hydrolysis by adsorption of cellulose degrading enzymes [24,25].

In both grasses and woody plant species, the secondary cell wall of cellulose fibrils and hemicellulosic polysaccharides that are bound to the encrusting lignin can be described as a composite matrix. The ultrastructure of this complex matrix can be compared to that of fibreglass, where the glass fibres with their high tensile strength are embedded in a hard crust of epoxy. The spatial distribution as well as extent and nature of cross-linking are import factors in the accessibility and degradability of cell walls. However, knowledge about the dynamics and specificity of interactions between carbohydrates, lignin and proteins in the cell wall is relatively small [26].

The composition, micro-structural heterogeneity and complexity of the cell wall are often significantly determined by the cell type. Thus, the degradability of the lignocellulose is also governed by these factors. Although often ignored, the macro-structural characteristics of tissue type must be considered when investigating recalcitrance [27]. The diversity and number of plant tissues and cell types is enormous but can be separated into three general categories of dermal, vascular and fundamental tissues [16]. The epidermis cell surface layer is the first barrier of the plant and protects the cell from intrusion. It varies from being a thick bark layer in woody species to a wax-covered cuticle in grasses. Phloem, xylem and their associated cell types make up the vascular tissues. These highly specialised cells are responsible for transport of water and nutrients in the plants and can be heavily lignified and therefore difficult to break down [21,28]. From a processing point of view, the most interesting categories of cells are the fundamental tissues, as these generally make up the bulk of the mass of the lignocellulosic material. This is particularly the case for the supportive tissue of sclerenchyma, composed of sclereids and fibres. Not to be confused with fibrils, fibres are long, slender cells that make up the principal supportive tissue. When fully mature, the dead cells of sclerenchymatous tissue have thick secondary walls that are lignified to various extents depending on species [29]. For an example of the distribution and occurrence of various cell types found in grass species, see Fig. 3.

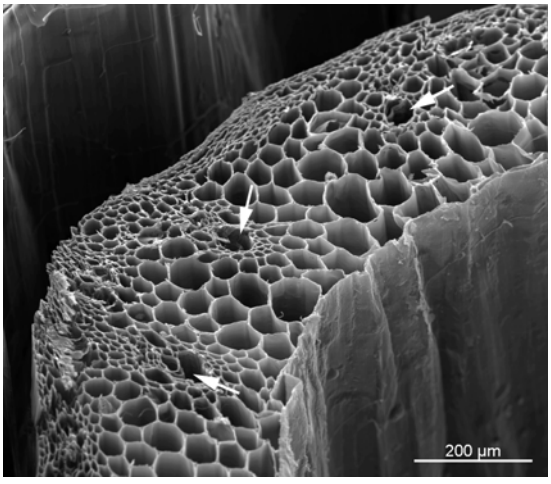

Figure 3: *Scanning electron microscopy (SEM) image showing a number of different cell types in a cross section of a wheat straw stem. White arrows indicate the location of three vascular bundles made up by phloem, xylem and surrounding thick-walled sclerenchyma cells (fibres). Between the vascular bundles, easily-digestible large parenchymatic cells with thin walls can be seen. Towards the outer edge of the straw stem (left side of image) there is a layer of small diameter fibres with thick secondary walls. On the inside of the stem towards the pith is a thin, smooth layer of rudimentary parenchyma cells. This layer of unlignified cells has proven beneficial in studying the ultrastructure of primary cell wall microfibrils (**Paper II**).*

Recently, microscopic investigations have indicated that the secondary cell wall of sclerenchymatous tissue is not a homogeneous matrix but rather constructed of concentric lamellae of cellulose aggregates [30,31]. As secondary cell wall architecture affects lignin distribution and in particular enzyme accessibility - two factors that play a key role in cell wall degradability - a better understanding of the macro-structural characteristics of the secondary cell wall is likely to increase our ability to degrade it. As discussed in Chapter 3, enzyme accessibility of the cell wall substrate is most often determined by the pretreatment that the biomass undergoes before enzymatic hydrolysis.

Most agricultural crops have been optimised for producing starch (rather than biomass) over many centuries and as already touched upon; plants have evolved to successfully resist microbial degradation. Reducing production and especially conversion costs through plant genetic engineering is believed to be able to significantly improve the potential for plant biofuel production [7,32]. Traits that may be improved upon include cellulose or biomass yield

and compositional characteristics such as reduced or altered lignin and hemicellulose contents, and even plants that produce cell wall degrading enzymes themselves [33,34].

EFFECT OF BIOMASS PRETREATMENT

In order to efficiently hydrolyse hemicellulose and cellulose to fermentable monomeric sugars, a form of pretreatment is necessary. The chemical, physical and morphological characteristics of lignocellulose are important to the digestibility of the substrate. Pretreatment changes these characteristics and in particular makes the material more accessible for the saccharifying enzymes. Thus, pretreatment is considered one of the most crucial steps in bioethanol production since it has a large impact on all other steps in the conversion process. Also, pretreatment is usually an energy-intensive process step, significantly affecting the cost of the process.

Over the last decades, much research has gone into a range of pretreatment technologies but as of yet, no single technology has proven ideal or superior for all substrates. The optimal pretreatment technology should have a number of features. The digestibility and recovery of both cellulose and hemicellulose should be high and at the same time, no or little lignin and hemicellulose-derived inhibitors should be generated. Also, water, chemical and energy usage should be minimised and the method must be scalable to industrial size. Finally, the downstream process method must be considered when evaluating the best pretreatment method. Often, pretreatment is a balancing act of improving digestibility while keeping hemicellulose loss and inhibitor formation at a minimum.

Pretreatment methods utilise the following methods of modification: Physical (e.g. milling, grinding), chemical (e.g. alkali, dilute acid), thermal (e.g. steam treatment) and biological methods (e.g. wood degrading fungi). Often, technologies will be comprised of a combination of the above. Pretreatments such as steam pretreatment, acid-catalysed pretreatment and various treatments under alkaline conditions are among the most widely reported and most promising technologies (**Paper I**). A detailed description and comparison of the various technologies is beyond the scope of this thesis and the reader is referred to a number of reviews [8,35-37]. Instead, focus will be on the effect of pretreatment in terms of how it improves the enzymatic degradability through a modification of the lignocellulosic substrate. This modification has a large influence on the interaction between substrate and enzymes and is therefore an important aspect to consider in substrate-enzyme interactions.

Different pretreatment technologies rely on different mechanisms or principles for lowering the recalcitrance of the substrate. For example, dilute acid and steam explosion methods partially hydrolyse and solubilise hemicelluloses where other methods aim at removing lignin from the material. Both approaches have been proven to be effective in increasing cellulose hydrolysis [38-41]. The general idea of pretreatment is to increase surface area and enzyme accessibility through changes in porosity and

particle size, and to a lesser degree decrease cellulose crystallinity. As visualised in Fig. 4, the current model of pretreatment explains that it results in partial removal of lignin and hemicellulose and decomposition of the cell wall matrix.

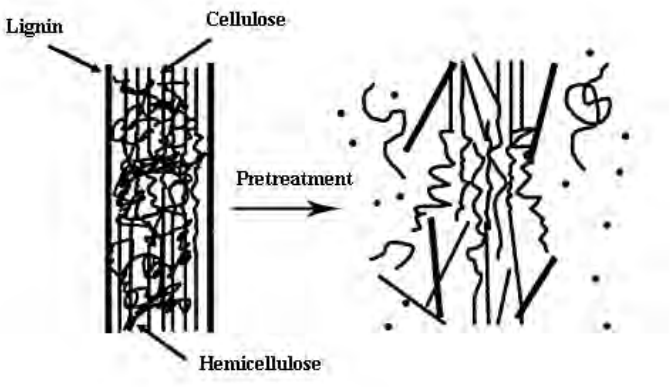

Figure 4*: Simplified representation of pretreatment. This model has previously been used to explain to effects or results of pretreatment. Lignin and hemicellulose is seen to be released while a decomposition of the structural network of the plant cell wall takes place. It has later been found that a structural decomposition is not necessarily a requirement for making the substrate digestible. Schematic figure adapted from [36] who adapted it from [42].*

When developing and comparing pretreatment methods a severity factor based on temperature, duration of treatment and pH can be calculated [8]. However, this rather empirical way of assessing a certain method does not tell anything about the actual effect on the cell wall structure and chemistry. In **Paper II** , we investigated the structural changes from hydrothermal pretreatment of wheat straw. The reasoning behind the investigation was that a better understanding of the effect or mechanism of the pretreatment would allow us to optimise the energy-intensive pretreatment process. The hydrothermal pretreatment is a form of steam pretreatment and is described in detail elsewhere [43-45]. Atomic force microscopy (AFM), scanning electron microscopy (SEM) and attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) was used for the investigation. AFM in particular has over the last decade been shown to be an effective tool in studying the ultrastructure of plant cell wall [46,47].

We found that neither the overall cell wall nor the fibrillar structure of the cell wall was ruptured or decomposed (**Fig. 2D-2I, Paper II**). This was not in accordance with the general perception that pretreatments must disrupt the skeletal cell wall structure (Fig. 4). Similarly, no signs of cell wall rupture in steam explosion pretreated wheat straw were identified (**Fig. 2J-L, Paper**

II). This reinforces the view that the "explosive" action of a quick pressure release is not necessary to render the material more suitable for enzymatic hydrolysis [8]. Interestingly, more than 90% of the surface of the hydrothermally pretreated fibres was covered with lignin. Although the pretreatment likely increases accessibility and porosity through solubilising a significant portion of the hemicelluloses, this re-localisation of lignin is believed to be important for the digestibility of the material. With such a large percentage of the surface masked by lignin, it may seem surprising that the digestibility of the substrate has been significantly increased. However, the lignin re-deposited on the surface likely exposes cellulose fibril surfaces inside the cell wall matrix. It is also possible that the re-deposited lignin is not as strongly attached to polysaccharides as in the native material and can be removed by shear forces during mixing. In conclusion, hemicellulose and lignin modification or re-localisation appears to be more important for enzymes accessibility and digestibility than actually disrupting the cell wall.

The need to examine the spatial or structural arrangement of the components and not just the overall composition when investigating the effect of pretreatment was recently reinforced when looking at samples pretreated at different temperatures (**Paper VII**, manuscript in Appendix). It was found that the lack of changes in overall chemical composition does not necessarily correspond to a lack of effect on conversion.

CELLULASES AND ENZYMATIC HYDROLYSIS

Cellulose in nature is mostly decomposed by a range of cellulolytic fungi and bacteria. It is the cellulases of these microorganisms that are being used for a number of industrial purposes, e.g. cotton processing, detergent enzymes and paper recycling. If (or when!) cellulosic biofuel becomes a major transportation fuel, cellulases will become the most produced industrial enzyme [48]. In this thesis, only the well-characterised enzyme system of the aerobic fungus *Trichoderma reesei* (an asexual, clonal derivative of the ascomycete *Hypocrea jecorina* [49]) will be considered. For information on bacterial and thermostable cellulase systems, see [50,51] and [52,53] respectively.

The cellulase enzyme system

Although cellulose is a homopolymer, a number of enzymes are needed to degrade it. These can be divided into the following three types: Endoglucanases (EG) (EC 3.2.1.4), which hydrolyse internal β-1,4-D-glucosidic linkages randomly in the cellulose chain; Cellobiohydrolases (CBH, also known as exoglucanases) (EC 3.2.1.91), which progress along the cellulose line and cleave off cellobiose units from the ends; β-glucosidases (BG) (EC 3.2.1.21), which hydrolyse cellobiose to glucose and also cleave off glucose units from cello-oligosaccharides. These three groups of enzymes work synergistically to degrade cellulose by creating new sites for each other and preventing product inhibition [54,55]. The concerted action of cellulases ensures an efficient hydrolysis and diminishes product inhibition. A simplified diagram of enzymatic hydrolysis can be seen in Fig. 5.

In total, *T. reesei* produces two CBHs, five EGs and two BGs. Several of these apparently redundant enzymes have been shown to exhibit synergy by either hydrolysing different ends of the cellulose chain or exhibiting different affinities for different sites of attack [56,57]. Cellulases are classified in glycosyl hydrolase families based on their sequence homology and hydrophobic cluster analysis according to the CAZy classification system (carbohydrate active enzymes). An updated list of these families is kept in the CAZy-database [58]. *T. reesei* also produces a range of hemicellulases. Being a diverse group of heterogeneous polymers with various side groups, the hemicellulosic system is more complex and involves for example xylanases, mannanases, arabinanases and various esterases. Depending on substrate and pretreatment, hemicellulases can be crucial for efficient hydrolysis of the cell wall. Hemicellulases are not the focus of the thesis but are reviewed elsewhere [59].

Enzymatic hydrolysis

Cellulases distinguish themselves from most other classes of enzymes by being able to hydrolyse an insoluble substrate. To facilitate sufficient contact and orientation between the catalytic domain (CD) and the substrate, CBHs and EGs have a cellulose-binding domain (CBD - or the broader term carbohydrate-binding module, CBM). The CBD is connected to the catalytic domain with a glycosylated flexible linker. This modular feature of cellulases is critical to their ability to dock with and degrade crystalline cellulose. CBDs have little impact on the activity of cellulases on a soluble substrate (such as carboxymethyl cellulose), but removal of the CBD from the enzyme significantly impairs the hydrolysis of crystalline cellulose, demonstrating the importance of CBDs [60]. CBDs of cellobiohydrolases are able to move laterally along the cellulose chain while the CD cleaves off cellobiose units [61]. Interestingly, CBDs appear to have a disruptive effect on cellulose fibrils, an ability that is still being discussed [62-64]. Furthermore, different CBDs appear to promote hydrolysis at different sites on cellulose [65]. However, despite extensive research on the mechanisms of CBDs, such as how the aromatic residues of the CBD are able to interact with the cellulose crystal structure [66], little is known about their ability to desorb from the substrate and re-attach.

Due to the insoluble nature of native cellulose and anchoring of CBDs, cellulases primarily work in a two-dimensional environment involving the uni-directional movement of cellobiohydrolases along the cellulose chain. Thus, with this lack of parameters normally used for evaluating enzyme kinetics (substrate concentration, freely diffusing enzymes) it is not surprising that the synergistic degradation of lignocellulose does not follow classic Michaelis-Menten kinetics. The understanding of mechanisms involved in the interfacial solid-liquid hydrolysis system is further complicated by a number of factors that include the heterogeneous nature of lignocellulose. Even when working with model substrates (e.g. filter paper), the interaction between enzyme system and substrate creates innumerable factors, which influence the rate and extent of the hydrolysis. These factors often cannot be fully isolated, even under lab conditions. Much research has gone into cellulase kinetics, resulting in various models [55,67,68]. Unfortunately, few have been able to extensively predict limiting factors or process design optima through kinetic models.

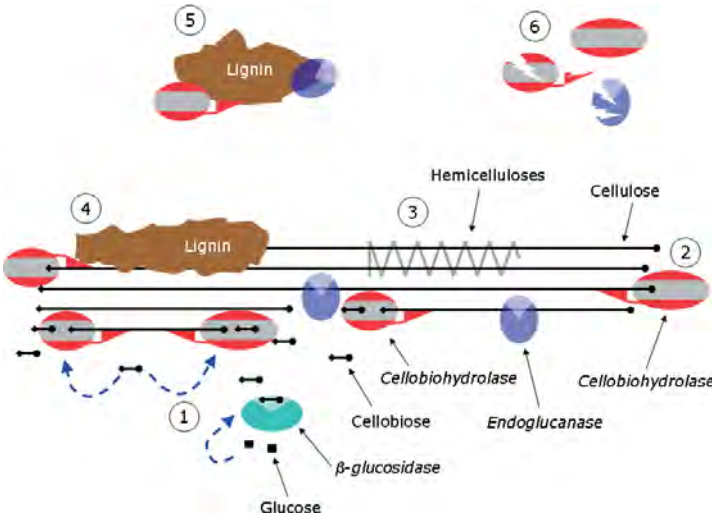

Figure 5: *Simplified diagram of lignocellulose hydrolysis showing synergism and limiting factors. Cellulose is symbolised by straight lines. 1: Released glucose and cellobiose cause product inhibition of β-glucosidase (BG) and cellobiohydrolase (CBH). 2: CBH hydrolysing from the end of a cellulose chain. Due to the processivity of CBH and the binding of the cellulose chain in their catalytic core, obstacles are thought to be able to halt the enzymes or cause them to become unproductively bound. Endoglucanases (EG) hydrolyse internal linkages in cellulose strands, thereby creating new sites for CBH hydrolysis. 3 and 4: Depending on pretreatment type and efficiency (and type of substrate), lignin and hemicelluloses can mask cellulose surfaces and prevent cellulases from reaching the substrate. 5: Both cellulases and hemicellulases can unproductively adsorb onto lignin particles or surfaces. 6: Mechanical shear, proteolytic activity or low thermostability can cause denaturation or loss of enzyme activity. Figure from* ***Paper I****.*

With the advent of SEM and in particular AFM, it has become possible to directly follow or see the effects of cellulases on cellulosic substrate. For example, Lee and co-workers found that endoglucanase has a smoothing effect on cellulose surfaces whereas cellobiohydrolase caused grooves or tracks along the macrofibril [62]. Our initial investigations have shown that unlignified cell walls (e.g. pith parenchyma cells) are easily hydrolysed and that the degradation generally appears to occur on the cell surfaces, essentially making the cell walls gradually thinner and thinner (unpublished). This is in accordance with the findings of Wang and co-workers [69]. As methods continue to improve, ultramicroscopy is likely to give more insight into the mechanisms of enzymatic hydrolysis.

SURFACTANTS

Enzymatic hydrolysis of lignocellulose into fermentable sugars is one of the most costly steps in bioethanol production, mainly due to the price of cellulases and the high dosages needed. Being able to reduce enzyme loading is thus very desirable. It has been shown that certain additives, in particular surfactants, have a positive effect on enzymatic hydrolysis of lignocellulose. Surfactants (**surf**ace **act**ing **a**ge**nts**) are amphiphilic compounds that usually contain a hydrophilic head and a hydrophobic tail. Surfactants are able to self-assemble into micelles and will adsorb onto surfaces. The assembly and degree of adsorption depends on the surfactant structure and the polarity of the surface. The linear polymer poly(ethylene glycol) (PEG) is an example of a non-ionic surfactant with numerous medical and industrial applications.

The effects of non-ionic surfactants and non-catalytic protein in enzymatic hydrolysis have been investigated for more than two decades. It has been shown that such surfactants increase hydrolysis efficiency significantly, allowing for either a faster hydrolysis rate or lower enzyme dosage [70-75]. Addition of surfactants also allows for better recycling of cellulases [76,77]. The positive effect of surfactants has been observed in enzymatic hydrolysis of both cellulose [73] and lignocellulose, as well as in simultaneous saccharification and fermentation (SSF) [78] and even pretreatment [79].

Only recently have the mechanisms of surfactant additives in enzymatic hydrolysis been investigated more intensively [25,80]. If surfactants are to be used commercially in the hydrolysis of lignocellulose, then it is important to fully understand how they work in order to optimise process designs and enzymes. The mechanisms that have been proposed responsible for the effects can be categorised in the following three groups: 1) Surfactants may act as enzyme stabilisers and prevent denaturation. 2) Surfactants may have an effect on substrate structure, i.e. a surface structure modification or disruption that increases enzyme accessibility. 3) Surfactants may affect enzyme-substrate interactions, in particular by preventing non-productive adsorption of enzymes.

Results indicate that surfactants do act as stabilisers, in particular at elevated temperatures [81-83]. Likewise, there have been indications that surfactants could promote the number of reaction sites through surface disruption, in turn increasing the hydrolysis rate [81]. However, more recent research has not supported this theory [25]. Some experiments have shown little or no effect on the hydrolysis of pure cellulose substrates [25,80,83]. This is in contrast to lignin-containing substrates where the effect of surfactant addition is significant, in some specific cases resulting in almost doubling of the yield [83]. Lignin thus appears to be involved in the primary

mechanism behind the surfactant effect. As already touched upon, lignin is known to have adverse effects on cellulases and their performance, in particular due to its ability to adsorb enzymes [84].

More specifically, there is general consensus that the primary mechanism behind the increased hydrolysis efficiency is due to the hydrophobic interaction between lignin surfaces and surfactants [80]. The hydrophilic portions of the bound surfactant protrude into the aqueous solution and sterically hinder the non-productive adsorption of cellulases, and in this way helps increase cellulose conversion. In accordance with this theory, adding non-catalytic protein such as bovine serum albumin (BSA) has a similar effect to the addition of non-ionic surfactants [25,85]. BSA is known to adsorb to surfaces, reducing unspecific binding by "filling up" adsorption sites on lignin surfaces [86]. The CBDs of cellobiohydrolases have been shown to be the major contributing factor responsible for lignin adsorption [87], but both the structure and properties of the CBD as well as the catalytic domain are involved in the binding affinity [80].

In **Paper III**, we investigated the use of surfactants in enzymatic hydrolysis of pretreated wheat straw, and in particular the relationship between the type of pretreatment and the increase in hydrolysis caused by the surfactant. We found that despite a lower content of hemicelluloses in straw compared to softwoods, the additives generally had a positive effect on both glucose and xylan conversion, of up to a 70% increase. Interestingly, the various surfactants had the most pronounced effect on straw that had been pretreated with sulfuric acid. Without surfactant, the acid-treated straw showed the lowest conversion (**Fig. 1, Paper III**). This shows that although lignin is known to be a limiting factor in lignocellulose conversion, the content of lignin is not directly proportional to the effect of surfactants on the hydrolysis of the material. In our experiments, the lignin content of the differently pretreated wheat straw varied from 19.6% to 24%. It is possible that sulfuric acid-treated wheat straw has a high capacity for (non-productive) enzyme adsorption, which would be in agreement with recent studies on corn stover [85]. These results indicate that the acid pretreatment renders the lignin more receptive to cellulase adsorption, possibly through a change of surface properties such as hydrophobicity. It is also possible that the increased solution of hemicelluloses by acid exposes more lignin, increasing accessibility and adsorption. Comparison of the different surfactants revealed that no individual surfactant appeared to be optimal for all types of pretreatment. However, PEG 6000 generally performed well with all pretreatments. Also, our results showed an increase in enzyme activity in solution due to addition of surfactants, which was comparable to the increase in cellulose conversion (**Figs. 1A and 4, Paper III**). The finding of this relationship between bound enzyme and hydrolysis yield supports the

current theory that the main mechanism behind the surfactant effect is prevention of unproductive enzyme adsorption to lignin.

Although the surfactant-lignin mechanisms are still being investigated, it is important to also consider the enzyme-lignin relationship. In the light of the significance of non-productive adsorption of cellulases on lignin, Berlin and co-workers introduced the concept of weak lignin-binding enzymes [88]. Through protein engineering it may be possible to develop low lignin-affinity enzyme systems.

An extractive pretreatment where lignin is removed may appear to be the solution to the non-productive adsorption problem. However, such as pretreatment is usually chemical-intensive and involved the generation of hemicellulose-derived inhibitors [89]. The Canadian company Iogen has successfully tested an organosolv process with softwoods at pilot plant scale [90,91].

Whether surfactants are going to be used in full-scale industrial enzymatic hydrolysis of lignocellulose will depend on the price of enzymes and surfactants. Since cellulosic bioethanol is still in its infancy on an industrial scale, many of the factors involved are difficult to determine. For the possible implementation of surfactants it is however important to consider the process design. For example, the addition of surfactants is likely to facilitate efficient enzyme recycling, a process step that ideally needs to be considered before the construction of the plant.

HIGH SOLIDS ENZYMATIC HYDROLYSIS

Maintaining high solids concentrations throughout the conversion process from biomass to ethanol is important from an energy and economic viability viewpoint for several reasons. By increasing the solids loading, the resulting sugar concentration and consequently ethanol concentration increase, with significant effects on processing steps and costs, particularly distillation [92,93]. If the fermentation broth contains a minimum of 4% (w/w) ethanol, the energy required for the distillation is significantly reduced [94]. To reach this ethanol level, a sugar concentration of at least 8% (w/w) is required. Depending on the type of lignocellulosic substrate, this in turn requires a minimum initial solids (dry matter) content of approximately 20% (all solids concentrations are given as total solids on a *w/w* basis) [45].

Lower water content also allows for a larger system capacity, less energy demand for heating and cooling of the slurry as well as less waste water [95]. Model-based estimations have shown significant reductions in operating costs of simultaneous saccharification and fermentation (SSF) of pretreated softwood when the initial solids concentration was increased [93]. High-solids enzymatic hydrolysis has been defined as taking place at solids levels where initially there are no significant amounts of free liquid water present [96].

Unfortunately, there are also disadvantages by increasing the substrate concentration. Concentrations of end products and of inhibitors will increase, causing enzymes and the fermenting organism to function less optimally. High-solids loadings can also cause insufficient mixing or excessive energy consumption in conventional stirred-tank reactors as the viscosity of slurries increases abruptly at increased solids loadings [97,98].

High solids concentrations also present new challenges in measuring and determining the degree of hydrolysis (conversion yield). During the complex hydrolysis reaction where insoluble biomass is subjected to a liquefaction process, the solids level decreases while the density and volume of the liquid phase increases, although not at the same rate. Due to the water retention properties of lignocellulosic biomass, the initial cellulose concentration is often measured as weight per weight (e.g. g/kg), whereas the products are mostly measured as weight per volume (e.g. g/L). As shown in **Paper IV**, if these factors are not taken into consideration when calculating the percent-of-theoretical yield, the result is often a significant overestimation. To overcome this problem, we demonstrated a non-laborious method for approximating yields in high solids hydrolysis (**Paper IV**).

Water content in the hydrolysis slurry is directly correlated to rheology, i.e. viscosity and shear rate during mixing [99], which is important for the interaction between lignocellulose and cell wall degrading enzymes. Thus, water content is not only critical in enzymatic hydrolysis acting as a

substrate and a prerequisite for enzyme function, but is also important for enzyme transport mechanisms throughout the hydrolysis reaction as well as mass transfer of intermediates and end products [100]. *In situ* native cellulase systems have been reported to function at solids levels as high as 76% [101]. However, at both lab and industrial scale, 12-20% total solids is often considered the upper limit at which pretreated biomass can be mixed and hydrolysed in conventional stirred tank reactors [96,102,103]. Using other reactor designs, enzymatic hydrolysis at laboratory scale has been reported at up to 32% total solids [97,104]. A number of studies have utilised fed-batch operations in order to increase the final solids loading [96,98,105,106]. Jørgensen and co-workers have previously described a gravimetric mixing reactor design that allows batch enzymatic liquefaction and hydrolysis of pretreated wheat straw at up to 40% solids concentration [95].

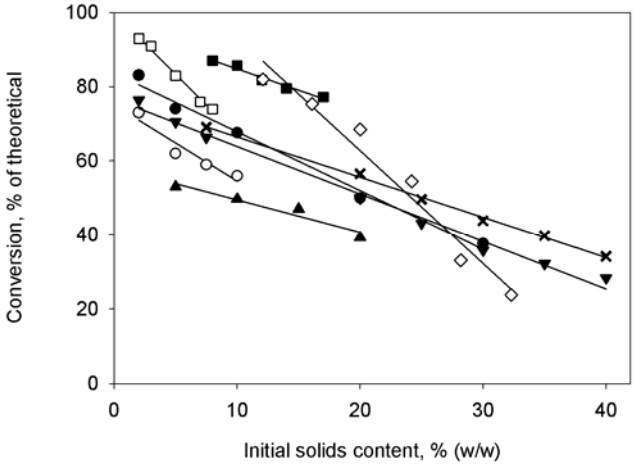

Figure 6*: Results collected from several publications indicate that decreasing conversion at increasing solids content is a general effect. Results are for different kinds of biomass and for both enzymatic hydrolysis and simultaneous saccharification and fermentation (SSF). Added trend lines show that for each experiment there is a near-linear relationships between initial solids content and yield. Data taken from the following publications: ○: Softwood, enzymatic hydrolysis [107], ●: Hardwood, enzymatic hydrolysis [104], □: Softwood, enzymatic hydrolysis [108], ■: Corn stover, SSF [106], ▼: Wheat straw, enzymatic hydrolysis [95],* ***X:*** *Wheat straw, SSF[95], ◊: Wheat straw, SSF [97] and ▲: Whatman no 1 filter paper, enzymatic hydrolysis, (own results, not published).*

This is a large increase from what has previously been possible, and thus significantly increases the techno-economic potential of the whole process. The gravimetric mixing principle has been up-scaled and used in a pilot plant for several years [43,45].

During the work with high solids loadings it was found that the enzymatic conversion (percent of theoretical) linearly decreased with increasing solids concentration despite using a constant enzyme-to-substrate ratio [95]. This decrease partly off-sets the advantages of working at high solids concentrations. As seen in Fig. 6, the effect has been observed in both enzymatic hydrolysis and SSF by several groups working with various kinds of biomass [97,104-110]. Although several of these studies were conducted at less than 10% initial solids content, the phenomenon appears to be an intrinsic effect of enzymatic hydrolysis at increasing solids levels. In this thesis, the decrease in yield at high solids concentrations is referred to as the *solids effect*.

Some groups have suggested that the mechanism responsible for the decreasing conversion is product inhibition [97,104,111] or inhibition by other compounds such as hemicellulose-derived inhibitors (furfural and hydroxymethylfurfural (HMF)) [112] and lignin [22]. Others have suggested it may be explained by mass transfer limitations or other effects related to the increased content of insoluble solids, such as non-productive adsorption of enzymes [103,113]. However, the specific mechanism(s) responsible for the decreasing hydrolytic efficiency are still uncertain [95,114]. In this thesis, the possible mechanisms have been divided into the following four categories:

- Compositional and substrate effects
- Product inhibition
- Water concentration
- Cellulase adsorption

Each topic has been investigated through experiments and studying the literature in order to identify the nature of this apparently intrinsic property of high-solids conversions of lignocellulose. Also, it is discussed how the decrease in yield can be alleviated in an effort to maximise bioethanol yield.

Compositional and substrate effects

As discussed, the heterogeneity and structure of lignocellulosic biomass means that high viscosity prevents efficient mixing at high solids concentrations [103,113,115]. The viscosity of lignocellulosic slurries increases sharply over a certain threshold (typically around 15-20% solids), where the mixture is better described as being in a semi-solid state than an actual slurry. However, despite the extreme difference in viscosity observed between 5% and 40% solids loading, the conversion of lignocellulosics as a

function of solids content appears to be near-linear (Fig. 6). Although mixing of substrate and enzymes is crucial for efficient liquefaction, the change in viscosity (and thus mixing) does not correspond to the decreasing conversion. Also, an increase of the relatively slow mixing speed of the liquefaction reactor does not significantly affect the cellulose conversion [95]. Hence, lack of mixing does not appear to be the cause of the falling yield, at least not at the solids levels documented. This is partly in accordance with the recent findings of Hodge and co-workers who concluded that possible mass transfer limitations caused by insoluble solids were not apparent at up to 20% insoluble solids content [111]. Hodge and co-workers did show a drop-off in conversion at 25% insoluble solids. This drop-off is however not apparent at below 40% total solids when using the gravimetric mixing described above. Furthermore, the linearity of the solids effect over a range of conditions with a number of substrates (wheat and barley straw [95,97,103], corn stover [106], softwood [107,110], hardwood [104,108] and an industrial ethanol fermentation residue (vinasse) [113]) indicates that a single factor may be responsible for the effect.

We initially wanted to establish that the solids effect is not caused by lignin adsorption or lignin-derived inhibitors (phenolics). It was thus decided to carry out the experiments with filter paper. This substrate has the advantage of containing no lignin yet still retains the secondary cell wall structure as opposed to Sigmacell or Avicel. The hydrolysis experiments with filter paper all displayed the characteristic profiles with a very high initial rate of conversion that decreases considerably after only six to eight hours (**Fig. 2A, Paper V**). When the conversion was displayed as a function of initial solids content, the characteristic downward curve was obvious (**Fig. 2B, Paper V**). Again, the relationship is near-linear with a decrease from 56.5% conversion at 5% initial solids content to 22.8% conversion at 25% initial solids content, both after 24h of hydrolysis. This shows that lignin or other phenolics are not involved in the solids effect.

The filter paper used in the experiments contained approximately 15% hemicellulose in the form of 14% mannan and 1% arabinan. However, our experiments with hydrolysis of Whatman filter paper (98% cellulose) (see Fig. 6) also displayed the same trend at increasing solids loadings. The same has been observed with hydrolysis of α-cellulose [109]. This clearly indicates that hemicellulose-derived sugars/inhibitors are not the cause of the solids effect.

Product inhibition

Numerous kinetic models have been developed for the enzymatic hydrolysis of lignocellulose [24,38,116-118]. The motivation of such models is usually two-fold. The first objective is to demonstrate a mechanistic understanding

of the process through fitting experimental data to a model. Another objective is to develop an application-based model that can be used to design and optimise various process parameters [96]. In enzymatic saccharification, a complex system of synergistic enzymes is applied to an insoluble and heterogeneous matrix of plant cell wall polymers. Furthermore, the substrate has been pretreated, modifying its structure and chemistry in various ways [37]. As already discussed, the interaction between enzyme system and substrate creates a range of factors influencing the rate and extent of the hydrolysis. Due to this complexity of the process, few investigators have been able to predict limiting factors or process design optima through kinetic models, or the characteristic decrease of enzymatic rate of hydrolysis over time [119].

End-product inhibition has been shown to play an important role in enzymatic hydrolysis as glucose, cellobiose and ethanol have been demonstrated to significantly inhibit endoglucanases, cellobiohydrolases and β-glucosidase [120,121]. However, when working with a insoluble substrate and kinetics that do not follow the Michaelis-Menten model, the exact type of inhibition can be difficult to determine [122]. The decrease in hydrolysis rate over time has been attributed to inhibition by the accumulated end-products [123]. Others conclude that when hydrolysing natural, lingocellulosic substrates, cellulases are more resistant to product inhibition than with amorphous reference materials and that the early stage decrease in hydrolysis rate is not caused by product inhibition [55,124]. In high-solids enzymatic hydrolysis of pretreated corn stover, Hodge and co-workers recently found that increased sugar concentrations were the primary cause of performance inhibition [111].

Based on the above, we wanted to investigate the inhibitory effect of increased sugar concentrations at high solids concentrations. To do this we added various amounts of sugar to a hydrolysis reaction of filter paper. An example of such an experiment is seen in **Fig. 3, Paper V**. With 50 g/L glucose added, the rate of hydrolysis during the first few hours was significantly reduced compared to the reference, in particular for the 5% solids hydrolysis where the initial phase of fast conversion was completely absent. As there is a constant enzyme dosage per gram of solids in the experiments, the ratio between glucose and enzyme is much higher at 5% than 20% solids (for hydrolyses with 50 g/L glucose added) and the stronger inhibition is therefore not surprising. Although eight hours often make up a small part of the whole hydrolysis time, the fast rate of hydrolysis in the first phase is responsible for conversion of a major part of the substrate. Interestingly, after approximately eight hours, the rate of hydrolysis (at each solids content) is nearly identical despite the significant difference in glucose level. This indicates that one of two things is happening. Either there are other and stronger factors inhibiting the hydrolysis after the first phase,

thereby “masking” the product inhibition, or there is a certain glucose level threshold, above which the enzymes are inhibited to a similar extent, thus resulting in a similar conversion rate. However, if the latter is the case and product inhibition is the major factor responsible for the solids effect, then one would not expect a linear relationship between solids level and conversion.

It is worth noticing that it is not only the concentration of the inhibitor that is important but that the inhibitor-to-enzyme ratio is equally so. This means that when running a hydrolysis reaction at different solids contents but with constant enzyme-to-substrate levels, the degrees of inhibition should theoretically be identical. Xiao and co-workers showed that in the hydrolysis of a cellobiose solution, addition of 20, 50 and 100 g/L of glucose to 2, 5 and 10% cellobiose (w/v) resulted in β-glucosidase inhibition of 53, 51 and 48%, respectively. The almost identical degree of inhibition at different concentrations proves that the inhibitor-to-enzyme ratio is essential in product inhibition [120]. As this is not the case in the experiments with increasing solids concentrations, it shows that inhibition of β-glucosidase (alone) is not the main cause of the solids effect. However, indirectly the cellulose-binding cellobiohydrolases are even stronger inhibited by glucose. The high glucose concentration leads to an accumulation of cellobiose, which acts as a particularly strong inhibitor on cellobiohydrolases [122].

Surprisingly, cellobiose concentrations in our experiments have generally been low. Normally, less than 10% of the converted material (glucose + cellobiose) is found as cellobiose, even at high solids concentrations (not shown). For comparison, during experiments with lower proportions of β-glucosidase, inhibition caused cellobiose proportions of over 35% of the converted material while still retaining a certain degree of hydrolysis (not shown).

SSF is normally used to offset the well-known effects of glucose and cellobiose inhibition but interestingly the solids effect has also been observed under those conditions [97,106]. Ethanol is also known to act as an inhibitor on cellulases (although less severe than cellobiose) [121,125], indicating that other factors may influence the conversion under these conditions.

To test if product inhibition was the sole cause of the solids effect, a new experiment was carried out. Filter paper was hydrolysed to approximately 45% but at three different enzyme loadings and lengths of time: 20 FPU per g dry matter (DM) for 22 hours, 10 FPU per g DM for 48 hours and 5 FPU per g DM for 84 hours. Interestingly, the slopes of the three curves are nearly identical (**Fig. 4, Paper V**). If product inhibition alone was the cause of the solids effect, one would expect the hydrolysis with the lowest enzyme-to-substrate ratio to display the strongest degree of inhibition and thus a steeper curve. In other words, it is not possible to bypass the solids effect by

using higher enzyme dosages, at least not within the normal range of dosages. This is an important consideration when trying to alleviate the solids effect.

Related to product inhibition at increased substrate concentration is the phenomenon of transglycosylation, where enzymes display not only hydrolytic activities but also transglucosidic activities [126]. Examples include the β-glucosidase-catalysed transglycosylation of cellobiose to form a trisaccharide [127], and transglycosylation by endoglucanase and cellobiohydrolase of *T. reesei*. [126,128]. It is well-known that the substrate concentration, and thus acceptor concentration, is one of the most important factors determining the degree of an oligomerization reaction. It is thus not unlikely that an increasing portion of cellulose is being converted into cellotriose, cellotetraose and other oligomers (with both α and β-linkages) at high solids concentrations, resulting in compounds that are not necessarily detected by HPLC and thus not included when determining the yield. However, results by Gruno and co-workers indicate that transglycosylation by cellobiohydrolase plays a minor role compared to product inhibition [122]. More research is needed to elucidate the extent of transglycosylation in high solids enzymatic hydrolysis.

In conclusion, product inhibition at increased solids concentrations was found to be a significant and potentially determining factor for the solids effect. However, the linearity over a large range of solids contents does not fit with the current model for product inhibition. Likewise, transglycosylation is likely to become more pronounced at high solids levels but is not thought to contribute significantly to the solids effect.

Water concentration

Low water content may directly affect enzyme performance. Not only is water a substrate for the hydrolysis but it is also the solvent that allows the enzymes to function [129]. Water also facilitates contact between enzymes and substrate and is important for the transfer of products. We have previously investigated the role of water in enzymatic hydrolysis [100] (**Paper VI** in Appendix). In the present study, it was investigated if the solids effect was related to a lower concentration of water in relation to solids. As mentioned, hydrolysis is possible even at very high solids concentrations, but the rate of reaction may be impaired under such conditions [101]. In order to investigate this, various amounts of the water was replaced with oleyl alcohol, an inert oil that does not directly affect the function of the enzymes [130,131]. The rationale behind these experiments is that by substituting part of the water, it is possible to run a hydrolysis reaction with an altered water-to-enzyme ratio but with a more or less constant viscosity of the slurry. If it is a lack of water that is causing the

solids effect, then the hydrolysis conversion where a certain amount of the water has been replaced should be lower, presumably at the level of the corresponding solids level (taking only the aqueous phase into consideration).

In the experiment shown in **Fig. 5, Paper V**, a quarter of the water (buffer) in an enzymatic hydrolysis of 20% solids filter paper was substituted with oleyl alcohol. At this level of substitution, the actual solids concentration in relation to water was therefore increased by 25%; from 20 to 25%. After 40 hours of hydrolysis, 5.6% less glucose was released compared to the reference (without oleyl alcohol addition). However, a 20% increase in solids usually leads to a decrease in conversion of over 12%. Thus, the decrease in conversion did not correspond directly to the lowered water content. However, the sugar concentration is not the only parameter that has been changed. Oleyl alcohol may act as a mixing agent, fully or partially replacing the effect of water in assisting mass transfer, even when neither cellulose nor enzymes are solubilised in the oleyl alcohol. As previously discussed, the interconnection of factors affecting the yield is very characteristic of lignocellulose hydrolysis, complicating the identification of limiting factors.

There is no doubt that water plays a number of important roles in enzymatic hydrolysis, and that these roles become even more crucial in systems with no free water. As cellulases can only break down cellulose when adsorbed onto the material, efficient mass transfer of enzymes is likely to increase conversion. It is important to note that mass transfer takes places at several different levels, e.g. in “bulk” around the cell wall surface, in cell wall pores and *inside* the cell wall matrix itself. In transfer of enzymes in the cell wall, the total solids content may not be the most important parameter. Similarly, diffusion of released sugars away from the catalytic sites will theoretically prevent local product inhibition. As discussed, water content also affects mechanical stirring, which in turn may directly change the size distribution of larger particles. Unfortunately, our understanding of these mechanistic interactions is limited and also depends on the cell wall structure of the substrate. It is likely that such factors affect the degree of conversion at very high solids loadings, essentially causing a decrease in yield when over a certain solids loading. However, as the observed solids effect is also seen at loadings as low as 2-5% solids, mass transfer at neither the macroscopic nor molecular level can be responsible for the solids effect.

Related to the diffusion of enzymes is the phenomenon of substrate inhibition, which has previously been described in connection with hydrolysis of cellulose [132]. At increased substrate concentrations, with a fixed enzyme loading, the lateral (two-dimensional) diffusion of bound enzymes is believed to be restricted, thus inhibiting the synergy between exo and endo-enzymes [133]. However, this form of synergistic inhibition relates

to a fixed enzyme load where the amount of substrate is increased, i.e. a decreasing enzyme-substrate ratio as opposed to a constant ratio used in our and other's experiments. Therefore, this phenomenon is not likely to be involved in the solids effect. Traditionally, substrate inhibition is explained as a situation where two molecules of substrate bind to the enzyme simultaneously, thereby blocking activity. However, this mechanism is not likely to be applicable to the hydrolysis of an insoluble substrate such as cellulose [134].

In conclusion, water itself as a substrate or diffusing agent in enzymatic hydrolysis does not appear to be the limiting factor responsible for the solids effect, nor is substrate inhibition involved.

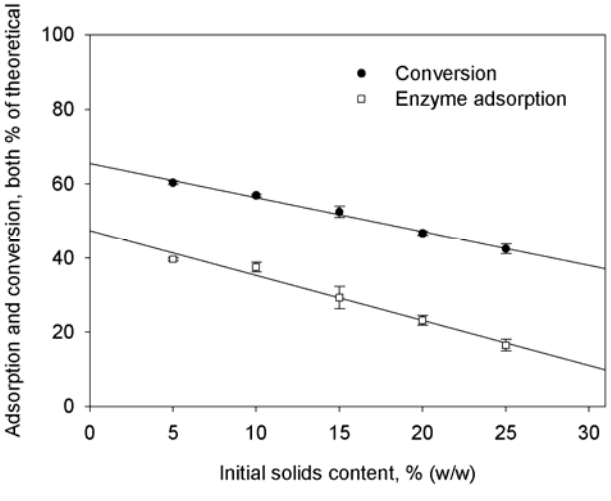

Figure 7*: Upper graph shows the decreasing conversion in enzymatic conversion of filter paper at increasing solids loading (20 FPU per gram dry matter (DM), 24 hours hydrolysis at small laboratory scale). Points are averages of three observations. The lower graph shows the adsorption of enzyme on the solid fraction based on total nitrogen content, also as a function of initial solids content. Values are averages of three observations and have been corrected for varying amounts of remaining solids.*

Cellulase adsorption

The degree of adsorption of cellulases is known to be a controlling or determining factor for conversion rates and yields [135,136]. It is also well-established that certain hydrolysis products are able to inhibit cellulase

adsorption [137]. Interestingly, it has recently been shown that glucose and especially cellobiose strongly inhibit cellulase adsorption in a near-linear fashion [138].

In order to investigate whether adsorption (or lack thereof) could possibly be involved in the observed solids effect, the adsorption of enzyme was measured in hydrolysis of filter paper at different solids contents. As seen in Fig. 7, there is a near-linear correlation between initial solids content and the amount of adsorbed enzyme (percentage of nitrogen adsorbed on solids of total nitrogen added). After 24 hours of hydrolysis of 5% solids filter paper, approximately 40% of the added enzyme was adsorbed onto the remaining solids. The adsorption decreases with increasing solids content and at 25% solids content, only approximately 17% of the added enzyme is adsorbed, even when at this point there is more solids remaining than at lower solids loadings. Even more interestingly, there is a statistically significant correlation between the decrease in conversion and the decrease in enzyme adsorption. In other words, it appears that the increasing concentrations of glucose and cellobiose in high-solids hydrolysis result in inhibition of adsorption of the enzymes. As adsorption is a requirement for hydrolysis of the insoluble substrate, this in return results in lower conversion at increasing solids concentrations.

Based on an experiment with a fixed cellobiose concentration, Kumar and Wyman argue that binding inhibition can be reversed using high substrate concentrations [138]. However, working with a fixed inhibitor concentration over a range of solids concentrations does not reflect the actual conditions since high solids loadings will invariably lead to higher product concentrations. At any degree of conversion, the ratio between substrate and inhibitor (product) in hydrolysis will be constant no matter the initial solids concentration. Xiao and co-workers also observed reduced impact of products on inhibition at higher solids loadings but again it was measured against a constant inhibitor concentration [120]. Based on our experiments we do not believe that increased solids concentrations can reverse binding inhibition, rather the opposite.

It can be argued that the adsorption inhibition phenomenon described above is a variant of product inhibition. However, in both competitive and non-competitive inhibition the catalytic site is affected, which is not necessarily the case with inhibition of adsorption. β-glucosidase does not bind to the substrate and is therefore not affected in this way. The combination of binding inhibition of endoglucanases and cellobiohydrolases and β-glucosidase being inhibited to a smaller extent may explain the relatively low cellobiose-levels under conditions where the hydrolysis is obviously affected.

It is not yet known to what extent inhibition of adsorption is responsible for the solids effect or if it can be partially avoided through SSF. It has been

shown previously that adsorption inhibition could not explain the decrease in cellulase activity [139]. In an attempt to learn more about the nature of the inhibition, we used the data from the experiment in **Fig. 2, Paper V** to investigate the relationship between the rate of reaction and glucose concentration. We found no direct relationship (not shown) - possibly due to the fact that different proportions of the substrate remained, i.e. when 60% of the substrate has been converted, the remainder is more difficult to hydrolyse than if only 40% has been converted.

It is likely that the binding inhibition is caused by the cellulose binding domains (CBDs) of the cellulases being affected by glucose and/or cellobiose but this issue remains to be much more extensively investigated. As previously described, binding of cellulases and clarification of the role of CBDs is an important topic in cellulosic biomass conversion, and has been the topic of numerous studies [64,140]. Unfortunately, very little information on inhibition and desorption of CBDs/CBMs appears to be available. Experiments with isolated CBDs are likely to reveal important information on this topic. Also, an improved understanding of the structural basis by which the CBDs bind to the target carbohydrate may be crucial in elucidating the underlying mechanisms and determining which products are causing the inhibition as well as the manner of the inhibition. It also remains to be investigated how adsorption inhibition functions over time. Differences in ability to desorb from the substrate have been shown for two cellobiohydrolases [86]. Based on this, it is possible that certain CBDs are more or less susceptible to binding inhibition. Being able to alter or change the CBD of cellulases to make them less susceptible to high concentrations of products could contribute to making high yields at high solids concentrations a reality.

SUMMARY

The increased interest in using cellulosic substrates as feedstocks for the production of ethanol has resulted in intensive research in enzymatic hydrolysis of lignocellulose. Despite important advances in substrate pretreatment and cellulase production in recent years, a better understanding of the factors and mechanisms involved in the interactions of substrate and enzymes is important for further developments in making bioethanol (and chemicals) a cost-effective and sustainable alternative.

This thesis has dealt with enzyme-substrate interactions in enzymatic hydrolysis of pretreated biomass. The required pretreatment step is crucial in preparing lignocellulosic substrate for hydrolysis. In **Paper II**, hydrothermally pretreated wheat straw was investigated. Contrary to common belief, it was found that the fibrillar structure of the cell wall was relatively intact, showing that a partial decomposition of the cell wall structure is not needed in order to render the material less recalcitrant. Significant re-localisation of lignin to the fibre surface was observed. Together with hemicellulose removal, this re-localisation is thought to increase enzyme accessibility, an important factor in substrate degradability.

Experiments with surfactants showed a significant positive effect on hydrolysis, resulting in either a reduced hydrolysis time or lower enzyme dosage requirement (**Paper III**). The results supported the current theory that surfactants function by preventing unproductive enzyme adsorption to lignin. Although lignin is a limiting factor in lignocellulose conversion, the surfactant effect was found to not directly correlate with the lignin content of the material but rather the pretreatment method. The effect was most pronounced with straw that had been pretreated with sulfuric acid.

Working at high solids loading is important for the efficiency of enzymatic conversion of lignocellulose. The work of this thesis has in particular focused on the issues and challenges related to high solids conversions (**Paper IV and V**). The extent of hydrolysis of cellulosic biomass was investigated at varying solids concentrations up to 40%. The conversion decreased at increasing solids concentration in a linear fashion, an effect that appears to be an intrinsic feature of lignocellulose conversion. This decrease is highly problematic as it partially off-sets the significant advantages of working at high solids concentrations. It was found that the solids effect was not caused by lignin content or hemicellulose-derived inhibitors. Insufficient mixing of the insoluble substrate was not causing the effect either. Rather, the increased concentrations of products (glucose and cellobiose) at high solids concentration are likely causing product inhibition, even when the enzyme-to-inhibitor ratio is constant. However, the solids effect has also been observed in SSF where much less sugar is present, although additional parameters are introduced when including fermentation.

Also, the linearity of the effect over a large range of solids contents did not fit with the current model for product inhibition.

Interestingly, it was found that at increasing solids concentrations, the proportion of adsorbed cellulase decreased. There was a statistically significant correlation between this adsorption inhibition and the decreasing yields at increasing substrate concentrations. Thus, the solids effect can be explained by inhibition of the binding of the cellulases. The exact extent and mechanism of the adsorption inhibition remain unknown.

Future perspectives

One of the defining aspects of scientific research is the generation of seemingly never-ending questions. Not surprisingly, the work and results this thesis is based on has generated many questions, some of which may be important for further increasing the conversion of cellulosic biomass into fermentable sugars.

A better understanding of how enzymes and lignocellulose interact is still needed. A prerequisite for the decomposition is the pretreatment step. Further investigations on the spatial or structural arrangement of the cell wall components of the pretreated material and how it is being hydrolysed is likely to provide insight, that can improve both pretreatment and enzymatic hydrolysis

In future studies it is important to look further into the mechanisms responsible for the performance of cellulase CBDs under conditions with a high concentration of solids and products. Precisely how is the adsorption inhibited and by which products? A key step may be a better understanding of the structural basis of CBDs. Engineering enzymes to be more resistant to high sugar concentrations would result in higher conversions at high solids concentrations, in turn significantly improving the viability of lignocellulosic biomass conversion and bioethanol production.

DANSK SAMMENFATNING

Den øgede interesse i at bruge cellulose-holdigt biomasse til produktion af bioethanol har resulteret i intensiv forskning i enzymatisk hydrolyse af lignocellulose. På trods af vigtige fremskridt i forbehandling af biomasse samt enzymproduktion er en forbedret forståelse af de faktorer og mekanismer, der er afgørende for interaktionen mellem substrat og enzymer, et vigtig skridt mod at gøre bioethanol et økonomisk og bæredygtigt alternativ til fossile brændstoffer.

Denne afhandling omhandler enzym-substrat-interaktioner i enzymatisk hydrolyse af forbehandlet lignocellulose. Forbehandling er et nødvendigt trin i processen og er afgørende for den enzymatiske nedbrydning. I **Paper II** blev det undersøgt, hvordan en hydrotermisk forbehandling ændrer substratet. Normalt beskrives effekten af forbehandling som en delvis nedbrydning af cellevæggen. Det viste sig dog at fibrilstrukturen i cellevæggen var stort set uændret, hvilket indikerede at en energi-intensiv, partiel nedbrydning af cellevæggen ikke er nødvendig for at gøre biomasse enzymatisk nedbrydelig. Det blev endvidere vist, at en stor mængde af halmens lignin var blevet transporteret til overfladen af fibrene. Sammen med den delvise opløsning af hemicellulose menes denne relokalisering at være afgørende for enzymernes adgang til cellulosen, en vigtig faktor i nedbrydningsprocessen.

Eksperimenter med overflade-aktive stoffer, såkaldte surfactanter, viste en signifikant positiv effekt på enzymatisk hydrolyse, hvilket resulterede i en enten reduceret hydrolysetid eller lavere enzymdosis (**Paper III**). Resultaterne underbyggede desuden teorien om at surfactanter fungerer ved at forhindre uproduktiv adsorption af enzymer på ligninoverflader. Selvom lignin er en begrænsende faktor i konvertering af lignocellulose og at effekten af surfactants er relateret af lignin, så viste resultaterne, at effekten af surfactanter ikke er direkte proportional med ligninindholdet, men i højere grad afhænger af forbehandlings-metoden.

Højt tørstofindhold under den enzymatisk hydrolyse er en yderst vigtig parameter for effektiviteten og rentabiliteten af konverteringsprocessen fra biomasse til ethanol. Arbejdet i denne afhandling har i høj grad fokuseret på dette emne samt de udfordringer, der er tilknyttet (**Paper IV** og **V**). Graden af hydrolyse ved varierende tørstofindhold op til 40% blev undersøgt. Konverteringen faldt lineært proportionelt med et stigende tørstofindhold, et forhold som ser ud til at være et naturligt forhold ved enzymatisk hydrolyse. Dette fald er problematisk, da det modvirker fordelene ved at arbejde ved højt tørstofindhold. Det blev vist, at den øgede koncentration af sukkerprodukter resulterer i produktinhibering, selv når forholdet mellem enzym og inhibitor var konstant. Lineariteten af fænomenet passede dog ikke med den nuværende model for produktinhibering. Derimod blev det konstateret at andelen af adsorberet enzym faldt ved stigende tørstof- og sukkerindhold.

Den lineære sammenhæng var statistisk signifikant og kan forklares ved adsorptionsinhibering af sukkerprodukter. Mekanismen bag denne adsorptionsinhibering er endnu ukendt.

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PUBLICATIONS

This thesis is based on the work presented in the following publications. They are referred to in the text by their roman numerals.

I Jørgensen, H., Kristensen, J.B., Felby, C.:
Enzymatic conversion of lignocellulose into fermentable sugars: Challenges and opportunities
Biofuels, Bioproducts and Biorefining 2007, 1(2):119-134.

II Kristensen J.B., Thygesen, L.G., Felby, C., Jørgensen, H., Elder, T.:
Cell-wall structural changes in wheat straw pretreated for bioethanol production
Biotechnology for Biofuels 2008, 1(5).

III Kristensen, J.B., Börjesson, J., Bruun, M.H., Tjerneld, F., Jørgensen, H.:
Use of surface active additives in enzymatic hydrolysis of wheat straw lignocellulose
Enzyme and Microbial Technology 2007, 40(4):888-895.

IV Kristensen, J.B., Felby, C., Jørgensen, H.:
Determining yields in high solids enzymatic hydrolysis of biomass
Applied Biochemistry and Biotechnology, DOI: 10.1007/s12010-008-8375-0.

V Kristensen, J.B., Felby, C., Jørgensen, H.:
Yield determining factors in high solids enzymatic hydrolysis of lignocellulose
Submitted for publication.

I

Review

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Enzymatic conversion of lignocellulose into fermentable sugars: challenges and opportunities

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Abstract: The economic dependency on fossil fuels and the resulting effects on climate and environment have put tremendous focus on utilizing fermentable sugars from lignocellulose, the largest known renewable carbohydrate source. The fermentable sugars in lignocellulose are derived from cellulose and hemicelluloses but these are not readily accessible to enzymatic hydrolysis and require a pretreatment, which causes an extensive modification of the lignocellulosic structure. A number of pretreatment technologies are under development and being tested in pilot scale. Hydrolysis of lignocellulose carbohydrates into fermentable sugars requires a number of different cellulases and hemicellulases. The hydrolysis of cellulose is a sequential breakdown of the linear glucose chains, whereas hemicellulases must be capable of hydrolysing branched chains containing different sugars and functional groups. The technology for pretreatment and hydrolysis has been developed to an extent that is close to a commercially viable level. It has become possible to process lignocellulose at high substrate levels and the enzyme performance has been improved. Also the cost of enzymes has been reduced. Still a number of technical and scientific issues within pretreatment and hydrolysis remain to be solved. However, significant improvements in yield and cost reductions are expected, thus making large-scale fermentation of lignocellulosic substrates possible. © 2007 Society of Chemical Industry and John Wiley & Sons, Ltd

Keywords: cellulases; hemicellulases; pretreatment; biorefinery

Introduction

The current dependence on oil for energy and production of numerous chemicals and products together with the climate changes caused by fossil fuels has put tremendous focus on finding alternative renewable sources for the production of fuels and chemicals. In this respect, biomass will be a major contributor in the future supply of energy, chemicals and materials.[1,2] At present, large quantities of bioethanol are produced from sugar cane and cereals

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like corn or grain; in 2006 this production was approximately 46 million m^3. In order to expand this production significantly and be able to produce other products and chemicals, the whole plant, including stalk and leaves (the lignocellulosic part), from both herbaceous and woody plants has to be efficiently utilized.

Lignocellulose is composed of up to 75% carbohydrates, and in the near future it will become an essential source for fermentable carbohydrates. These may form the basis for production of liquid biofuel for the transport sector as well as a large variety of commodity chemicals and biodegradable materials. It is expected that the present fossil-based petro-chemical industry will gradually be replaced by biorefineries, which produce a number of valuable products from lignocellulosic materials, including energy in the form of fuels, heat and electricity (Fig. 1). The use of carbohydrates will, in the future, provide a viable route to products such as alcohols, esters and carboxylic acids, which are expensive to produce in the petroleum industry.[2] A key issue for the biorefineries is the conversion of carbohydrates from lignocellulosic feedstocks into fermentable sugars – the so-called 'sugar platform'. Efficient and cost-effective hydrolysis of the carbohydrates cellulose and hemicellulose into monosaccharides is a challenge for their use and attention should be focused on this step.[2,3]

Several schemes for the conversion of lignocellulosics into sugars have been demonstrated in laboratory and pilot scale.[4–6] The general concept involves a pretreatment step that increases the digestibility of the material followed by enzymatic hydrolysis to liberate the monosaccharides. Improvement of pretreatment technologies and enzymatic hydrolysis gives scope for numerous ongoing research projects. Lignocellulose is a complex matrix of polymers, and efficient hydrolysis of the carbohydrates to monosaccharides requires not only efficient pretreatment and enzymes but also optimization of both steps in relation to each other.

This review will give an overview of the various challenges and opportunities that exists. Different approaches to optimize both process steps and improve their interactions are presented and discussed.

Characteristics of lignocellulosic materials

Plant biomass is composed primarily of cellulose, hemicelluloses and lignin and smaller amounts of pectin, protein, extractives and ash. Cellulose, hemicelluloses and lignin are present in varying amounts in the different parts of the plant and they are intimately associated to form the structural framework of the plant cell wall.[7,8] The composition of lignocellulose depends on plant species, age and growth conditions. Distribution of cellulose, hemicelluloses and lignin as well as the content of the different sugars of the hemicelluloses varies significantly between different plants (Table 1).

Cellulose, the most abundant constituent of the plant cell wall, is a homo-polysaccharide composed entirely of D-glucose linked together by β-1,4-glucosidic bonds and with a degree of polymerization of up to 10 000 or higher. The linear structure of the cellulose chain enables the formation of both intra- and intermolecular hydrogen bonds resulting in the aggregation of chains into elementary crystalline fibrils of 36 cellulose chains. The structure of

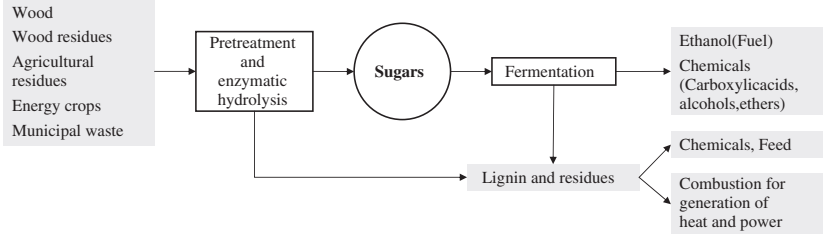

Figure 1. Overview of an integrated biorefinery producing fuel, chemicals and energy from a variety of lignocellulosic materials.

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Table 1. Composition of different lignocellulosic materials.

Material	Glucose[a]	Xylose[b]	Arabinose[b]	Mannose[b]	Lignin	Reference
	[% of total dry weight]					
Hardwood						
Birch	38.2	18.5	–[c]	1.2	22.8	136
Willow	43.0	24.9	1.2	3.2	24.2	31
Softwood						
Spruce	43.4	4.9	1.1	12.0	28.1	34
Pine	46.4	8.8	2.4	11.7	29.4	137
Grasses (Poaceae)						
Wheat straw	38.2	21.2	2.5	0.3	23.4	137
Rice straw	34.2	24.5	n.d.[d]	n.d.[d]	11.9	137
Corn stover	35.6	18.9	2.9	0.3	12.3	136

[a]Glucose is mainly coming from cellulose.

[b]Xylose, arabinose and mannose make up hemicelluloses.

[c]Below detection limit.

[d]Not determined.

the elementary fibril is crystalline; however, some sources claim that the surface could be viewed as amorphous.[9] The structure of cellulose along with the intermolecular hydrogen bonds gives cellulose high tensile strength, makes it insoluble in most solvents and is partly responsible for the resistance of cellulose against microbial degradation.[10] The hydrophobic surface of cellulose results in formation of a dense layer of water that may hinder diffusion of enzymes and degradation products near the cellulose surface.[11]

Hemicelluloses are complex heterogeneous polysaccharides composed of monomeric residues: D-glucose, D-galactose, D-mannose, D-xylose, L-arabinose, D-glucuronic acid and 4-*O*-methyl-D-glucuronic acid. Hemicelluloses have a degree of polymerization below 200, side chains and can be acetylated.[8] Hemicelluloses are classified according to the main sugar in the backbone of the polymer, e.g. xylan (β-1,4-linked xylose) or mannan (β-1,4-linked mannose). Plants belonging to the grass family (Poaceae), e.g. rice, wheat, oat and switch grass have hemicelluloses that are composed of mainly glucuronoarabinoxylans.[12] In softwoods such as fir, pine and spruce, galactoglucomannans are the principal hemicelluloses, while arabinoglucuronoxylans are the second most abundant.[13] In hardwood species, such as birch, poplar, aspen or oak, 4-*O*-methyl-glucuronoxylans are the most abundant hemicelluloses with glucomannans being the second most abundant.[14] Due to these differences in hemicellulose composition, agricultural waste products like straw and corn stover as well as hardwood materials are rich in the pentose sugar xylose, whereas softwoods are rich in the hexose sugar mannose (Table 1).

Lignin is a complex network formed by polymerization of phenyl propane units and constitutes the most abundant non-polysaccharide fraction in lignocellulose. The three monomers in lignin are *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol and are joined through alkyl–aryl, alkyl–alkyl and aryl–aryl ether bonds. Lignin embeds the cellulose thereby offering protection against microbial and chemical degradation. Furthermore, lignin is able to form covalent bonds to some hemicelluloses, e.g. benzyl ester bonds with the carboxyl group of 4-*O*-methyl-D-glucuronic acid in xylan. More stable ether bonds, also known as lignin carbohydrate complexes (LCC), can be formed between lignin and arabinose or galactose side groups in xylans and mannans.[8] In general, herbaceous plants, such as grasses, have the lowest content of lignin, whereas softwoods have the highest lignin content (Table 1).

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Pretreatment technologies

Due to the robust structure of lignocellulosic biomass, pretreatment is a prerequisite for enzymatic hydrolysis into fermentable sugars to be completed within an industrially acceptable time frame. Over the years a number of different technologies have been developed for pretreatment of lignocellulose (Table 2). There is an overall consensus that the successful pretreatment should:[15]

- maximize the enzymatic convertibility
- minimize loss of sugars
- maximize the production of other valuable by-products, e.g. lignin
- not require the addition of chemicals toxic to the enzymes or the fermenting microorganisms
- minimize the use of energy, chemicals and capital equipment
- be scalable to industrial size.

In reality it is difficult to fully accomplish all the above-mentioned issues in any process. However, the last two points are important for economical and practical viability of a given industrial process.

Various technologies use different strategies to increase the enzymatic convertibility. The general ideas are to alter or remove hemicelluloses and/or lignin, increase surface area and decrease the crystallinity of cellulose.[6,16] Removal of lignin, and to a less extent hemicelluloses, has been proven to be effective in increasing the cellulose hydrolysis.[17–19] The main principles in the various technologies are listed in Table 2, but a comprehensive review of all technologies is beyond the scope of this article. The reader is referred to other review articles.[6,16,20,21] The following section will concentrate on the most widely reported pretreatment technologies along with those tested in pilot scale and potential technologies for industrial scale.

Steam pretreatment and acid-catalysed pretreatment technologies

For many years steam pretreatment/explosion technologies have been used for fractionation of wood or straw for the production of fibre boards and paper. Facilities for batch or continuous operation have been operated in both pilot and commercial scale, proving the reliability of the technologies.[22] Examples are the batch digester developed for the Masonite process[23] or the continuous Stake tech digester from SunOpta (http://sunopta.com).[22]

In steam pretreatment, the material is heated rapidly with steam to 180–210 °C for typically 1–10 min. Often the steam pretreatment is combined with an explosive discharge of the material after the pretreatment – steam explosion.[21,24]

During the steam pretreatment, hemicellulose acetyl groups are cleaved off and the acids will catalyse partial hydrolysis of the hemicelluloses to mono- and oligosaccharides.[25] Removal of hemicelluloses from the microfibrils is believed to expose the cellulose surface and increase enzyme accessibility to the cellulose microfibrils.[26] Lignin is only to a limited extent removed from the material during the pretreatment but rather redistributed on the fibre surfaces due to melting and depolymerization/repolymerization reactions.[27,28] The removal and redistribution of hemicellulose and lignin increases the pore volume of the pretreated material. Rapid flashing to atmospheric pressure and turbulent

Table 2. List of pretreatment methods and main mechanisms involved.

Pretreatment method	Main principle
Dilute acid Steam explosion (auto hydrolysis) Acid-catalysed steam explosion	Partial hydrolysis and solubilization of hemicelluloses, redistribution of lignin on fibre surfaces, fractionation of fibres
Hot water flow through	Removal of hemicelluloses and some lignin
Lime	Removal of lignin
Wet oxidation Wet explosion	Removal and partial degradation of lignin, solubilization and oxidation of some hemicelluloses
AFEX	Cleavage of lignin and partially depolymerization of hemicelluloses and cellulose
Organosolv/Alcell	Removal of lignin and some hemicelluloses

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flow of the material work to fragment the material, thereby increasing the accessible surface area.[21] The microfibrils do not seem to be affected significantly by the pretreatment.[27] The fragmentation itself is believed to be of less importance to the digestibility of the material.[16] Depending on the severity of the pretreatment, some degradation of the cellulose to glucose will also take place.

Steam (explosion) pretreatment without addition of acid as a catalyst has been used for pretreatment of agricultural materials like straw and corn stover and hardwoods. In general it is found that addition or impregnation of the material with H_2SO_4 or SO_2 (typically 0.3 to 3% (w/w)) prior to pretreatment can decrease time and temperature and at the same time increase the recovery, reduce formation of inhibitors and improve the enzymatic hydrolysis.[29–31] For pretreatment of softwoods, the addition of an acid catalyst (H_2SO_4or SO_2) is a prerequisite to make the substrate accessible for enzymes.[21,32–34] Impregnation with gaseous SO_2 has the advantage that it can quickly penetrate into the material. Corrosion problems are also less using SO_2 compared to H_2SO_4.[24]

Steam pretreatment with addition of a catalyst is the technology that has been claimed to be closest to commercialization. It has been tested extensively for pretreatment of a large number of different lignocellulosic feedstocks. In relation to bioethanol production, the technology has been up-scaled and operated at pilot scale at the Iogen demonstration plant in Canada. The technology is also being implemented in a plant operated by the company Abengoa in Salamanca in Spain.

Another acid-catalysed pretreatment technology is dilute acid pretreatment. In dilute acid pretreatment, the lignocellulosic material is mixed with dilute acid (typically H_2SO_4) and water to form a slurry, heated by steam to the desired temperature, and after a given residence time flashed to atmospheric pressure. This is in essence very similar to the steam pretreatment and in the literature there is not always a clear distinction between the two methods. Generally, the material used in steam pretreatment is only moist whereas a slurry with a lower dry matter content down to 5% is applied in dilute acid pretreatment. Another difference is that often the particle size is smaller in dilute acid pretreatment. Usually, acid concentration, pretreatment temperature and residence time in the reactor are rather similar to acid-catalysed steam pretreatment, although lower temperatures and longer residence times are sometimes employed.[21,35]

The dilute acid pretreatment can be performed in plug-flow reactors, but other reactor designs have been used.[21,36] Counter-current or continuous counter-current shrinking-bed reactors have been tested in laboratory and bench scale.[37] The National Renewable Research Laboratory in the USA (NREL) has been operating continuous dilute acid pretreatment of corn stover in a pilot scale process development unit (1 ton per day) for up to 15 days.[36] In Örnsköldsvik in Sweden, the company SEKAB is operating a pilot plant using dilute acid hydrolysis for complete hydrolysis of wood residues but they will also run the process as a pretreatment combined with enzymatic hydrolysis (http://sekab.com). Since 2005, the company DONG Energy in Denmark has operated a 100–1000 kg h^{-1} pilot plant for semi-continuous counter-current pretreatment (http://bioethanol.info). The plant is designed for testing various pretreatment methods, to operate with large particles and at dry matter concentrations up to 50% in the reactor. Pretreatment of wheat straw has been performed with water only (hydrothermal pretreatment), with addition of dilute H_2SO_4 or alkaline (see methods below).[5]

Pretreatment under alkaline conditions

A number of methods for pretreatment under alkaline conditions have also been successfully tested: Lime pretreatment, wet oxidation and ammonia fibre/freeze explosion (AFEX). As opposed to the acid-catalysed methods, the general principle behind alkaline pretreatment methods is the removal of lignin whereas cellulose and a major part of the hemicelluloses remain in the solid material.

Lime pretreatment with calcium carbonate or sodium hydroxide is usually employed at low temperatures (85–150°C) but at rather long residence times, from 1 h and up to several days.[6,16] From a processing perspective such long residence times are less attractive and lime pretreatment has not been implemented in large scale.

Wet oxidation has been used as a pretreatment method for a number of lignocellulosic materials; straw, corn stover, bagasse and softwood.[38–41] Wet oxidation is a thermal pretreatment at 180–200°C for 5–15 min with addition of

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an oxidative agent such as H_2O_2 or over-pressure of oxygen. The pretreatment is performed at 5–20% dry matter, and often the initial pH is increased by the addition of alkali such as sodium carbonate. Performing wet oxidation under acidic conditions has not proven beneficial.[39,41] In wet oxidation, some of the lignin but also hemicelluloses are partially oxidized to low molecular weight carboxylic acids, CO_2 and water. The degradation products from lignin (phenolics) and sugars (furans), which are inhibitory to microorganisms, can be oxidized to carboxylic acid.[42]

A modification of the wet oxidation capable of handling large particles and a dry matter up to 30% has recently been developed, termed wet explosion. In wet explosion the oxidizing agent is introduced during pretreatment after a certain residence time. The pretreatment is then terminated by flashing to atmospheric pressure.[43] A pilot scale facility for using this technology was build at the Technical University of Denmark in 2006.

Ammonia fibre explosion (AFEX) has shown good results on pretreatment of corn stover, rice straw and switchgrass.[44–46] In AFEX the lignocellulosic material is treated with liquid ammonia at moderate temperatures (90–100°C) and high pressure (17–20 bar) for 5–10 min. A unique feature of AFEX is the possibility to efficiently process material with a dry matter content of up to 60%.[47] The ammonia can be recovered after the pretreatment and unlike most other methods no liquid fraction with dissolved products is generated by the AFEX pretreatment as ammonia is evaporated. Consequently, no lignin or other substances are removed from the material but lignin–carbohydrate complexes are cleaved and deposition of lignin on the surface of the material is observed. Furthermore, AFEX results in depolymerization of the cellulose and partially hydrolysis of the hemicelluloses.[45] Only little degradation of sugars occurs and therefore low concentrations of inhibitors are formed.[47] The AFEX method enables operating the process at high solids concentrations. However, so far no large-scale or pilot plant operation with AFEX has been reported.

Extractive pretreatment

Organosolv pretreatment or the Alcell® pulping process is a technology in which the lignocellulosic material is delignified by an extraction process involving the use of aqueous ethanol (40–60% ethanol) at 160–200°C for 30–60 min with a wood-to-liquid ratio of 1:7–10 (w/v).[18,48] Sulphuric acid is usually added as a catalyst. The majority of the hemicelluloses are removed and to some extent degraded, for example to furfural.[48] Sodium hydroxide has also been used as a catalyst.[15] From the organosolv process lignin of high quality can be isolated, and therefore potentially add extra income to the biorefinery.[18] The Canadian company Lignol has a pilot plant based on the organosolv process and it has been tested successfully with softwoods.[18,49]

Challenges for the development of pretreatment technologies

One of the challenges for pretreatment technologies is energy efficiency. With respect to utilization of biomass for bioethanol production a lot of attention has been on the energy balance of the process.[50] All of the pretreatment technologies presented above in general rely on the principle of heating the material to temperatures in the area of 100 to 200°C. Pretreatment is therefore energy intensive. Furthermore, some technologies require or have only been tested with small particle sizes but grinding or milling is also an energy-intensive unit operation.[51] A variety of technologies is on the market or has been described but most research has so far been on optimizing the technologies towards optimum convertibility of the material and reduced formation of degradation products. To make biorefineries cost effective more focus has to be on energy efficiency of the processes and minimized water usage. This means that the processes have to: (1) operate with large particle sizes to reduce the energy used for size reduction; (2) operate at high solids concentrations to reduce water and energy usage; (3) be integrated to use surplus heat/steam from other processes in the pretreatment. In our opinion this also means that pretreament technologies relying on extensive usage of electricity, e.g. heating by microwaves,[52] are not viable, as electricity generation often result in waste heat production that cannot be efficiently or practically utilized.

Pretreatment technologies must be developed with robust reactor systems capable of operating at high solids concentration with large particles and harsh biomass types such as straw and rice straw, which contain high amounts of silica, introducing severe wear and tear on moving parts.

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Even steam explosion technologies that have been used in the pulp and paper industry for many years need to be validated with other types of lignocellulosic materials than wood.

Enzymes for hydrolysis of lignocellulose

Cellulose comprises the largest fraction of the sugars in lignocellulose (Table 1) and glucose is for many microorganisms the preferred carbon source. However, development of microorganisms fermenting hemicellulose sugars efficiently is rapidly progressing.[4,53] Many of the pretreatment methods also partially remove and degrade the hemicelluloses. Most focus has therefore traditionally been put on improving cellulases and decreasing the costs associated with the enzymatic hydrolysis of cellulose.[26,54,55] However, some pretreatment methods leave the hemicelluloses in the material and efficient hydrolysis of these materials therefore also requires the use of hemicellulases. As hemicelluloses vary between different plant species, the optimal enzyme mixture is most likely to be tailor made or adjusted to each different kind of material.

The enzyme system

Efficient hydrolysis of cellulose requires a number of enzymes. According to the traditional enzyme classification system the cellulolytic enzymes are divided into three classes; exo-1,4-β-D-glucanases or cellobiohydrolases (CBH) (EC 3.2.1.91), which move processively along the cellulose chain and cleave off cellobiose units from the ends; endo-1,4-β-D-glucanases (EG) (EC 3.2.1.4), which hydrolyse internal β-1,4-glucosidic bonds randomly in the cellulose chain; 1,4-β-D-glucosidases (EC 3.2.1.21), which hydrolyse cellobiose to glucose and also cleave of glucose units from cellooligosaccharides. All these enzymes work synergistically to hydrolyse cellulose by creating new accessible sites for each other, removing obstacles and relieving product inhibition.[56,57]

Hemicelluloses are heterogeneous with various side groups and as such the hemicellulolytic system is more complex. The hemicellulase system includes among others endo-1,4-β-D-xylanases (EC 3.2.1.8), which hydrolyse internal bonds in the xylan chain; 1,4-β-D-xylosidases (EC 3.2.1.37), which attack xylooligosaccharides from the non-reducing end and liberate xylose; endo-1,4-β-D-mannanases (EC 3.2.1.78), which cleave internal bonds in mannan and 1,4-β-D-mannosidases (EC 3.2.1.25), which cleave mannooligosaccharides to mannose. The side groups are removed by a number of enzymes; α-D-galactosidases (EC 3.2.1.22), α-L-arabinofuranosidases (EC 3.2.1.55), α-glucuronidases (EC 3.2.1.139), acetyl xylan esterases (EC 3.1.1.72) and feruloyl and *p*-cumaric acid esterases (EC 3.1.1.73).[58,59]

Although cellulose *per se* is a simple polysaccharide most cellulose-degrading fungi produce multiple enzymes that are apparently functionally redundant.[60] In *Trichoderma reesei*, the most well characterized cellulase producer, two cellobiohydrolases, five endoglucanases and two β-glucosidases have been characterized and in addition three putative endoglucanases and five β-glucosidases have been identified based on similarity of gene sequence to known enzymes.[61] Among the hemicellulases there are also several enzymes with similar enzymatic properties within each class, e.g. four endoxylanases are produced by *T. reesei*.[61] Due to this a detailed classification system with enzyme families based on sequence homology and hydrophobic cluster analysis was developed and is now accepted for grouping cellulases and hemicellulases.[62,63] An updated list of the glycosyl hydrolase families (GHF) is maintained at the web address www.cazy.org/fam/acc_GH.htlm (the CAZy database).[64]

Most carbohydrate hydrolases are modular proteins with a catalytic and a carbohydrate-binding module (CBM). CBMs are divided into families similarly to the enzymes as mentioned above. At present there are 49 families (www.cazy.org/fam/acc_CBM.html).[64] CBMs were first discovered on cellulases but it is now evident that many carbohydrate hydrolases acting on insoluble but also soluble polysaccharides, e.g. xylan, mannan and starch, have CBMs. The function of the CBM is to bring the catalytic module in close contact with the substrate and ensure correct orientation. Furthermore, for some CBMs a disruptive effect on the cellulose fibres has also been shown.[65,66] As cellulose is an insoluble substrate, the adsorption of the cellulases onto the cellulose surface is the first step in the initiation of hydrolysis. Therefore, the presence of CBMs is essential for fast and correct docking of the cellulases on the cellulose. Removal of CBMs significantly lowers the hydrolysis rate on cellulose.[67]

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Factors affecting the enzymatic hydrolysis

Enzymatic hydrolysis of lignocellulose is confronted by a number of obstacles that diminish the enzyme performance (Fig. 2). Although enzyme price has decreased due to intensive research by, for example, Novozymes and Genencor,[55] enzyme loading should be minimized in order to reduce production costs. This, however, increases the time needed to complete hydrolysis. The use of high substrate concentrations increases the problem of product inhibition, which results in lower performance of the enzymes. The presence of lignin, which shields the cellulose chains and adsorbs the enzymes, is also a major obstacle for efficient hydrolysis. Furthermore, the activity of some enzymes might be lost due to denaturation or degradation. Other factors are closely linked to the substrate composition and thus the pretreatment method employed.

Operating hydrolysis at high substrate concentrations

For almost any application, high sugar concentrations after the hydrolysis are preferable for the fermentation process. This will increase the product concentration and facilitate the downstream processing and product recovery. Operating hydrolysis with high initial substrate concentrations has been faced by the problem of product inhibition of especially the cellulolytic enzyme system (Fig. 2). The β-glucosidases from typical cellulase-producing microorganisms are to some extent inhibited by glucose (K_i of most β-glucosidases is 1–14 mmol L^{-1} glucose).[68,69] This results in accumulation of cellobiose, which is a potent inhibitor of the cellobiohydrolases.[70,71] Inhibition of the cellulases by hemicellulose-derived sugars has also been shown.[72] The competitive product inhibition of the β-glucosidases can to some extent be overcome by addition of a surplus of β-glucosidase activity. Another strategy is to screen for β-glucosidases with high glucose tolerance. β-glucosidases with K_i up to 1400 mmol L^{-1} have been reported,[69,73] and these could be cloned into the cellulase-producing microorganisms to produce a more efficient enzyme mixture.

Other compounds have also been shown to inhibit the enzymes. Among these are degradation products formed during the pretreatment. It has been shown that washing the pretreated material results in faster conversion of cellulose due to removal of inhibitors.[74] The effect of typical degradation products formed during pretreatment has been tested on the activity of cellulases and hemicellulases. Cellulases were only significantly inhibited by formic acid, whereas compounds like vanillic acid, syringic acid and syringylaldehyde in addition to formic acid caused significant inhibition of xylanases.[75,76]

Removal of end product (glucose) is also one possibility. Operating the lignocellulose to bioethanol process as simultaneous saccharification and fermentation (SSF) is frequently employed.[77,78] In this way glucose is fermented into ethanol thereby alleviating the inhibition by glucose. However, the optimum conditions for enzymes and fermenting microorganism are usually not the same. Cellulases have an optimum around 50°C whereas yeast and

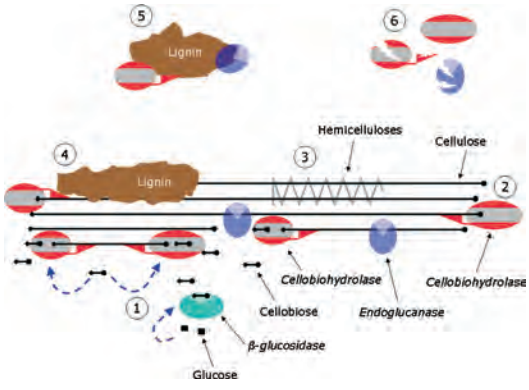

Figure 2. Simplistic overview of factors limiting efficient hydrolysis of cellulose (symbolised by the straight lines). 1: Product inhibition of β–glucosidases and cellobiohydrolases by glucose and cellobiose, respectively. 2: Unproductive binding of cellobiohydrolases onto a cellulose chain. Due to the processivity of cellobiohydrolases and their strong binding of the cellulose chain in their catalytic core, obstacles can make the enzymes halt and become unproductively bound. 3 and 4: Hemicelluloses and lignin associated with or covering the microfibrils prevent the cellulases from accessing the cellulose surface. 5: Enzymes (both cellulases and hemicellulases) can be unspecifically adsorbed onto lignin particles or surfaces. 6: Denaturation or loss of enzyme activity due to mechanical shear, proteolytic activity or low thermostability.

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bacteria have growth optima around 32–37°C. The operation of SSF is therefore at suboptimal conditions for enzymatic hydrolysis. Inhibition by fermentation products should also be taken into account. Ethanol is inhibitory to cellulases, although less compared to glucose.[70,79] In a biorefinery other products, such as organic acids, could be the end product. Lactic acid is also inhibitory to cellulases.[80] Consequently, the effect of end product(s) on the enzymes has to be evaluated before selecting the hydrolysis and fermentation strategy.

The fermentable sugars can also be separated by employing membrane reactors using ultra filtration with a cut off of 50 KDa or less. This way, product inhibition from glucose and cellobiose can be reduced and the service life of the enzymes increased. Membrane reactors can be built with separated or combined reaction and separation zones.[81] The technology works well using pure cellulose substrates but once lignocellulosic substrates are used, membrane fouling is increased. Vigorous stirring may be used to prevent fouling but is energy intensive and the shear forces may cause inactivation of the enzymes.[82] Conversion of cellulose in the range 50–90% has been reported, but none on solid levels above 15%,[83] although higher substrate concentrations in the reaction zone can be obtained.[84]

Operating hydrolysis at initial substrate concentrations above 10–15% (w/w) has also been technically difficult, especially at laboratory scale. The initial viscosity of the material at these concentrations is very high, which makes mixing difficult and inadequate and the power consumption in stirred tank reactors becomes high.[85,86,87] In pilot scale plants, 15–20% dry matter has often been reported as maximum that can be handled.[88] During the initial phase of hydrolysis, the material is liquefied and the viscosity drops significantly.[86,87,89] Operating the hydrolysis or SSF in batch-fed mode by adding fresh substrate when the viscosity has decreased has been used to increase final substrate concentrations added.[40, 90] A special reactor designed for operating the liquefaction and hydrolysis of lignocellulosic material with up to 40% (w/w) initial dry matter have also been investigated.[89] The reactor has been up-scaled to 11 m^3 at the pilot plant of DONG Energy in Denmark. Although it is possible to perform hydrolysis and also SSF with initial substrate concentrations up to 40% (w/w) it was shown that enzyme performance gradually decreased as substrate concentration increased. This was attributed to inhibition of the enzymes by end-products, other inhibitors, presence of high concentrations of lignin and mass transfer limitations.[89]

Optimizing enzyme composition

Extensive research is being done on improving the performance of the enzymes. This involves screening for new enzyme-producing microorganisms, random mutagenesis of fungal strains and genetic engineering of individual enzymes. The development is on increasing specific activity, modifying CBMs to alter interaction with cellulose, increasing tolerance towards end products, improving thermal stability to enable operation at higher temperatures and in some cases modifying pH optima.[55]

It has been argued that although companies like Novozymes and Genencor have reported substantial progress in developing more efficient and cheaper enzymes for cellulose hydrolysis, these have only been optimized for one specific substrate and cannot necessarily be applied to other substrates successfully.[55] Besides β-glucosidase activity, which is of uttermost importance to avoid accumulation of cellobiose and thus severe inhibition of the cellulases, other auxiliary enzyme activities can improve the performance of the enzyme preparation even when not directly involved in hydrolysis of cellulose.[91,92] Production of enzymes on the target lignocellulosic material for hydrolysis has shown that these enzyme preparations perform better than standard commercial enzyme preparations produced on substrates such as purified cellulose.[93–95] This could beneficially be employed in biorefineries to produce enzymes on-site. Moreover, on-site enzyme production could reduce enzyme costs due to less needs for purification and stabilization of enzyme preparations.[96] Another possibility is Consolidated BioProcessing (CBP) where the fermenting microorganism produces the enzymes necessary to hydrolyse cellulose and hemicelluloses.[97] Besides simplifying the process by performing everything in one step, synergism between enzyme and microbe have been observed.[98] CBP has been mostly developed towards ethanol production but not yet tested in industrial scale. So far no efficient microorganisms for CBP are available. A challenge is therefore to select suitable microorganisms, modification for efficient ethanol

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production and simultaneous enzyme production as well as proving the concept and stability in various lignocellulosic materials.

The presence of hemicelluloses has long been neglected when considering the performance of enzyme mixtures for hydrolysis of cellulose. Hemicelluloses are found in close association with the cellulose fibrils as well as lignin and do to some extent cover the fibre surfaces, thereby limiting the access of the cellulases to the cellulose surface (Fig. 2).[9,26] Even after acid-catalysed pretreatment some hemicellulose remains left in the material. It has been shown that addition of xylanases and pectinases significantly improves the performance of cellulases and increases cellulose conversion of pretreated corn stover, hardwoods and softwoods.[91,92,99] Material pretreated by methods such as AFEX, wet oxidation and organosolv has a relatively high hemicellulose content, and in these cases the supplementation with xylanases will probably have an even more pronounced effect.

Due to the complex structure of hemicelluloses a number of enzymes are needed. In wheat, arabinoxylan represents the largest fraction of the hemicelluloses. The arabinofuranosyls can be both (1→2) and (1→3) linked to xyloses and also to doubly substituted xyloses. These side groups have to be removed in order for the xylanases to hydrolyse the xylan backbone. In addition to xylanases, a number of α-L-arabinofuranosidases of various origin are needed in order to obtain efficient hydrolysis of wheat arabinoxylan.[100] Hydrolysis of xylan from hot-water pretreated corn fibre was improved by addition of feruloyl esterases, which removes side groups that limits the access of xylanases to the xylan backbone.[101]

Recently, non-catalytic proteins, the so-called expansins and swollenins, have attracted some attention. These proteins do not catalyse hydrolysis of cellulose but have been shown to disrupt the crystalline structure of cellulose, thus making it more accessible to enzymes. Expansins were first isolated from plants where they participate in weakening the non-covalent binding between cell wall polysaccharides during growth of the cell wall.[102] A protein termed swollenin with sequence similarity to the plant expansins has been isolated from *T. reesei*.[103] Similarly to the expansins this protein exhibits disruption activity on cellulosic materials. Some CBMs have also been shown to have a disruptive effect on cellulose fibres.[65] The applicability and feasibility of using these proteins to improve hydrolysis of lignocellulosic materials still remains to be demonstrated.

Enzyme adsorption and recycling

The presence of lignin in the lignocellulosic material is one of the major obstacles in enzymatic hydrolysis. Lignin forms a barrier that prevents the cellulases from accessing the cellulose[104] and moreover, the lignin is also capable of binding a large part of the enzymes (Fig. 2). The adsorption of cellulases and hemicellulases onto lignin is believed to be due to hydrophobic interaction but ionic-type lignin–enzyme interaction is also possible.[105–107] After almost complete hydrolysis of the cellulose fraction in lignocellulosic material, up to 60–70% of the total enzyme added can be bound to lignin.[93,108,109] Although CBMs participate in binding of cellulases onto cellulose, cellulases without CBM also adsorbs onto lignin. Degree of adsorption of various cellulases and their catalytic core is very different and some cellulases appear to have less affinity for lignin[93,109,110] This could be exploited in the development of new enzyme preparations.

The large fraction of cellulases and hemicellulases unproductively bound to lignin emphasizes that pretreatment methods that remove the majority of the lignin can be advantageous. Removal of 80% of the lignin in steam exploded softwood by an alkaline peroxide treatment has been shown to improve the digestibility of the substrate significantly. Furthermore, due to the lower lignin content a six-fold lower enzyme loading was needed to obtain the same degree of conversion.[111] However, extensive lignin removal and addition of extra steps after steam pretreatment add to the costs of conversion of lignocellulose into fermentable sugars. Furthermore, there is a risk of loss of sugars or degradation of sugars in these subsequent steps. The value of lignin for other applications including as fuel for combustion should also be taken into account before performing an oxidative removal of the lignin. The benefit of lignin removal should therefore be carefully evaluated.

The addition of various compounds to the material before hydrolysis has been shown to improve enzyme performance by reducing unproductive adsorption of enzymes onto lignin. Addition of other proteins or peptides will bind to the lignin and reduce the binding potential

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of enzymes by occupying the binding sites.[112] Addition of non-ionic surfactants like Tween 20 or Tween 80 can also reduce unspecific binding of enzymes, thereby improving the hydrolysis rate so that the same degree of conversion can be obtained at lower enzyme loadings.[106] Ethylene oxide polymers like poly(ethylene glycol) (PEG) show a similar effect.[113, 114] PEG is proposed to bind to lignin by hydrophobic and hydrogen bonding and thereby prevent the lignin from binding of enzymes. A stabilizing effect of PEG at elevated temperatures has been shown, supposedly due to reduced deactivation through exclusion of enzymes from the lignin surface.[115] Unlike Tween 20 or other non-ionic surfactants previously shown to reduce enzyme adsorption and improve hydrolysis, PEG is a low cost commodity product. Addition of 2.5–5 g PEG per kg of pretreated material increases the hydrolysis performance with 20–50% for both steam pretreated spruce and pretreated wheat straw.[113,114] The benefit achieved by the reduction of the enzyme loading should be compared to the costs of adding surfactants.

Recycling of the enzymes has also appeared to be an attractive way of reducing costs for enzymatic hydrolysis.[116–119] The recovery of enzymes is largely influenced by adsorption of the enzymes onto the substrate, especially the binding to lignin. Another constraint in the recycling of the enzymes is inactivation of the enzymes. Cellulases are stable around pH 5 and at a temperature of 50°C for up to 48 h or longer.[120,121] The presence of substrate can increase the stability even further.[71] However, presence of proteases and shear stress caused by pumps and stirring in the reactor might slowly degrade or denaturate the enzymes. The stability of the enzymes in the presence of substrate is, however, difficult to estimate due to adsorption of the enzymes.[122]

There are several strategies to recover and reuse the cellulases. The filtrate obtained after complete hydrolysis of the cellulose fraction can be concentrated by ultra-filtration to remove sugars and other small compounds that may inhibit the action of the enzymes. Depending on the lignin content of the substrate, only up to 50% of the cellulases can be recycled using this approach.[116,122] The saving is therefore low, taking costs of the recovery into account. Another approach takes advantages of the ability of cellulases to adsorb onto the material. By addition of fresh material after hydrolysis, enzymes in solution were readsorbed onto the new material. The new material retaining up to 85% of the enzyme activity free solution could then be separated and hydrolysed in fresh media eventually with supplementation of more enzyme.[123] Another method for recycling enzymes is by immobilization, which enables separation of the enzymes from the process flow. The principle of immobilization is to fixate the carbohydrolytic enzymes onto a solid matrix either by adsorption or grafting.[124] Apart from extending the service life of a given enzyme, immobilization will also affect activity and stability with regard to temperature and pH optima. Typically broader optima of both pH and temperature are observed.[125,126] The stability can be significantly increased but *Km* levels are generally higher, thus the specific activity is lower.[127,128] Reported work shows that immobilization does not hinder access to insoluble cellulose, nor the hydrolysis of it. However, few studies use industrially relevant substrates and none are done under high solids loading with, for example, high lignin levels.[129, 130] The use of immobilized enzymes for lignocellulose is a technically difficult task, in part because a multitude of enzymes are required for breakdown of the carbohydrates. An opportunity could be the combination of membrane separation and immobilized enzymes.[131]

All of these techniques for recycling and reducing enzyme adsorption have so far only been tested at laboratory scale. Furthermore, most of the studies do not include cost calculations to evaluate the feasibility of addition of different compounds to reduce enzyme binding or the costs of recycling. Therefore, the ability to scale up the techniques, the robustness and feasibility still needs to be demonstrated.

Opportunities for conversion of lignocellulose into fermentable sugars

The focus on biofuels from lignocellulose has attracted a lot of research, development and optimization in a number of fields related to conversion of lignocellulose into fermentable sugars. More and more pilot-scale facilities for pretreatment are being constructed, facilitating much better evaluation of the technologies, their constraint and opportunities. As outlined above, many technologies are available and an evaluation of a number of pretreatment technologies

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revealed that they all performed very similarly and at almost identical costs, on corn stover.[132, 133] The ideal pretreatment therefore depends on local conditions like type and costs of raw material and the need for extraction of by-products such as lignin as well as costs of enzymes. Future research needs to focus on development and validation of large-scale reactor systems. In particular handling of biomass at high solids concentrations, large particle sizes and with high amounts of silica from agricultural residues has to be tested and verified to ensure long-term stability of a future biorefinery plant. Of course this should not exclude evaluation of radically new concepts such as the use of supercritical water for pretreatment and hydrolysis of cellulose and biomass.[134, 135]

Development of more efficient enzymes is also the subject of extensive research. Many opportunities exist for improving the enzyme mixtures, the stability and specific activity of the enzymes but also to reduce their costs and cost of application. Depending on raw material and pretreatment technology, the enzyme mixture must be designed to the specific substrate, e.g. through addition of substrate-specific auxiliary enzymes. Finally, there are a number of other possibilities to not only improve the enzyme proteins but to also improve the enzyme/substrate interaction. Significant improvements in yield and cost reduction can therefore be expected, thus making large-scale hydrolysis and fermentation of lignocellulosic substrates possible.

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II

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Cell-wall structural changes in wheat straw pretreated for bioethanol production

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Abstract

Background: Pretreatment is an essential step in the enzymatic hydrolysis of biomass and subsequent production of bioethanol. Recent results indicate that only a mild pretreatment is necessary in an industrial, economically feasible system. The Integrated Biomass Utilisation System hydrothermal pretreatment process has previously been shown to be effective in preparing wheat straw for these processes without the application of additional chemicals. In the current work, the effect of the pretreatment on the straw cell-wall matrix and its components are characterised microscopically (atomic force microscopy and scanning electron microscopy) and spectroscopically (attenuated total reflectance Fourier transform infrared spectroscopy) in order to understand this increase in digestibility.

Results: The hydrothermal pretreatment does not degrade the fibrillar structure of cellulose but causes profound lignin re-localisation. Results from the current work indicate that wax has been removed and hemicellulose has been partially removed. Similar changes were found in wheat straw pretreated by steam explosion.

Conclusion: Results indicate that hydrothermal pretreatment increases the digestibility by increasing the accessibility of the cellulose through a re-localisation of lignin and a partial removal of hemicellulose, rather than by disruption of the cell wall.

Background

Research in bioethanol production from lignocellulosic plant materials has grown significantly over the last few decades as the depletion of non-renewable fuels and increasing greenhouse gas emissions continue to create an increasing need for an alternative non-fossil transportation fuel. Enzymatic hydrolysis of lignocellulosic biomass, such as agricultural residues, with subsequent fermentation of sugars into ethanol has long been recognised as an alternative to the existing starch and sucrose-based ethanol production, especially considering recent improvements in yields and enzyme prices [1-3]. Furthermore, lignocellulose may be used as a feedstock for biorefineries, and full-scale plants for cellulosic bioethanol production are planned or under construction in several countries.

Two process steps are involved in the conversion of lignocellulose into bioethanol: (1) enzymatic hydrolysis of the cell-wall carbohydrates, cellulose and in some cases hemicellulose, into monomers; and (2) fermentation of the monomers into ethanol. Often the two processes are integrated into simultaneous saccharification and fermentation (SSF). A common feature of the enzymatic hydrolysis step is the need for pretreatment of the lignocellulosic material resulting in a more efficient reaction despite the recalcitrant nature of the plant cell wall [4].

While a costly step in production, optimal pretreatment is important from an economic viewpoint, as it has an impact on product yields and concentration, the rate of hydrolysis and fermentation, enzyme loading, waste products and fermentation toxicity [5]. The effect of the pretreatment has been described as a disruption of the cell-wall matrix including the connection between carbohydrates and lignin, as well as depolymerising and solubilising hemicellulose polymers [6]. This improves access for the saccharifying enzymes and alleviates mass-transport limitations [5]. Pretreatment is also able to change the degree of cellulose crystallinity [7].

There are several different ways of pretreating biomass, depending on the type, composition and subsequent processing technology that will be applied. The most widely investigated pretreatment technologies are thermochemical treatments such as dilute acid treatment (with or without rapid steam decompression (explosion)) [8-10] and ammonia pretreatment [11,12]. Hydrothermal pretreatment without the use of chemicals has also proven to be effective [13,14]. For a review of the most important pretreatment methods, see [5,15].

Recently, an EU-funded project on the co-production of bioethanol and electricity (Integrated Biomass Utilization System - IBUS) has resulted in a hydrothermal pretreatment process for wheat straw that has proven to be effective at preparing straw for enzymatic hydrolysis [16]. The process is designed to handle large particles (pieces of straw over 5 cm in length) and run at high dry-matter levels (exceeding 30% w/w) [16]. In the process, the straw is treated with water while being moved through a countercurrent reactor at a temperature of 190-200°C. The wash water can be recycled and salt and solubilised hemicellulose sugars can be isolated [16]. A pretreatment pilot plant with a capacity of up to 1000 kg/hour has been working since 2006. As described in [16] and [17], the pretreated straw can be enzymatically liquefied, saccharified and subsequently fermented into ethanol at initial dry-matter levels of up to 40% w/w. Recent SSF experiments with an initial dry-matter content of 27% (w/w) have produced ethanol levels of over 60 g/kg slurry [18]

Atomic force microscopy (AFM) has proven to be a powerful tool for visualising the surface of plant cell walls [19-22] including modification of plant fibres and pulp [23-25]. In the present study, AFM and scanning electron microscopy (SEM) investigations of the effects of hydrothermal treatment on straw cell wall disruption, composition, ultrastructure and surface properties were carried out in order to better understand the increased susceptibility to enzymatic hydrolysis. Chemical decomposition into constituent polymer classes was carried out for all sample types. Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy was used as an analytical tool to qualitatively determine the chemical changes in the lignocellulosic material upon pretreatment. For comparison, analyses were also carried out on SO_2-impregnated steam-explosion pretreated wheat straw. Steam explosion is a widely recognised pretreatment [8].

Results and discussion

Straw composition

As seen in Table 1, the main effect of the hydrothermal pretreatment on the composition of the biomass is the partial but substantial removal of hemicelluloses. All measurable arabinan is removed and the xylan content is reduced from 24.5% to 5.2%. Consequently, the overall cellulose content increases. After delignification of the pretreated material, no Klason lignin can be detected. The composition of the straw that has undergone SO_2-impregnated steam explosion is similar to that of the hydrothermally pretreated straw except for a slightly higher xylan content at 7.8%.

ATR-FTIR spectroscopic analysis

ATR-FTIR spectroscopy was used as an analytical tool to qualitatively determine the chemical changes in the sur-

Table 1: Compositions

	Cellulose	Xylan	Arabinan	Klason lignin	Ash
Straw, untreated	39.8	24.5	2.8	22.6	4.2
Pretreated straw	59.0	5.2	0.0	25.5	5.6
Delignified, pretreated straw	75.1	9.8	0.0	0.0	8.8
Steam-exploded straw	56.7	7.8	0.7	23.6	6.3

Contents expressed as percentages, based on dry matter.

face of pretreated straw to complement and understand the microscopic investigations. The FTIR spectra of untreated, hydrothermally pretreated, delignified hydrothermally pretreated and steam-exploded straw samples are shown in Fig. 1A. Excerpts of the four spectra are presented in Fig. 1B.

One of the effects of the pretreatment is the removal of wax from the straw: Fig. 1A shows that the CH_2- stretching bands at approximately 2850 and 2920 cm^{-1} (see [26]) are reduced for the pretreated straw sample, signifying a reduction in the amount of the aliphatic fractions of waxes.

Two interesting features are shown in Fig. 1B. First, it can be seen that the carbonyl band at 1735 cm^{-1}, which has been ascribed to hemicelluloses [27-29] is reduced for the pretreated straw. This is expected as the pretreatment is known to remove a large portion of the hemicelluloses as shown in Table 1 and in Thomsen et al. [16]. Second, lignin bands at approximately 1595 and, in particular, 1510 cm^{-1} (aromatic ring stretch) [30] are strongly enhanced in the hydrothermally pretreated sample compared with both untreated wheat straw and delignified hydrothermally pretreated straw, where these peaks are reduced (Fig. 1B). One explanation for this could be a relative increase in the amount of lignin due to the removal of hemicelluloses. Another reason could be that lignin is

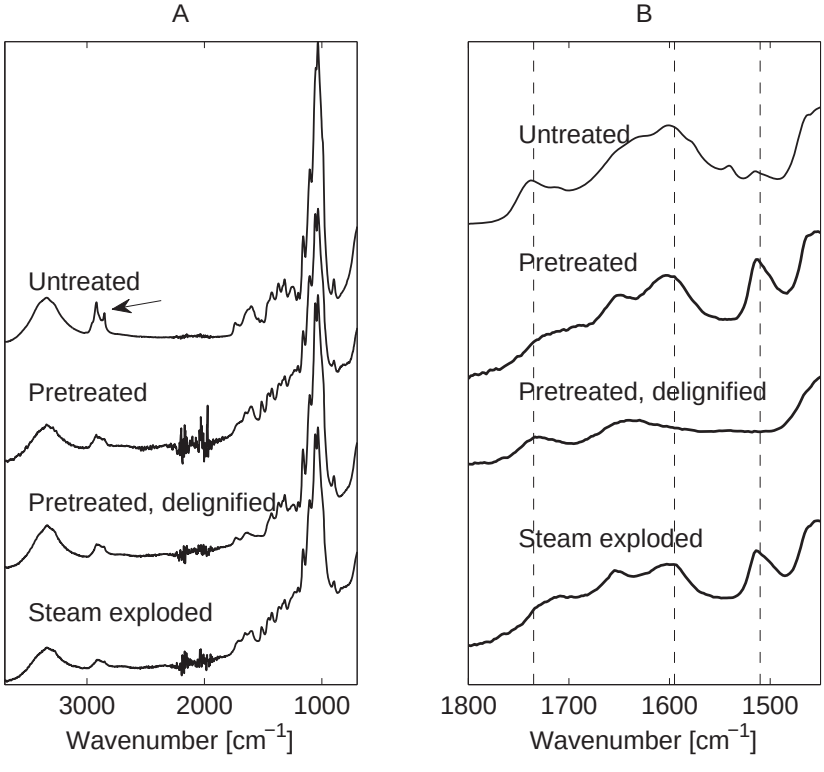

Figure 1
Spectroscopy. ATR-FTIR spectra of untreated, hydrothermally pretreated, delignified hydrothermally pretreated and steam-exploded wheat straw. **(A)** Complete spectra of all treatments. **(B)** Excerpt of spectra. All spectra are separated to ease comparison. The arrow in A points to the bands at 2850 and 2920 cm^{-1} (CH_2- stretching bands ascribed to wax). The vertical lines in B mark the positions of the bands at 1735 (carbonyl, ascribed to hemicellulose), 1595 and 1510 cm^{-1} (aromatic ring stretch, ascribed to lignin).

released and re-deposited on the surface (ATR-FTIR spectroscopy is a surface technique; according to [26,31] the penetration depth in straw is approximately 0.5-3 μm with the signal intensity exponentially decreasing with penetration depth). The increase in lignin is believed to be too significant to be only due to the hemicellulose removal.

One of the strategies employed in increasing enzymatic convertibility is to decrease cellulose crystallinity [15]. Differences between samples with regard to the relative amounts of amorphous and crystalline cellulose have earlier been described through infrared peak ratios. At least four different peak pairs have been proposed [32,33]. Of these, only the peak pair 1429 cm^{-1} (crystalline) and 893 cm^{-1} (amorphous) is seen for the samples of the present study. The peak ratio for the untreated straw was 0.56, while it was 0.52 for the pretreated straw. In the study by Wistara et al. [33], values from 0.46 to 0.56 were reported, and from this and other results the authors claimed that there was no difference in crystallinity between their samples. When comparing their results with ours, it appears that the pretreatment does not adversely affect the degree of cellulose crystallinity. More precise measurements of cellulose crystallinity are needed to confirm this result.

SEM and AFM images

Based on the results from ATR-FTIR spectroscopy, SEM and AFM were used to gather information on the effect of the hydrothermal pretreatment on the ultrastructure and possible disruption of the cell wall.

When untreated, the anatomy of the harvested, chopped wheat straw is easily recognisable, with sheath leaves surrounding the straw itself (Fig. 2A). The various cell types of the straw wall can be seen, including epidermis cells, parenchyma cells, vascular bundles (phloem and xylem) as well as thick-walled fibre cells, as seen in the SEM micrograph presented in Fig. 2B. Imaging by AFM of parenchyma cells lining the straw cavity reveals the appearance of interwoven cellulose microfibrils of the primary wall (Fig. 2C). These particular cells are largely unlignified [34] but microfibrils are partially embedded in what is believed to be hemicellulosic polymers (left-hand side of Fig. 2C).

Initially, the most apparent effect of the hydrothermal pretreatment apart from a colour change from yellow into dark brown is the partial defibration, or separation of individual fibres and cell types of the wheat straw. Although the pretreated material is quite heterogeneous and contains larger pieces (up to about 1 cm) that are easily recognised as straw, a significant fraction consists of cells that are either completely or partially separated from each other (Fig. 2D).

All individual fibres (and most other cell types) seem to be intact despite the hydrothermal treatment, rather than being broken or otherwise disrupted (Fig. 2D and 2E). When looking more closely at the pretreated fibres it becomes apparent that the surface is covered with 'debris' and a thin layer of deposits that seems to be covering the whole surface (Fig. 2E). This debris could be fractions of middle lamellae. When further investigating the pretreated fibre surfaces through AFM, it was not possible to identify any primary or secondary wall cellulose microfibrils (such as seen in untreated fibre cell walls; Fig. 2C). Instead, an uneven surface of spherical and globular shapes was seen (Fig. 2F). These globular shapes (diameter approximately 20-100 nm) are characteristic of lignin deposits as reported in the literature [22,25,35], and this interpretation is in accordance with the spectroscopic findings of higher surface lignin concentrations.

Initially, delignification did not have a great effect on the overall structure of the pretreated material apart from a change in colour; the straw was still only partially defibrated (Fig. 2G), presumably due to the hemicellulose content of the middle lamella [34]. However, upon closer observation, the surface of the individual fibres had changed drastically. The uneven surface now appeared smooth and cellulose aggregates (macrofibrils) running in the direction of the fibre could be seen, as in the SEM image in Fig. 2H. When investigating the delignified fibre surfaces with AFM, the globular shapes of deposited lignin were not seen. Instead, intact surfaces believed to be primary and secondary wall lamellae were observed. Due to the mixing of fibres and other cell types during the pretreatment it was not possible to investigate the same straw cavity parenchyma cells as with the untreated straw. However, numerous scans of different cells revealed several surfaces with similar primary walls to the parenchyma cells. The microfibrils of these primary walls displayed the same interwoven structure as previously seen and were partially embedded in non-cellulosic polymers (Fig. 2I). It should be added, that with AFM only relatively smooth surfaces are successfully imaged.

Surprisingly, neither the overall or fibrillar structure of the individual fibres seems to show large structural changes such as the rupture of fibres or a visible increase of porosity, which are believed to be associated with thermal pretreatments. No holes or cracks were seen in the fibres and AFM did not indicate that the accessibility of the internal parts of the cell wall matrix had been improved due to structural dislocations. Rather, the primary and secondary cell walls appeared to be fully intact, except for the pits and simple perforations that already exist in certain cell types [36]. Despite these observations of a substrate where the skeletal structure is intact and the crystallinity of the cellulose does not appear to have been lowered, the

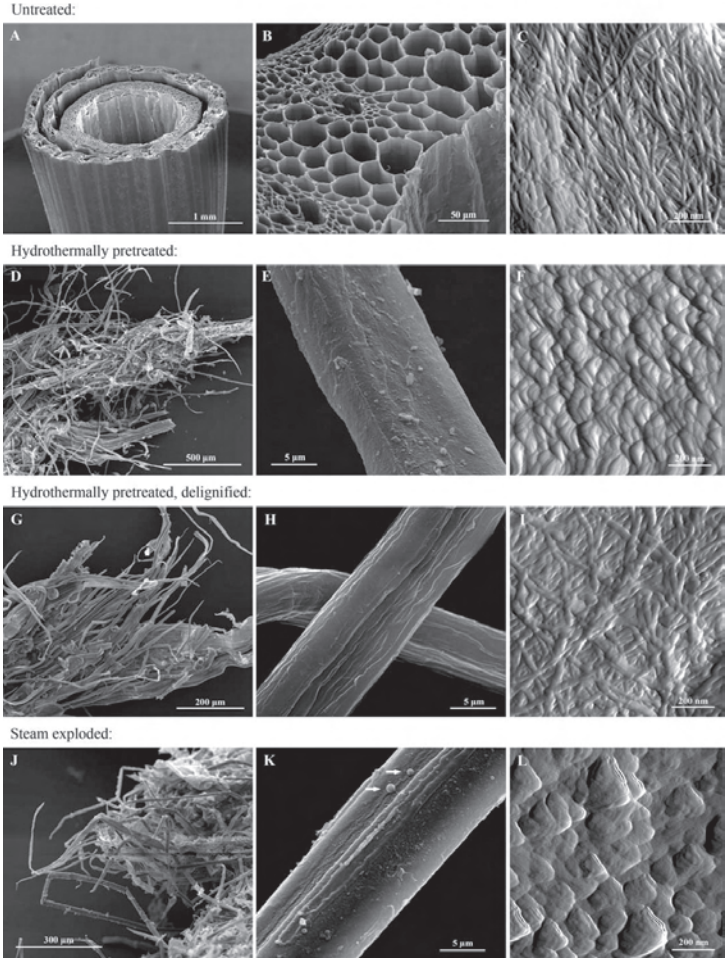

Figure 2

Microscopy images. SEM and AFM images of untreated **(A)-(C)**, hydrothermally pretreated **(D)-(F)**, delignified hydrothermally pretreated **(G)-(I)** and steam-exploded wheat straw **(J)-(L)**. In untreated wheat straw, the straw itself is surrounded by a sheath leaf (A, SEM image) and at slightly higher magnification the individual cells of the straw wall can be identified (B, SEM image). A high-resolution AFM scan (amplitude image) of a primary cell wall lining the straw cavity shows interwoven cellulose microfibrils, partially imbedded in non-cellulosic polymers (left-hand side of C). In hydrothermally pretreated wheat straw, the defibrating effect of the pretreatment causes the individual fibres to partially separate, as can be seen in D (SEM image). The pretreatment leaves a surface layer of debris and re-deposited cell-wall polymers on the individual fibres (E, SEM image). An AFM scan (amplitude image) of fibre surface shows the 'globular' deposits characteristic of lignin (F). No microfibrils are visible. Delignification of pretreated fibres causes no further separation of fibres (G and H, SEM images) but removes most of the surface layer/deposits seen in (E). Cellulose lamellae/agglomerates are now visible (H). An AFM scan (amplitude image) shows that delignification exposes intact, interwoven cellulose microfibrils (I). Steam explosion causes partially separated fibres with 90° compression bends (J, SEM image) and a surface layer with debris and droplets (K, SEM image). Droplets are indicated with arrows. High-resolution imaging of AFM shows globular surface deposits (L, amplitude image), similar to those seen on hydrothermally pretreated straw (F).

hydrothermally pretreated straw has been shown to be easily digestible by enzymes [16,17]. Consequently, the effectiveness of the pretreatment must be related to hemicellulose removal and lignin re-localisation. This is in spite of the fact that lignin is not removed by the pretreatment and that lignin is known to be responsible for unproductive adsorption of cellulases [37,38]. It is well known that lignin encases the cellulose in the cell-wall matrix, hindering cellulases from reaching cellulose fibrils. We hypothesise that the migration of lignin to the outer surface exposes internal cellulose surfaces. More investigations are needed in order to confirm this. Selig et al. [39] have also observed the formation and migration of spherical lignin deposits onto the surface of fibres as a result of pretreatment. They also suggest that the deposited lignin can have a negative impact on the enzymatic cellulose hydrolysis. It is possible, however, that the surface lignin layer is easily removed by simple mechanical forces through mixing, due to lignin being less strongly bound to carbohydrate polymers compared with its native linkages. Furthermore, we theorise that the re-located lignin has exposed cellulose inside the cell wall, thus increasing the enzyme accessibility.

Based on these observations, we therefore propose that the re-localisation of lignin as well as partial hemicellulose removal are likely to be important factors in increasing the enzymatic digestibility of wheat straw through hydrothermal pretreatment. It seems that exposing cellulose through manipulation of hemicelluloses and lignin are equally as important as altering the crystallinity and rupture of the skeletal structure of the cell wall.

Comparison with conventional steam explosion

In order to understand whether the factors affecting biomass digestibility through hydrothermal pretreatment are of a more general nature, steam-exploded straw was also investigated microscopically and spectroscopically. Steam explosion is considered one of the most promising pretreatment technologies and is often combined with the addition of chemicals [5,6]. In our case the straw was impregnated with SO_2 prior to steam explosion. In principle, steam explosion is not unlike hydrothermal pretreatment. As such, the effect on compositional changes is also similar (Table 1).

As seen in Fig. 1 the FTIR spectra of the steam-exploded straw are similar to those of hydrothermally treated straw, both in general and in spectral ranges related to wax, hemicellulose and lignin. SEM investigations (Fig. 2J and 2K) showed that steam-exploded straw was more heterogeneous than hydrothermally pretreated straw, containing larger pieces of almost intact straw but also a larger fraction of individual fibres that had been compacted together. Some SEM images also showed droplets on the surface of the fibres (see the arrows in Fig. 2K). These droplets are also believed to be lignin, possibly formed through coalescence of smaller sized lignin deposits during the pretreatment as described in [39]. The difference in amount of larger lignin droplets between the different pretreatments may be due to varying water contents and pH during the treatment. AFM showed globular deposits similar to, but larger than those seen on hydrothermally pretreated straw (Fig. 2L).

Conclusion

Hydrothermal pretreatment has proven to be an effective way of increasing the enzymatic digestibility of wheat straw for conversion into fermentable sugars for bioethanol production. However, it has been unclear how the pretreatment affects the ultrastructure and molecular organisation of the biomass.

It was found that the hydrothermal pretreatment had a partial defibrating effect on wheat straw, producing a heterogeneous substrate of semi-separated fibres. Interestingly, in contrast to what might be expected, individual fibres were intact with no evidence of disruption. It was found that the vast majority of all fibre surfaces (more than 90%) were covered with a layer of globular deposits. The deposits were established to be re-localised lignin. Upon delignification of pretreated fibres, the cellulose fibrillar structure of the cell walls was found to be intact. The conservation of the skeletal structure of the cell wall through pretreatment is not in accordance with the general perception that pretreatments must disrupt the structure of the cell wall in order to increase its accessibility to enzymes.

Partial hemicellulose removal and lignin re-localisation are important factors in increasing the digestibility of hydrothermally pretreated wheat straw, possibly more important than rupture of the skeletal cell-wall structure and modification of cellulose crystallinity. Results show that it is possible to pretreat wheat straw sufficiently without disrupting the cell wall. Thus, only a modest pretreatment is necessary in order to enzymatically digest the carbohydrates, provided that mixing is efficient [17].

Although much is known about the chemical changes caused by pretreatments of lignocellulose, little seems to be known of the physical changes. We believe that research and development of technologies must be accompanied by structural and molecular investigations of the biomass in order to achieve substantial progress.

Methods

Pretreatments

The hydrothermal pretreatment was carried out at the IBUS pilot plant at Fynsværket in Odense, Denmark [16].

Pretreatment was performed at a feed rate of 75 kg of chopped wheat straw (0-5 cm long pieces) per hour (or approximately 67.5 kg dry matter per hour), which is presoaked in water at 80°C for 6 minutes prior to being transported into the reactor. Residence time in the reactor averaged 6 minutes with the reactor temperature maintained at 195°C by injection of steam, and a counter-current flow of water of 250 litres per hour. No chemicals were added to the water or the steam. The dry-matter content of the pretreated straw out of the reactor was between 25% and 32% (w/w). The pretreated straw was collected in plastic bags containing 30-50 kg of material and stored at 4°C for up to 5 months. The pretreated straw is used for hydrolysis and fermentation into bioethanol without any further treatment such as washing. For more information on this particular pretreatment technology as well as hydrolysis and fermentation studies with the pretreated material, see [16] and [17].

The steam-exploded straw was a gift from the Center for Chemistry and Chemical Engineering, Lund University, Sweden. The straw was pretreated as described in [38].

Compositional analysis of straw

The typical compositions of straw, pretreated straw, delignified pretreated straw and conventionally steam-exploded straw were analysed using two-step acid hydrolysis according to the procedure published by NREL [40]. Before hydrolysis, the samples were dried at 45°C for 1 day. The dried samples were milled in a Braun coffee grinder. Dry matter was determined using a Sartorius MA 30 moisture analyser at 105°C. The content of monosaccharides in the hydrolysed samples (D-glucose, D-xylose and L-arabinose) was quantified on a Dionex Summit high-performance liquid chromatography (HPLC) system equipped with a Shimadzu RI-detector. The separation was performed in a Phenomenex Rezex RHM column at 80°C with 5 mM H_2SO_4 as an eluent at a flow rate of 0.6 ml min^{-1}. Samples were filtered through a 0.45 μm filter and diluted with eluent before analysis on HPLC.

Straw sample preparation for analyses

The straw samples analysed in this study consisted of an untreated control, hydrothermally pretreated material and steam-exploded straw, all of which were oven dried at 50°C for 24 hours. The hydrothermally pretreated straw was subsequently delignified by mixing approximately 25 g of dried straw with 800 ml MilliQ water, 40 ml of 98% glacial acetic acid and 20 g of sodium chlorite ($NaClO_2$). The mixture was placed in a water bath at 80°C for 1 hour. The sodium chlorite and acetic acid additions were repeated twice, the second time with the addition of glacial acetic acid only. The reaction was terminated by cooling to 10°C. The holocellulose was isolated by filtration through a glass filter and rinsing with ice-cold MilliQ water, followed by oven-drying at 50°C for 24 hours. For SEM, the straw was lyophilised without prior oven-drying.

ATR-FTIR spectroscopic analysis

ATR-FTIR spectra (4000-700 cm^{-1}) were obtained using an ABB Bomem FTIR spectrometer equipped with a SensIR/Durascope diamond. An ATR accessory was used to qualitatively identify chemical changes in the pretreated wheat straw. Spectra were obtained with 4 cm^{-1} resolution, and 128 scans for the background spectrum and 64 scans for each sample spectrum were performed. After drying, the straw sample was pressed against the diamond surface using a spring-loaded anvil to obtain the same pressure for each sample. To ensure that the surfaces measured were similar to those investigated by microscopy, the samples were not homogenised prior to spectral analysis. The risk taken when selecting this procedure was that the surface cells of the untreated straw were not representative for the bulk material. In order to check whether this was the case, some untreated material was ground to a fine powder, and ATR-FTIR spectra were obtained from the homogenised material. No significant differences were found between these spectra and those from the non-homogenised samples.

Spectra were recorded from three different sub-samples per sample type, and all spectra were corrected according to the standard normal variate (SNV) method [41]. The mean spectrum of the three corrected spectra is presented for each sample type.

SEM analysis

SEM analysis was performed with a FEI Quanta 200 (FEI Company, Eindhoven, The Netherlands) operated at 20 kV. The samples were coated (gold/palladium) with a SC7640 Suto/Manual High Resolution Sputter Coater (Quorum Technologies, Newhaven, UK).

AFM analysis

All AFM measurements were made with a MultiMode scanning probe microscope with a Nanoscope IIIa controller (Veeco Instruments Inc, Santa Barbara, CA). Images were acquired in TappingMode with an etched silicon probe (MPP-12100, Veeco NanoProbe, Santa Barbara, CA). An auto-tuning resonance frequency range of approximately 150-300 kHz with a scan rate of 0.5-3 Hz (usually around 2 Hz) was used. The drive amplitude and amplitude set-point were adjusted during measurements to minimise scanning artefacts. Height, amplitude and phase images were captured simultaneously. Scan size varied from 500 nm to 5.0 μm but was usually 1 μm.

Samples were fixed on metal discs with double-sided adhesive tape. All images were measured in air. Images were collected from a minimum of 20 different fibres for

each treatment with representative images displayed in the present paper. To eliminate external vibration noise, the microscope was placed on an active vibration-damping table. All AFM images were recorded in a 512 × 512 pixel format and analysed and processed (contrast, illumination and plane fitting) by the accompanying Veeco Nanoscope software.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JBK carried out the AFM and SEM work, participated in the spectroscopic measurements and drafted the manuscript. LGT carried out the spectroscopic measurements, analysed the spectra and drafted most of the spectroscopically related parts of the manuscript. CF participated in the AFM work as well as design and coordination of the study. HJ carried out the composition analyses, participated in the SEM work and helped to draft the manuscript. TE participated in the AFM work and the experimental design. All authors suggested modifications to the draft, commented on several preliminary versions of the text and approved the final manuscript

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Use of surface active additives in enzymatic hydrolysis of wheat straw lignocellulose

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Abstract

Monocot residues such as corn stover and straw are often not fully exploited and constitute a potential substrate for bioethanol production. However, a number of factors such as high enzyme loadings make large-scale utilization economically difficult. Addition of non-ionic surfactants and poly(ethylene glycol) to enzymatic hydrolysis of various lignocellulosic substrates has been found to increase the conversion of cellulose into soluble, fermentable sugars. We have shown that surfactants are able to increase cellulose conversion with up to 70%. This provides an opportunity of decreasing enzyme loading while retaining the same degree of hydrolysis. Investigations of five wheat straw substrates produced with different pretreatment methods revealed that surfactants have a more pronounced effect on acid and steam treated straw than, e.g. ammonia and hydrogen peroxide treated straw. Thus, lignin content is not directly proportional with the potential surfactant effect. Studies of adsorption of cellulases support the theory that the main mechanism behind the surfactant effect is prevention of unspecific adsorption of enzyme on the substrate lignin. This is believed to be due to hydrophobic interaction between lignin and the surfactant, causing steric repulsion of enzyme from the lignin surface. More research is needed to reveal which factors influence enzyme and surfactant adsorption.
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Keywords: Cellulose; Cellulase; Hemicellulose; Surfactant; Adsorption; PEG

1. Introduction

For more than a decade lignocellulose has been recognized as a potential substrate for ethanol production [1]. Despite intensive research, several factors still prevent a large-scale utilization of lignocellulose for liquid fuel production. The main obstacle is the need of high enzyme concentrations in order to obtain a high rate of cellulose conversion into glucose along with long process times due to rapid decrease of the hydrolysis rate [1,2]. In addition, enzyme recycling is difficult as enzymes adsorb to residual lignocellulosic material. In order to make cellulose hydrolysis for ethanol production economically feasible it is important to identify methods to increase enzyme effectiveness.

It has been shown that addition of surfactants such as non-ionic detergents and protein significantly increases the enzymatic conversion of cellulose into soluble sugars [3–7]. Various mechanisms have been proposed and investigated for the positive effect of surfactant addition on the enzymatic hydrolysis of lignocellulose. Recent studies on steam-treated softwood substrate propose that the dominating mechanism responsible is the influence of surfactants on cellulase interaction with lignin surfaces [7]. Surfactant adsorption to lignin is believed to prevent unproductive binding of enzymes to lignin, thereby producing higher yields and better recycling of enzymes. This is in accordance with other results showing less adsorption of enzymes to lignocellulose during hydrolysis in the presence of a surfactant [3,6]. Added protein such as BSA is also believed to bind to lignin, preventing unproductive binding of cellulases [7,8].

Other mechanisms proposed include the surfactant being able to change the nature of the substrate, thereby increasing the available cellulose surface; in turn promoting reaction sites for cellulases to adsorb onto [6,9]. Surfactants may also have a stabilizing effect on the enzymes, effectively preventing enzyme denaturation during the hydrolysis. This possible binding of the

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surfactants to the tertiary structure of the enzyme-proteins is known from other enzymes [10].

The first step in the enzymatic hydrolysis where soluble cellulases convert solid cellulose into soluble sugars is the adsorption of the enzymes onto the cellulosic surface. It has been shown that the rate of adsorption is rapid compared to the actual hydrolytic activity of the enzymes, thus making the amount of adsorbed cellulase an important factor in the effectiveness of the reaction [11]. The pretreatment or processing of the lignocellulosic substrate has a significant effect on the rate and extent of cellulase adsorption [12].

Previous studies have focused on wood materials and especially softwood lignocellulose due to the regional abundance of it. However, corn stover and straw are agricultural residues, which are today not fully exploited and therefore interesting as raw material for bioethanol production. The lignin content of herbaceous materials is in general lower and has a different composition. These differences are likely to influence the interaction between substrate and enzymes and therefore also the effect of the surfactants.

The main focus of this study is to investigate if the conversion of straw cellulose into sugar can be increased with various surface active additives as effectively as is the case with, e.g. steam-treated spruce lignocellulose [7]. Furthermore, the relationship between the type of pretreatment process applied and the increase of hydrolysis caused by the surfactant is investigated. This was carried out by hydrolyzing five different types of pretreated wheat straw, using a commercial enzyme mixture. Hydrolysis was performed with various non-ionic surfactants added and with protein (BSA) for comparison. To our knowledge, this is the first time the relationship between the pretreatment type and the effect of various non-ionic surfactants on cellulose hydrolysis has been investigated. This relationship has helped shed more light on the mechanism of the surfactant effect. In order to clarify this mechanism and the important role of lignin further, the endoglucanase activity in the hydrolysis solutions was measured.

Another limiting factor in converting lignocellulose into bioethanol is the lack of pentose-fermenting microorganism. The main sugar of monocot hemicelluloses is the pentose xylose, which often makes up a substantial part of the total sugar content of the cell wall. However, industrial yeast strains for this purpose are currently being produced [13]. Therefore, the effect of surfactants on xylan conversion was also investigated in the hydrolysis experiments.

2. Materials and methods

2.1. Substrates

Wheat (*Triticum aestivum*) was grown and harvested in Denmark in 2003. The straw was left to dry on the field and then pressed into big bales. The bales were stored dry at ambient temperature. Before use, the straw was cut into pieces up to 6–8 cm long by a forage harvester and stored in containers at ambient temperature. The dry matter (DM) content was approximately 90% (w/w).

Fresh, chipped spruce (*Picea abies*) free of bark was provided by a saw mill in southern Sweden. The chip size was 2–10 mm.

Table 1
Chemical composition of surface active additives

Surfactant/polymer	Composition
Berol (alcohol ethoxylate)	Berol ox 91-8: $CH_3–(CH_2)_{8–10}–O–(CH_2–CH_2–O)_8$; Berol 08: $CH_3–(CH_2)_{15–17}–O–(CH_2–CH_2–O)_{80}$
Poly(ethylene glycol) (PEG) (molecular masses: 2000, 4000 and 6000)	$HO–(CH_2–CH_2–O)_n–H$ (n = 45, 91 and 136)
Tween 80	Polyoxyethylene sorbitan monooleat
Bovine serum albumine (BSA)	

2.2. Surface active additives

The tested additives were: bovine serum albumine (BSA, Sigma–Aldrich, St. Louis, USA), poly(ethylene glycol): PEG 2000, PEG 4000 and PEG 6000 (Merck & Co., St. Paul, USA), Berol 08 and Berol ox 91-8 (Akzo Nobel, Stenungsund, Sweden). All surface active additives will be referred to as surfactants for the sake of convenience. Names and chemical compositions of the surface active additives used are listed in Table 1.

2.3. Pretreatment methods

Four different batches of pretreated straw were produced on the IBUS pilot plant at Fynsværket in Odense, Denmark [14]. In addition, one batch of pretreated straw and one batch of spruce were pretreated by steam explosion at Center for Chemistry and Chemical Engineering, Lund University, Sweden. The pretreatment conditions are summarized in Table 3.

Pretreatment on IBUS pilot plant. The straw was pretreated according to [14] using a feeding rate of 50 kg straw per h (= 45 kg DM h^{-1}), 250 l h^{-1} of counter-current water flow and a residence time of 6 min in the reactor. The reactor temperature was maintained at 190 or 195 °C (Table 3) by injection of steam. The pretreatment was performed using water or water with the addition of ammonia, sulfuric acid or hydrogen peroxide (Table 3). Pretreated straw had 23–26% DM. The pretreated straw was collected in plastic bags and stored at −20 °C until use.

Steam explosion pretreatment. The straw was treated with steam to reach a DM content of 59%. The straw was impregnated with SO_2 (2.7% (w/w)) for 1.5 h at room temperature in plastic bags. The amount of SO_2 absorbed was determined by weighing the plastic bags before and after impregnation. The impregnated material (750 g) was steam pretreated at 215 °C for 5 min in a steam pretreatment unit equipped with a 10 l reactor [15]. The material was stored at 4 °C. Before use the material was washed with two volumes of water to remove soluble sugars. The spruce was pretreated by similar means, but impregnated with 3% (w/w) SO_2 for 20 min.

2.4. Straw composition analysis

Dry matter (total dry matter including soluble and insoluble solids) was determined using a Sartorius MA 30 moisture analyzer at 105 °C (Sartorius AG, Goettingen, Germany). Samples were dried at 35 °C for 1–2 days and then cut and strained through a 1.5 mm sieve on a Retsch SM 2000 cutting mill (Retsch, Inc., Newtown, USA).

The composition of the straw was analyzed using two-step acid hydrolysis according to the procedure published by the National Renewable Energy Laboratory (NREL) [16]. The dried samples were treated with 3 ml of 72% H_2SO_4 and placed in a water bath with a temperature of 30 °C. The samples were diluted with 84 ml of Milli-Q water to give a H_2SO_4 concentration of 2.5%. The samples were autoclaved for 1 h at 121 °C. After cooling, 20 ml of the sample was neutralised with $CaCO_3$ to pH 5–6. Monosaccharide concentration was analyzed by HPLC.

Results are given as *glucan* (nearly all D-glucose originates from cellulose) and *hemicellulose*: D-arabinose and D-xylose in straw (mainly arabinoxylan) and

Table 2
Pretreatment conditions

Type	Temperature [°C]	Additive concentration [g (kg straw)$^{-1}$]	Residence time [min]
Water[a]	190	–	6
Alkaline[a]	195	NH_3, 25	6
Acid[a]	190	H_2SO_4, 35	6
Hydrogen peroxide[a]	190	H_2O_2, 25	6
Steam explosion[b]	215	SO_2, 18	5
Steam explosion spruce[b]	215	SO_2, 20	5

[a] Pretreated on the IBUS pilot plant.
[b] Pretreated at Lund University.

D-arabinose, D-xylose, D-mannose, D-galactose in spruce (mainly galactoglucomannan).

2.5. Hydrolysis experiments

The pretreated straw was dried at 35 °C for 1–2 days and then cut and strained through a 1.5 mm sieve on a Retsch SM 2000 cutting mill. The hydrolysis was performed using an enzyme mixture of Celluclast 1.5 l and Novozym 188 (weight ratio 5:1, from Novozymes A/S, Bagsvaerd, Denmark) with a filter paper activity of 74 FPU g^{-1}, as measured by the filter paper assay [17].

The hydrolysis was performed in 50 ml Falcon tubes (total reaction volume 40 g), at 5% DM (w/w) in a 50 mM sodium citrate buffer pH 4.80 and using an enzyme loading of 5 FPU (g DM)$^{-1}$. In the screening studies, the surfactant concentration was 0.05 g (g DM)$^{-1}$. In the concentration effect study, Berol 08 was tested at 0.005, 0.025, 0.05 and 0.10 g (g DM)$^{-1}$, and PEG 6000 was tested at 0.005, 0.01, 0.025 and 0.05 g (g DM)$^{-1}$. The test tubes where placed in a heated (50 °C), shaking water bath (80 rpm) for 24 h. All experiments were performed in triplicate. Samples for sugar analysis were boiled for 10 min to terminate the reaction and stored at −20 °C until analysis. Samples for determination of enzyme adsorption were frozen immediately after hydrolysis.

2.6. Sugar analysis by HPLC

Samples were filtered through a 0.45 μm filter and diluted appropriately by eluent (5 mM H_2SO_4). The content of monosaccharides (D-glucose, D-xylose and L-arabinose) was quantified on a Dionex Summit HPLC system (Dionex Corporation, Sunnyvale, USA) equipped with a Shimadzu refractive index detector (Shimadzu, Kyoto, Japan). The separation was performed in a Phenomenex Rezex RHM column (Torrance, USA) at 80 °C with 5 mM H_2SO_4 as eluent at a flow rate of 0.6 ml min^{-1}.

2.7. Determination of enzyme adsorption

Adsorption of enzyme onto the remaining solid material was determined by measuring residual endoglucanase activity in the liquid phase. Solids were removed by centrifugation for 10 min at 15,000 × *g*. Endoglucanase activity was measured using azo-carboxymethyl cellulose (Megazyme, Wicklow, Ireland) as substrate. The measurement was performed as described by [18]; except absorbance was measured at 590 nm. Standard curves were prepared using the same enzyme mixture of Celluclast and Novozym 188 as used in the hydrolysis experiments. Adsorption was calculated as the measured endoglucanase activity subtracted from the initial endoglucanase added.

2.8. Stabilization effect of surfactants

The direct effect of surfactants on enzyme stability was determined by preparing mixtures containing the same enzyme activity (250 FPU l^{-1}) and ratio between surfactant and FPU (0.01 g FPU^{-1}). The activity of the solutions (with and without surfactant) were measured by the filter paper assay [17] and by azo-carboxymethyl cellulose at $t = 0$ and 24 h. The solution was incubated at 50 °C for both the azo-carboxymethyl cellulose assay and the filter paper assay.

3. Results

3.1. Substrate composition

The lignin fraction of lignocellulose has been proved to be responsible for unspecific adsorption of cellulases [7]. However, the influence of the pretreatment method and conditions on this adsorption is less clear. To investigate this, five different types of pretreated wheat straw was produced. Four types were produced using a pilot scale pretreatment reactor [14] and one type using SO_2-catalyzed steam explosion [15]. In addition, spruce was pretreated using SO_2-catalyzed steam explosion. The conditions are summarized in Table 2. The composition of the resulting materials is shown in Table 3. The hemicellulose content in straw is based on content of xylose, arabinose, and in spruce on xylose, arabinose, mannose and galactose. The lignin content in the wheat straw pretreated in the pilot scale reactor varied only little, irrespective of the conditions applied. On average the lignin content in wheat straw was 22.6%. Steam exploded spruce contained significantly more lignin than pretreated straw (50.9%, see Table 3). Acid catalyzed pretreatment methods significantly lowered the xylan content and therefore also the total hemicellulose content.

3.2. Effect of pretreatment and surfactant on hydrolysis

The surfactants and pretreatments were compared by hydrolyzing a 5% substrate solution containing 0.05 g (g DM)$^{-1}$ of surfactant for 24 h using an enzyme loading of 5 FPU

Table 3
Composition of materials used in hydrolysis experiments

Pre-treatment	Klason lignin	Ash	Glucan	Hemicellulose[a]
Untreated straw	17.7	7.0	34.8	25.2
Water, straw	19.6	2.5	54.3	18.8
H_2O_2, straw	24.0	2.6	54.0	19.2
H_2SO_4, straw	22.7	5.8	56.8	8.2
NH_3, straw	23.0	1.8	50.6	20.0
Steam explosion, straw	23.6	6.3	56.7	8.5
Untreated spruce	27.0	0.1	43.0	19.7
Steam explosion, spruce	45.0	0.1	48.0	∼0

All values are in percent of total content on a dry matter basis.
[a] The hemicellulose fraction includes a number of polymers. In straw the main hemicellulose polymer is arabinoxylan. In spruce the main hemicellulose polymer is galactoglucomannan.

$(g\,DM)^{-1}$. The cellulose and xylan conversion was measured by quantifying the amount of released glucose and xylose, respectively, by HPLC. Although the hemicellulose in straw is present as arabinoxylan, only xylose was used as an estimate of the hydrolysis of the hemicellulose as the arabinose content was only 1–2%. Thus, in the following, xylan conversion refers to the release of xylose from arabinoxylan. Seven different surfactants were initially tested for their ability to enhance enzymatic hydrolysis of the pretreated wheat straw. Three types of PEG with an average molecular mass of 2000, 4000 and 6000 Da were tested. The results revealed a trend of slightly higher cellulose conversion with increasing molecular weight, which has also been observed when used with spruce lignocellulose [19]. Therefore, PEG 6000 was chosen for further studies. The results of Berol ox-91-8 and Berol 08 were not statistically different (not shown), and Berol 08 was selected for further studies.

Hydrolysis without the addition of surfactant resulted in glucose concentrations ranging between 5.0 and 15.7 g/l, depending on pretreatment. The acid pretreatment resulted in the lowest conversion of cellulose whereas the highest conversion was obtained using the steam-exploded straw. The degree of cellulose conversion or hydrolysis, defined as amount of glucose released relative to the maximum theoretical, was 36% for the water pretreated wheat straw but only 16% for the acid pretreated wheat straw (Fig. 1A). For the steam-exploded straw the cellulose conversion was 51%.

With surfactants added, the glucose concentration increased in all experiments, although not all of them being a statistically significant increase. Interestingly, the increase in cellulose conversion of acid treated straw was substantially higher than any of the other pretreatments, ranging from 58% (BSA) to 70% (Berol 08) (Fig. 1B). This increase brings the cellulose hydrolysis of the acid treated straw to the same level as for the other pretreatments. For the other types of substrate, the effect was in the range of 3–23% improvement in cellulose hydrolysis.

The xylan conversion varied between 36 and 60% of the theoretically possible, depending mainly on pretreatment method (Fig. 2A). Interestingly, the xylan conversion of the steam exploded and acid pretreated straw was comparable to the other pretreatment methods despite containing less than half the xylan (Table 3). The effect of surfactants on the hydrolysis of the xylan was not as pronounced as seen with glucose with improvements in the order of 0–10%, except for steam exploded straw where the increase in xylan conversion was 11–17% (Fig. 2B).

3.3. Surfactant concentration

The correlation between amount of surfactant added and effect on hydrolysis was investigated for Berol 08 and PEG 6000 on water and acid pretreated wheat straw (Fig. 3). For both substrates and both surfactants, the effect of increasing the surfactant concentration on the cellulose hydrolysis leveled off above 0.025 g $(g\,DM)^{-1}$. The optimum ratio between surfactant and substrate was approximately 0.05 g $(g\,DM)^{-1}$. Although the effect of Berol 08 on cellulose hydrolysis was higher on acid pretreated wheat straw compared to water pretreated straw

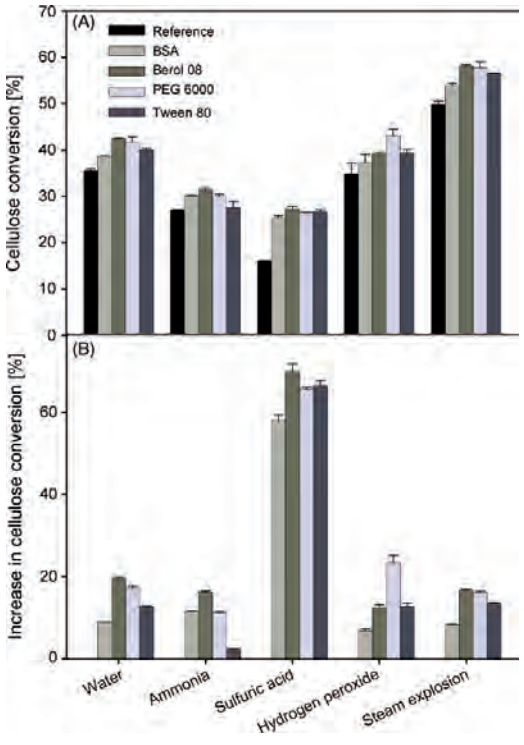

Fig. 1. (A) Cellulose conversion of straw after 24 h of hydrolysis in percentage of theoretical maximum as a function of pretreatment method. Substrate concentration was 5% (w/w). Reference was without addition of 0.05 g/g DM surfactant. Results are averages of triplicates. (B) Results from (A) calculated as percent increase in cellulose conversion compared to the reference. Results are averages of triplicates.

(Fig. 1B), the optimum concentration for both substrates (Fig. 3) was 0.025–0.05 g $(g\,DM)^{-1}$. At lower concentrations, PEG 6000 had a slightly higher effect on hydrolysis of water pretreated straw compared to Berol 08, but above 0.025 g $(g\,DM)^{-1}$ the difference was negligible.

No significant effect of surfactant concentration was seen on xylan hydrolysis (data not shown).

3.4. Enzyme activity in solution

It has been suggested that the surfactant effect is due to hydrophobic interaction between the surfactant and lignin on the lignocellulose, thereby either releasing unspecifically bound enzyme or preventing unproductive enzyme adsorption [4,7]. The effect of substrate and surfactants on the adsorption of enzyme was studied by measuring the endoglucanase activity remaining free in solution after the hydrolysis. Endoglucanases may become deactivated during hydrolysis for other reasons than adsorption. However, due to the stability of the enzymes, remaining endoglucanase activity is used as a measure of adsorption of cellulases.

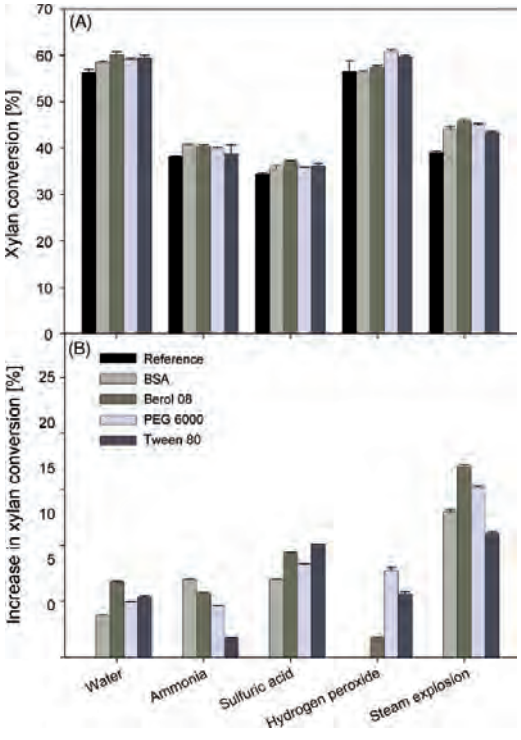

Fig. 2. (A) Xylan conversion of straw after 24 h of hydrolysis in percentage of theoretical maximum as a function of pretreatment method. Substrate concentration was 5% (w/w). Reference was without addition of 0.05 g/g DM surfactant. Results are averages of triplicates. (B) Results from (A) calculated as percent increase in xylan conversion compared to the reference.

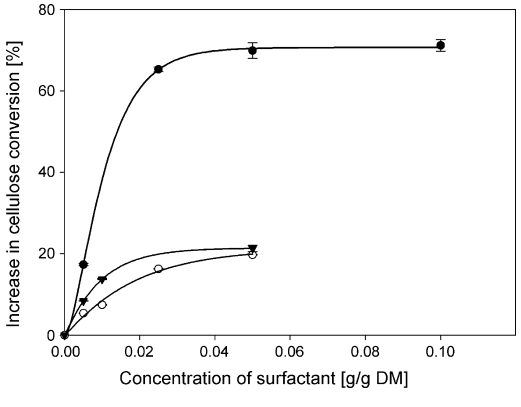

Fig. 3. Effect of surfactant concentration (Berol 08 and PEG 6000) on cellulose conversion of water and acid pretreated wheat straw. (●) Sulfuric acid treated straw and Berol 08; (◯) water treated straw, PEG 6000; (▼) water treated straw, Berol 08. Results are averages of triplicates.

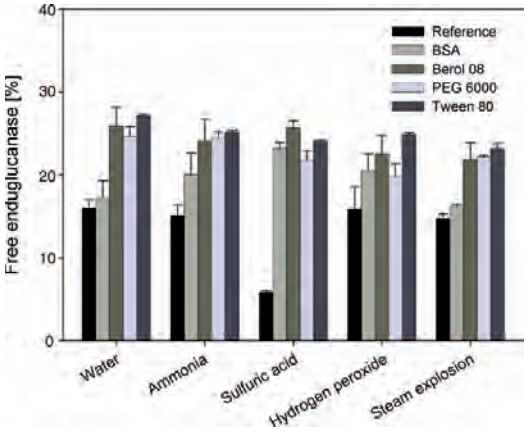

Fig. 4. Endoglucanase activity free in solution after 24 h hydrolysis of wheat straw (5%, w/w) depending on pretreatment and surfactant type, respectively. The enzyme activity was calculated as percentage of initially added activity. Reference was without addition of 0.05 g/g DM surfactant. Results are averages of triplicates.

The endoglucanase activity in the solution after hydrolysis of straw pretreated by water, ammonia, hydrogen peroxide and steam explosion without addition of surfactants were all around 15% of the activity initially added, except for the solution of the acid pretreated straw without surfactant which was only 6% (Fig. 4). Assuming that the endoglucanase activity can be used to estimate adsorption of cellulases, the results reveal that 85–94% of the cellulase enzymes are adsorbed onto the substrate after 24 h of hydrolysis.

The addition of surfactant increased the endoglucanase activity in solution by a minimum of 25% with the exception of BSA, which was less efficient (endoglucanase activity between 20 and 26% for all substrates, Fig. 4). The increase in endoglucanase activity by addition of surfactants correlated well with the concurrent improvement observed in the hydrolysis of cellulose (Fig. 1A and B). Interestingly, the addition of all surfactants, including BSA, increased the low enzyme activity measured in the acid pretreated straw solution to a point where the activity was equal to that of the other pretreatment types with surfactant added (Fig. 4). The endoglucanase activity in the solution of the acid treated straw was between 2.7 and 3.4 times higher than the reference without surfactant.

3.5. Spruce hydrolysis

Surfactants have previously been reported to improve the cellulose hydrolysis of steam pretreated spruce significantly [7]. In order to compare the effect of surfactants on materials from different origins, hydrolysis studies were performed on spruce pretreated by steam explosion under similar conditions as used with wheat straw (Fig. 5A). Conversion of steam exploded spruce cellulose was close to 80% with surfactant addition. This is equivalent to an improvement of the conversion from 59%

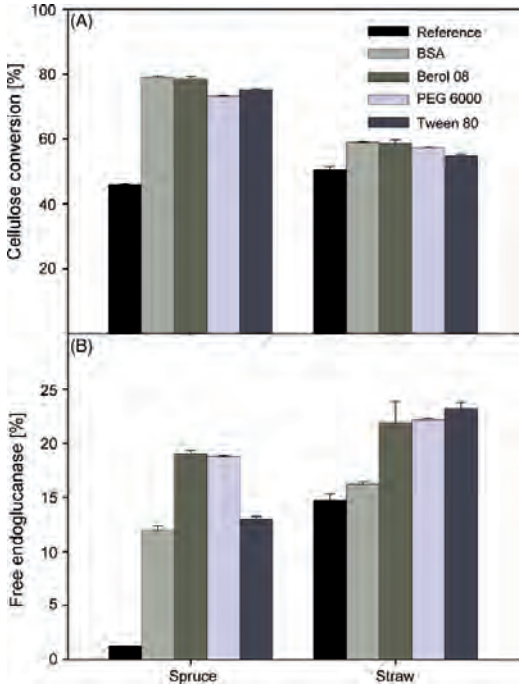

Fig. 5. (A) Effect of surfactants (0.05 g/g DM) on hydrolysis of wheat straw and spruce, respectively (substrate concentration 5% (w/w), 24 h hydrolysis). Both raw materials were pretreated by SO_2-catalyzed steam explosion. Results are averages of triplicates. (B) Percentage of added endoglucanase found free in solution of the hydrolyzed substrates in (A).

(Tween 80) to 72% (Berol 08). In contrast, hydrolysis of cellulose was only improved by 8–17% using the steam exploded wheat straw as substrate (cellulose conversion approximately 60%, see Fig. 5A).

3.6. Stabilizing effect of surfactants

As poly(ethylene glycols) and other surfactants have been reported to have a stabilizing effect on some enzymes [10], it was investigated if this was also the case with the tested surfactants and cellulases. The endoglucanase activity was measured on azo-carboxymethyl before and after 24 h incubation using the same enzyme mixture as used previously for the hydrolysis. Activity was measured on solutions containing no surfactant, PEG 6000, BSA and Berol 08, respectively. The decrease in enzyme activity in solutions with surfactant added was found to be slightly less (1.7–5.2%, data not shown) than the reference (5.4% decrease). However, the difference was not statistically significant. The FPU assay (measuring the overall cellulase activity) did not confirm the possible stabilizing effect as it showed no difference between the solutions with and without surfactant (data not shown).

4. Discussion

4.1. Combined effect of surfactants and pretreatment

Lignocellulose is a highly complex structure with a whole range of characteristics that influence and limit the hydrolysis of carbohydrate polymers into fermentable sugars [20]. As lignin is generally believed to be one of the most limiting factors of enzymatic hydrolysis of lignocellulose [7,21,22], it was interesting to investigate the relationship between lignin and convertibility of the pretreated materials.

Even though only the lignin content of the water pretreated wheat straw was slightly lower (19.6%) than that of the other pretreated wheat straw (22.7–24.0%, see Table 3), the cellulose conversion varied from 16 to 51%. This strongly suggests that although lignin content has been proven to be an important factor for degradability [7,21], other factors are perhaps equally important. The various pretreatments had a more differentiated effect on the xylan content, ranging from 7.8 to 18.4%. This is likely due to hemicelluloses being dissolved in some pretreatments, such as acid catalyzed pretreatment.

Surfactants were found to increase the cellulose hydrolysis significantly. Interestingly, the added surfactants had the most pronounced effect on the straw treated with sulfuric acid (increase in cellulose conversion more than 60%). Without surfactant, the acid treated straw showed lowest conversion. It is possible that the acid pretreatment makes the lignin more receptive to cellulose adsorption through a change of surface properties, e.g. increased hydrophobicity or hydrogen bonding capacity. It is also possible that the treatment dissolves hemicelluloses associated with or covering lignin, thereby increasing the accessibility of lignin and hence the adsorption. The low xylan content (7.5%) supports the last theory. However, the steam explosion pretreated straw has similarly low xylan content (7.8%) yet the increase is more modest. This could be due to the cellulose hydrolysis of the steam exploded straw already being closer to the theoretical maximum. More research is needed in order to establish a clear relationship between effect of surfactants and substrate characteristics.

Unlike previous studies, which have focused on materials with little hemicellulose content [7,23], the effect of surfactants on xylan hydrolysis was also studied. Xylan conversion was determined as xylose released from arabinoxylan, the main hemicelluloses in straw. Steam exploded and acid pretreated straw had low xylan content, yet the xylan conversion was still more than 30%. Addition of surfactant also had an effect on xylan hydrolysis, although not as pronounced as seen with cellulose. It is not known if this is due to the properties of the xylanases (lower tendency to unspecific adsorption) or perhaps the smaller content of xylan in the material compared to cellulose. When pentose-sugar-fermenting microorganisms become industrially available, the utilization of xylan will be an important factor in lignocellulose hydrolysis and add to the effectiveness of bioethanol production.

Comparison of the five tested surfactants revealed that no individual surfactant seems to be particularly well suited to a certain type of pretreatment. Regarding cellulose conversion,

Berol 08 and PEG 6000 have a tendency to perform the best, whereas all the used surfactants performed equally well on xylan hydrolysis. PEG 6000 had a tendency to outperform PEG 2000 and PEG 4000 with shorter chain lengths.

4.2. Mechanism of surfactant effect

The positive effect of surfactants on enzymatic hydrolysis has been reported a number of times. Various explanations to the surfactant effect have been proposed including increase of enzyme stability and increasing accessibility of the substrate. However, the most recent research suggests that prevention of unproductive enzyme adsorption to lignin is the major mechanism behind the surfactant effect [7]. The mechanism has been explained by hydrophobic sites on lignin being occupied by surfactant. The hydrophilic portions of the surfactant will in turn protrude into the aqueous solution and cause steric repulsion of enzyme from the lignin surface. It has also been shown that surfactant is able to displace already adsorbed enzyme [7].

The endoglucanase activities measured in the substrate solutions have been used to calculate adsorption of the enzyme mixtures, assuming that non-adsorbed enzyme is still active in the solution. The enzyme mixtures used for the hydrolysis have been found to be highly resistant to degradation and inactivation over a period of several days. Hence, the enzyme activity measured in solution can be correlated to the degree of adsorption.

Although not identical, there is a clear connection between the cellulose conversion of pretreated straw (Fig. 1A) and the endoglucanase activity in solution (Fig. 4). In both cases the acid pretreated substrate is below that of the other substrates. Similarly, with surfactants added, both the cellulose conversion and the free endoglucanase activity of the acid treated straw experiment increase significantly. Although the variation is higher, the increase in enzyme activity due to addition of surfactants is also comparable to the increase in cellulose conversion with surfactants. Likewise, the measured enzyme adsorption on spruce corresponds well with the improvement in cellulose conversion when surfactants were added. Consequently, there seems to be a clear relationship between cellulose conversion of pretreated straw lignocellulose and enzyme adsorption. Furthermore, the higher surfactant effect on spruce substrate compared to straw may be explained by the higher lignin content of spruce (51% compared to 20–24%, Table 3). These relationships strongly support the current theory on the dominating effect of surfactants being due to steric hindrance of enzyme interaction with lignin surfaces.

The correlation between surfactant concentration and increase in cellulose conversion showed that the effect leveled off at concentrations above approximately 0.025 g $(g\,DM)^{-1}$. An explanation as to why the leveling off and optimum concentration were equal for different substrates may be that all possible binding sites on lignin are occupied by surfactant when it reaches a certain concentration, irrespective of the ability of the substrate to unspecifically bind enzymes. Thus, there may be a number of potential sites on the lignocellulose that may either adsorb enzyme or surfactant. When these sites are all associated with surfactant, further addition will not increase hydrolysis.

However, this does not explain why the optimum surfactant concentration is equal for the two substrates tested, irrespective of the varying increase in cellulose conversion. In other words, it seems that the type of material or pretreatment does not have any influence on the amount of adsorption sites, yet the lignin content and adsorption can be correlated to the degree of conversion.

It is possible that the lignin interaction discussed above is not the only mechanism responsible for the positive surfactant effect. It has been suggested that surfactants have a stabilizing effect on some enzymes [10]. The experiments performed indicated that this mechanism was not responsible for the increased enzyme performance. However, the experiments were carried out without addition of substrate. It is possible that surfactants may have a stabilizing effect on an enzyme/substrate complex.

5. Conclusions

We have shown that addition of surface active additives, such as non-ionic surfactants and PEG, increased enzymatic conversion of pretreated straw lignocellulose for bioethanol purposes. The degree of surfactant effect varied depending on type of pretreatment. Although the surfactant effect was not as high as seen with spruce lignocellulose, it is most likely possible to lower the enzyme loading by adding, e.g. PEG 6000, while retaining the same degree of cellulose conversion. However, due to the lack of industrial scale prices of surfactants and enzyme, it has not been possible to perform economic calculations on the feasibility of surfactant addition.

Surfactants were also found to increase xylan conversion moderately.

Enzyme adsorption was measured and could be correlated to cellulose conversion of pretreated straw substrates with and without surfactant added. The results strongly support the prevalent theory that the main mechanism of the surfactants is prevention of unproductive enzyme adsorption with lignin surfaces.

The optimum surfactant concentration was found to be similar, irrespective of pretreatment type. Furthermore, as seen with the acid pretreated wheat straw substrate where surfactant addition improved conversion dramatically, lignin content and surfactant effect is not always directly proportional. Further research is needed to fully understand the factors influencing surfactant and enzyme adsorption.

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Determining Yields in High Solids Enzymatic Hydrolysis of Biomass

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Abstract As technologies for utilizing biomass for fuel and chemical production continue to improve, enzymatic hydrolysis can be run at still higher solids concentrations. For hydrolyses that initially contain little or no free water (10–40% total solids, *w*/*w*), the saccharification of insoluble polymers into soluble sugars involves changes of volume, density, and proportion of insoluble solids. This poses a new challenge when determining the degree of hydrolysis (conversion yield). Experiments have shown that calculating the yield from the resulting sugar concentration in the supernatant of the slurry and using the assumed initial volume leads to significant overestimations of the yield. By measuring the proportion of insoluble solids in the slurry as well as the sugar concentration and specific gravity of the aqueous phase, it is possible to precisely calculate the degree of conversion. The discrepancies between the different ways of calculating yields are demonstrated along with a nonlaborious method for approximating yields in high solids hydrolysis.

Keywords Enzymatic hydrolysis · Biomass · Bioethanol · High solids · High dry matter · Yield

Introduction

The enzymatic saccharification of biomass to fermentable sugars is a well-known bottleneck in the production of bioethanol in an economically viable manner [1]. An important process parameter in enzymatic hydrolysis is the ability to work at high solids concentrations. A high substrate concentration allows for the production of a concentrated sugar solution, which in turn is beneficial for the subsequent fermentation and, in particular, distillation. The energy requirement for distillation is significantly reduced if the solution contains more than 4% (*w*/*w*) ethanol [2]. Furthermore, working at high solids concentrations lowers heating requirements and increases the volumetric productivity of the plant.

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To reach an ethanol concentration of more than 4% (*w*/*w*), a sugar level of at least 8% (*w*/*w*) is needed. For most types of lignocellulosic biomass, this requires an initial solids content above 20% [3]. Recently, much research has gone into being able to perform and handle enzymatic hydrolyses at high substrate concentrations [4–7]. High solids content could be defined as initial concentrations where little or no free water is present. One solution to increase solids levels has been to replace conventional stirred-tank reactors with so-called gravimetric mixing, enabling liquefaction, saccharification, and fermentation of pretreated biomass at up to 40% initial solids content [3, 7].

Lignocellulosic bioethanol is on the verge of commercial reality [8], and as processes improve and the solids content in enzymatic hydrolysis increase, so does the need to consider the most accurate way of determining the yield in order to compare various enzyme systems, processes, and technologies. Hydrolysis of biomass is a complex reaction where multiple, insoluble polymers are broken down and their constituents dissolved in the liquid phase. During the reaction, the content of insoluble solids decreases, the density of the liquid phase increases as does the volume of the liquid phase. When working at high solids concentrations, it is practical to measure the initial biomass or cellulose concentration in weight per weight (e.g., 25% *w*/*w*). However, usually, sugars are measured by high performance liquid chromatography (HPLC) and only in the aqueous phase, free of insoluble components, and are reported in weight per volume (e.g., 70 g/L).

Often, the above-mentioned factors (including the solids content) are not taken into consideration when calculating the percent-of-theoretical yield. In the following, it is shown how high solids hydrolysis yields are overestimated, when based on the initial volume. Also, we suggest a nonlaborious method for approximating the correct yield.

Materials and Methods

Compositional Analysis

The compositions of hydrothermally pretreated straw (pretreated at 195 °C for approximately 6 min as described in [3]) and filter paper (AGF 725, 140 g/m^2 from Frisenette ApS, Knebel, Denmark) were analyzed using two-step acid hydrolysis according to the procedure published by the National Renewable Energy Laboratory (NREL) [9]. Before hydrolysis, the samples were dried at 45 °C for 1 day. The dried samples were milled in a Braun coffee grinder. Dry matter was determined using a Sartorius MA 30 moisture analyzer at 105 °C. The released sugars were quantified with HPLC as described below.

Enzymatic Hydrolysis

Hydrolysis was performed using an enzyme mixture of Celluclast 1.5 L and Novozyme 188 (weight ratio 5:1, from Novozymes A/S, Bagsværd, Denmark) with a filter paper activity of 75 FPU g^{-1}, as measured by the filter paper assay [10].

The hydrolyses were performed in 100 mL plastic bottles (total reaction mass 50 g), at 5–30% solids content (*w*/*w*) in a 50-mM sodium citrate (pH 4.80) buffer and using an enzyme loading of 5–20 FPU g DM^{-1}. The bottles were placed in a heated (50 °C), horizontally placed drum, rotating at 60 rpm for 24 h. The 80-cm diameter drum was equipped with two inside paddles that lifted and dropped the plastic bottles during rotation, mimicking the gravimetric mixing described in [3, 7]. All experiments were performed in duplicate. Samples for sugar analysis were boiled for 10 min to terminate the reaction.

Samples were spun down in 50 mL falcon tubes (4,223×*g* for 10 min) and the density of the supernatant was measured. The remaining solids were washed with MilliQ water five times to remove any water-soluble material. The solids were then dried at 105 °C and weighed in order to calculate the amount of insoluble solids.

Sugar Analysis

The content of monosaccharides and disaccharides in the hydrolyzed samples (D-glucose, D-xylose, L-arabinose, and D-cellobiose) was quantified on a Dionex Summit HPLC system equipped with a Shimadzu RI-detector. The separation was performed in a Phenomenex Rezex RHM column at 80 °C with 5 mM H_2SO_4 as eluent at a flow rate of 0.6 mL min^{-1}. Samples were filtered through a 0.45-μm filter and diluted with eluent before analysis on HPLC.

Results and Discussion

In the standard for enzymatic saccharification of lignocellulosic biomass proposed by the NREL [11], it is assumed that the specific gravity of all components of the hydrolysis is 1.000 g/mL. The equation for determining the yield can be written as:

$$\text{Percent hydrolysis} = \frac{[\text{Glc}] + 1.0526 \times [\text{Cel}]}{1.111 \times F_{\text{cellulose}} \times [\text{Ini. sol}]} \times 100\% \quad (1)$$

where [Glc] is the glucose concentration in the supernatant of the slurry (in grams per liter), [Cel] is the cellobiose concentration in the supernatant of the slurry (in grams per liter), $F_{\text{cellulose}}$ is the fraction of cellulose in the substrate, and [Ini. sol] is the initial solids concentration (in grams per liter) with the assumption that all solutions and biomass have a specific gravity of 1.000 g/mL. The volume of the reaction is assumed not to change during the hydrolysis and is thus omitted from the equation.

When working with a fixed mass reaction (e.g., 50 or 100 g assays) above a certain solids content, the assumption that all components are of the same specific gravity becomes invalid. Furthermore, calculating the yield by using the "initial" volume of the reaction (assuming that a 50-g assay equals 50 mL) to find the amount of cellulose digested (using the sugar concentration), usually leads to an overestimation of the yield. The reason is that the released sugar is dissolved in less than the "initial" volume, i.e., part of the fixed mass of the reaction is solid matter and thus, the liquid volume is significantly less than assumed.

Although the mass of the reaction is constant during the hydrolysis, there are significant changes to the volume of the reaction. As solids are hydrolyzed, the mass and density of the aqueous phase increases, although not at the same rate. This makes it difficult to calculate the precise amount of cellulose consumed, based purely on the resulting sugar concentrations. However, it is possible to measure the amount of insoluble solids remaining after hydrolysis as well as the specific gravity of the aqueous phase. As the mass is constant, the exact volume of the aqueous phase can be calculated, and based on the concentration of sugars, the exact amount of cellulose that has been converted. The equation for determining the yield then becomes:

$$\text{Percent hydrolysis} = \frac{\frac{m_{\text{reac}} - m_{\text{ins. sol}}}{\text{SG}_{\text{aq. phase}}} \times ([\text{Glc}] + 1.0526 \times [\text{Cel}])}{1.111 \times m_{\text{sub}} \times F_{\text{cellulose}} \times \text{DM}} \times 100\% \quad (2)$$

where m_{reac} is the mass of the whole reaction (in grams), $m_{ins.\ sol}$ is the mass of insoluble solids after hydrolysis (in grams), $SG_{aq.\ phase}$ is the specific gravity of the aqueous phase (in grams per liter), [Glc] is the glucose concentration in the supernatant of the slurry (in grams per liter), [Cel] is the cellobiose concentration in the supernatant of the slurry (in grams per liter), m_{sub} is the mass of the substrate (in grams), $F_{cellulose}$ is the fraction of cellulose in the substrate, and DM is the initial dry matter content (*w*/*w*).

We used this rather laborious method of measuring the fraction of insoluble solids and aqueous phase density to establish the exact yield at various time points/enzyme dosages for hydrothermally pretreated wheat straw at 5%, 10%, 15%, 20%, and 30% initial solids content. The yields were also calculated using Eq. 1 and compared with the exact yields. To illustrate the difference in yield between the two equations, the values for hydrolysis of hydrothermally pretreated straw at 30% initial solids content (50 g assay) are: [Glc]=136.22 g/L, [Cel]=20.31 g/L, $F_{cellulose}$=0.532, and [Ini. sol]=300 g/L.

Using Eq. 1, this gives a yield (percent hydrolysis) of 88.9%. The additional values needed for Eq. 2 are: m_{reac}=50.00 g, $m_{ins.\ sol}$=10.32 g, $SG_{aq.\ phase}$=1085.7, m_{sub}=39.28 g, and DM=0.3819.

With Eq. 2, this equals a yield (percent hydrolysis) of 65.0%. This means that at 30% initial solids content, the proposed standard (Eq. 1) gives a yield that is more than 36% too high. This is a considerable and unwanted overestimation, in particular when comparing new technologies for bioethanol production and biorefineries.

As seen in Fig. 1, for every initial solids content, there is a near linear relationship between the actual yield and the yield based on initial volume/supernatant sugar concentration. This means that the error of the noncorrected yield (Eq. 1) is not dependent on the degree of hydrolysis but rather on the initial solids content. As expected, the degree of error increases with solids content. When the ratio between the actual (Eq. 2) and noncorrected (Eq. 1) yields is plotted as a function of initial solids content (Fig. 2), the relationship is also near linear. Thus, the slope of this graph can be used to calculate the actual yield from the noncorrected yield at each initial solids content.

The correction factor is substrate-dependent as it correlates to the composition of the biomass used, mainly the cellulose content. The experiment was repeated with filter paper containing a higher proportion of cellulose (for the composition of pretreated wheat straw

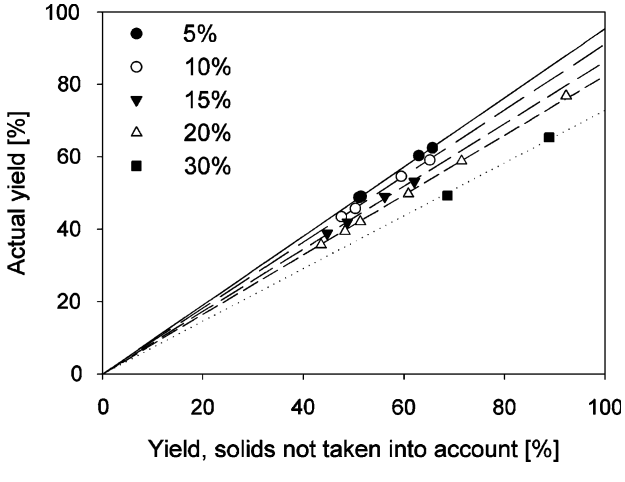

Fig. 1 Relationship between actual yield and yield calculated without taking solids content into account, when hydrolyzing pretreated straw at various initial solids contents (*w*/*w*). Each point is average of duplicates

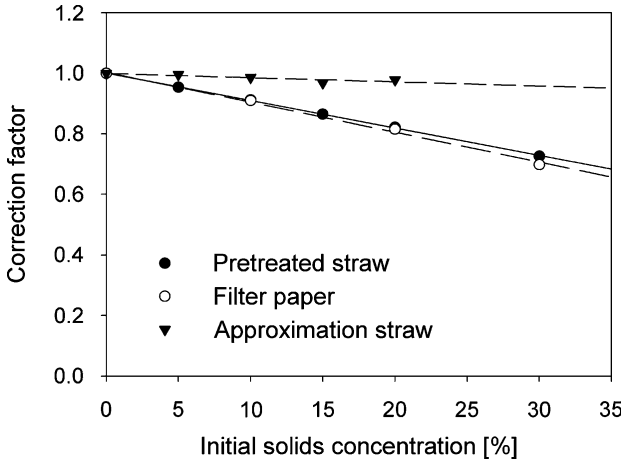

Fig. 2 Correction factor (for correcting yield overestimation) is the ratio between actual yield and yield calculated without taking solids content into account. The correction factor is shown as a function of initial solids content for pretreated straw and filter paper, respectively. The *upper graph* depicts the correction factor for pretreated straw when calculated from whole slurry sampling, thereby approximating the actual yield. Each point is the average of duplicates

and filter paper, see Table 1) and it was found that the correction curve for filter paper was slightly steeper. Thus, a correction factor must ideally be established for each substrate.

The errors seen for high solids enzymatic hydrolysis also apply for simultaneous saccharification and fermentation. Since the specific gravity of the aqueous phase starts decreasing when the released and dissolved sugars are converted to ethanol, the situation is more complex. The error of the yield is less than for enzymatic hydrolysis only, at least when a certain amount of the released sugar has been converted. Experiments with hydrothermally pretreated straw show that at 30% initial solids content, the yield was overestimated by 23% (results not shown).

Due to the inconsistency of pretreatments and natural substrates, calculating correction factors can be a laborious task. An alternative is to use the following approximation method: A representative slurry sample is weighed, e.g., 1.000 g, and diluted to 10.000 g (ten times dilution). It is then spun down and the amount of sugars in the supernatant is measured by HPLC. By knowing how large a fraction of the whole slurry is sampled, it can be calculated how much of the cellulose that has been hydrolyzed, as per Eq. 1. As the slurry was diluted ten times, the error caused by the solids content has been significantly reduced. Experiments have shown that the error (overestimate) for hydrolyses at up to 30% initial solids was reduced to a maximum of 3–5% (see Fig. 2).

Although it can be difficult to collect a representative slurry sample, especially early in the hydrolysis, we believe that this approximation method is more practical than measuring the fraction of insoluble solids and the density of the aqueous phase. Most importantly, it is better for calculating high solids yields rather than simply measuring the sugar concentration in the aqueous phase of the slurry and using the "assumed" initial volume, as prescribed by, e.g., NREL.

Table 1 Composition of pretreated straw and filter paper.

	Cellulose	Xylan	Arabinan	Klason lignin	Ash	Mannan
Pretreated straw	59.0	5.2	0.0	25.5	5.6	0.00
Filter paper	80.63	0.00	0.97	0.42	0.27	14.43

Contents expressed in percent, based on solids

Conclusions

It was found that, when working at high solids concentration (10–40% *w*/*w*), it is necessary to reconsider the way the yield is calculated in order to avoid significant overestimation in the order of up to 36%. As enzymatic saccharification of biomass is a complex and dynamic process, it is difficult to theoretically calculate the yield purely based on an assumed initial volume and sugar concentration in the aqueous phase.

By measuring the amount of insoluble solids, aqueous phase density, and sugar concentration, it is possible to precisely calculate the yield. This is, however, a laborious process and an alternative would be to dilute a representative portion of the slurry prior to measurement.

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V

Yield determining factors in high solids enzymatic hydrolysis of lignocellulose

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Abstract

Background
Working at high solids (substrate) concentrations is advantageous in enzymatic conversion of lignocellulosic biomass as it increases product concentrations and plant productivity while lowering energy and water input. However, for a number of lignocellulosic substrates it has been shown that at increasing substrate concentration, the corresponding yield decreases in a fashion which can not be explained by current models and knowledge on enzyme-substrate interactions. This decrease in yield is undesirable as it off-sets the advantages of working at high solids levels. The cause of the "solids effect" has so far remained unknown.
Results
The decreasing conversion at increasing solids concentrations was found to be a generic or intrinsic effect, describing a linear correlation from 5-30% initial total solids content (w/w). Hydrolysis experiments with filter paper showed that neither insufficient mixing, lignin content nor hemicellulose-derived inhibitors caused the decrease in yields. Product inhibition by glucose and in particular cellobiose (and ethanol in SSF) at the increased concentrations at high solids loading plays a role but could not completely account for the decreasing conversion. Adsorption of cellulases was found to decrease at increasing solids concentrations. There was a strong correlation between the decreasing adsorption and conversion, indicating that the inhibition of cellulase adsorption to cellulose is causing the decrease in yield.
Conclusion
Inhibition of enzyme adsorption by hydrolysis products was found to be the main cause of the decreasing yields at increasing substrate concentrations in the enzymatic decomposition of cellulosic biomass. In order to facilitate high conversions at high solids concentrations, the understanding of the mechanisms involved in high-solids product inhibition and adsorption inhibition must be improved.

Keywords: Enzymatic hydrolysis; biomass; bioethanol; high solids; high dry matter; yield

Background

Climate changes and shortage of fossil fuels have sparked a growing demand of liquid biofuels which in turn has increased the research in production of lignocellulose-derived bioethanol [1,2]. However, being an insoluble and highly heterogeneous substrate, lignocellulosic materials pose several challenges in conversion to fermentable sugars. In addition to understanding complex enzyme system kinetics, these biomass-related challenges include recalcitrance to hydrolysis [3] and mixing difficulties [4]. Water content in the hydrolysis slurry is directly correlated to rheology, i.e. viscosity and shear rate during mixing [5], important for the interaction between lignocellulose and cell wall degrading enzymes. Thus, water is not only critical in hydrolysis being a substrate and a prerequisite for enzyme function, but is obviously also important for enzyme transport mechanisms throughout the hydrolysis as well as mass transfer of intermediates and end-products [6].
Maintaining high concentrations of solids throughout the conversion process from biomass to ethanol is important for the energy balance and economic viability of bioethanol production. High solids enzymatic hydrolysis can be defined as taking place at solids levels where initially there are no significant

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amounts of free liquid water present [7]. By increasing the solids loading, the resulting sugar concentration and consequently ethanol concentration increase; having significant effects on processing costs, in particular distillation [8-10]. Furthermore, lower water content allows for a larger system capacity, less energy for heating and cooling of the slurry as well as less waste water [4]. Model-based estimations have shown significant reductions of operating costs of simultaneous saccharification and fermentation (SSF) of pretreated softwood when the initial solids concentration was increased [8]. Unfortunately, there are also disadvantages by increasing the substrate concentration. Concentrations of end products and inhibitors will increase, causing enzymes and fermenting organism to not function optimally. Also, high-solids loadings can cause insufficient mixing or mixing can be too energy-consuming in conventional stirred-tank reactors as the viscosity of slurries increases abruptly at increasing solids loadings, in particular over 20% solids [11,12].

In situ native cellulase systems have been reported to function at solids levels as high as 76% (all concentrations are given as total solids on a w/w basis) [13], indicating that enzymatic hydrolysis may be limited by the laboratory or industrial process set-up. 12-20% total solids is often considered the upper limit at which pretreated biomass can be mixed and hydrolysed in conventional stirred tank reactors [7,14,15]. However, at laboratory scale, enzymatic hydrolysis at up to 32% total solids has been reported [12,16]. A number of studies have utilised fed-batch operations in order to increase the solids loading [7,11,17,18]. We have previously described a gravimetric mixing reactor design that allows batch enzymatic liquefaction and hydrolysis of pretreated wheat straw at up to 40% solids concentration [4]. This is a significant increase from what has previously been possible, and thus significantly increases the techno-economic potential of the whole process. The gravimetric mixing principle has been up-scaled and used in a pilot plant for several years [19,20].

During the work with high solids concentrations we found that the enzymatic conversion (percent of theoretical) linearly decreased with increasing solids concentration (constant enzyme to substrate ratio) [4]. This decrease partly off-sets the advantages of running at high solids concentrations. As seen in Fig. 1, the effect has been observed in both enzymatic hydrolysis and SSF by several groups working with various kinds of biomass [12,16-18,21-24]. Although several of these studies were conducted at less than 10% initial solids content, the phenomenon appears to be an intrinsic or generic effect of enzymatic hydrolysis at increasing solids levels. In this paper, the decrease in yield at high solids concentrations is referred to as the solids effect.

Some groups have suggested that the mechanism behind the decreasing conversion is product inhibition [12,16,25] or inhibition by other compounds such as hemicellulose-derived inhibitors (furfural and hydroxymethylfurfural (HMF)) [26] and lignin [27]. Others have suggested it may be explained by mass transfer limitations or other effects related to the increased content of insoluble solids, such as non-productive adsorption of enzymes [14,28]. However, the specific mechanism(s) responsible for the decreasing hydrolytic efficiency are still uncertain [4,29].

In this paper the possible mechanisms behind the solids effect have been divided into the following four categories: Compositional and substrate effects; product inhibition; water concentration; and cellulase adsorption. These four topics will be introduced below.

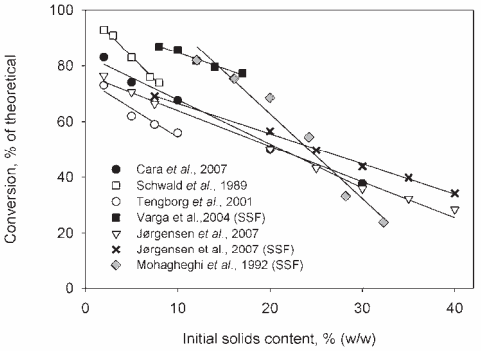

Fig. 1. Results collected from several publications indicate that decreasing conversion at increasing solids content is a general effect. Results are for different kinds of biomass and for both enzymatic hydrolysis and simultaneous saccharification and fermentation (SSF). Added trend lines show that for each experiment there is a near-linear relationships between initial solids content and yield. Data taken from [24] (enzymatic hydrolysis), [16] (enzymatic hydrolysis), [23] (enzymatic hydrolysis), [17] (SSF), [4] (enzymatic hydrolysis and SSF) and [12] (SSF).

Compositional and substrate effects

The heterogeneity and structure of lignocellulosic biomass means that high viscosity prevents efficient mixing at high solids concentrations when performed in conventional stirred-tank reactors [14,28,30]. The viscosity of lignocellulosic slurries increases sharply over a certain threshold (typically around 20% solids) but despite the extreme difference in viscosity between

e.g. 5% and 40% solids loading, the conversion of lignocellulosics as a function of solids content has previously appeared to be near-linear (Fig. 1). Although mixing of substrate and enzymes is crucial for an efficient liquefaction, it does not appear that lack of mixing is the cause of the decreasing conversion, at least not at the solids levels documented [4]. This is in accordance with the recent findings of Hodge and co-workers who concluded that possible mass transfer limitations caused by insoluble solids were not apparent at below 20% insoluble solids content [25]. Also, the linearity of the solids effect over a range of conditions with a number of substrates (wheat and barley straw [4,12,14], corn stover [17], softwood [22,24], hardwood [16,23] and an industrial ethanol fermentation residue (vinasse) [28]) indicates that a single factor may be responsible for the effect.

For this paper, experiments with filter paper were carried out in order to be able to establish that the solids effect is not caused by lignin adsorption or lignin-derived inhibitors (phenolics). Filter paper has the advantage of containing no lignin yet still retains the secondary cell wall structure as opposed to e.g. Sigmacell and Avicel.

Product inhibition

End-product inhibition plays an important role in enzymatic hydrolysis as glucose, cellobiose and ethanol have demonstrated their ability to significantly inhibit endoglucanases, cellobiohydrolases and β-glucosidase [31,32]. However, working with an insoluble substrate and kinetics that do not follow the Michaelis-Menten model, the exact type of inhibition is difficult to determine [33]. The decrease in hydrolysis rate over time has been attributed to inhibition by the accumulated end-products [34]. Others conclude that when hydrolysing natural, lignocellulosic substrates, cellulases are more resistant to product inhibition than with amorphous reference materials and that the early stage decrease in hydrolysis rate is not caused by product inhibition [35,36]. In high-solids enzymatic hydrolysis of pretreated corn stover, Hodge and co-workers recently found that increased sugar concentrations were the primary cause of performance inhibition [25].

Based on the above, we have investigated the inhibitory effect of increased sugar concentration in connection with high solids enzymatic hydrolysis.

Water concentration

Working with a system with low water content may directly affect enzyme performance. Not only is water substrate for the hydrolysis but it is also the solvent that allows function of enzymes, contact between enzymes and substrate as well as transport of products [37]. We have previously investigated the role of water in enzymatic hydrolysis [6]. In this study, we wanted to investigate if the solids effect was related to a lower concentration of water in relation to solids. As mentioned, hydrolysis is possible at very high solids concentrations but the rate of reaction may be impaired under such conditions [13].

We have investigated the role of water concentration by replacing various amounts of the water in enzymatic hydrolysis with oleyl alcohol, an inert oil that does not directly affect the function of the enzymes [38,39].

Cellulase adsorption

Cellulose accessibility and degree of adsorption of cellulases are well-known as controlling factors for conversion rates and yields [40,41]. It has long been known that certain hydrolysis products are able to inhibit cellulase adsorption [42]. It has, however, recently been shown that glucose and especially cellobiose strongly inhibit cellulase adsorption in a near-linear fashion [43].

In order to investigate whether adsorption (or lack thereof) could possibly be involved in the observed solids effect, the adsorption of enzyme was measured in hydrolysis of filter paper at different solids contents.

Methods

Compositional analysis

The composition of filter paper (AGF 725, 140 g/m2 from Frisenette ApS, Knebel, Denmark) was analyzed using two-step acid hydrolysis according to the procedure published by NREL [49]. Dry matter content was determined using a Sartorius MA 30 moisture analyzer at 105°C. The released sugars were quantified by HPLC as described below. The filter paper was found to consist of 80.6% glucan, 0.42% Klason lignin, 14.4% mannan, 1.0% arabinan, and 0.24% ash.

Enzymatic hydrolysis

The hydrolyses were performed using an enzyme mixture of Celluclast 1.5 L and Novozym 188 (weight ratio 5:1, both from Novozymes A/S, Bagsværd, Denmark) with a filter paper activity of 75 FPU per gram of dry matter (DM), as measured by the filter paper assay [50]. Enzyme loadings of 5-20 FPU per gram of DM and a hydrolysis times from 24 to 84 hours were used. Hydrolysis temperature was 50 ± 1ºC. Initial total solids content ranged from 5% to 35% (w/w) and pH was kept constant by adding sodium citrate buffer (pH 4.80, 50mM final concentration).

Hydrolysis experiments were performed at one of two scales. The 'large' scale hydrolyses were done in a horizontal, five-chambered liquefaction reactor where each chamber is 20 cm wide and 60 cm in diameter as described in [4]. In this reactor, a total reaction mass (solids and liquids) of 5 kg was used. The rotational speed was approximately 6 rpm.

The 'small' scale hydrolysis was performed in 100 mL plastic bottles (total reaction mass 50 g), also at 5-25% solids content (w/w); buffer concentration and enzyme loadings as described above. The bottles were placed in a heated, horizontally placed drum, rotating at 60 rpm. The 80 cm diameter drum was equipped with two inside paddles that lifted and dropped the plastic bottles during rotation, mimicking the gravimetric mixing described in [4,20]. All small scale experiments were performed in either duplicate or triplicate.

Samples for HPLC sugar analysis were boiled for 10 min to terminate the reaction. Whole-slurry was sampled after vigorous shaking to ensure a representable mixture of solids and liquid. Samples were then diluted five to ten-fold with eluent before insoluble material was removed by centrifugation at 4,200x g for 10 min. The dilution factor was determined by measuring the weight of the sample before and after dilution. When working at high insoluble solids concentrations there is an increasing difference between the concentration in the liquid phase and the overall concentration of a component [7] The dilution step minimises the measurement error introduced by the content of insoluble material, which would otherwise result in an overestimation when calculating the conversion, as discussed in [44].

Sugar analysis

The content of monosaccharides in the hydrolyzed samples (D-glucose, D-xylose, L-arabinose and D-cellobiose) was quantified on a Dionex Summit HPLC system equipped with a Shimadzu RI-detector. The separation was performed in a Phenomenex Rezex RHM column at 80°C with 5 mM H2SO4 as eluent at a flow rate of 0.6 ml min-1. Samples were filtered through a 0.45 µm filter and diluted with eluent before analysis on HPLC.

Inhibition experiments

Before hydrolysis, various amounts of D-glucose (Sigma-Aldrich, Brøndby, Denmark) were added to the substrate. Conditions were as described above.

Water replacement experiments

Hydrolysis was run at 'large' scale, as described above, with 20% solids content (w/w) and an enzyme loading of 10 FPU $(g\ DM)^{-1}$. 25% (w/w) of the initial aqueous phase was substituted with oleyl alcohol. It was found that neither the enzyme nor the released sugars was present in the oleyl alcohol. Sugar concentration was measured in the aqueous phase only.

Adsorption experiments

For cellulase adsorption studies, samples were kept on ice after hydrolysis instead of boiling, in order to prevent any desorption of enzyme from the solids. Rather than estimating the adsorption indirectly with a colorimetric method, total nitrogen content of the biomass was determined on an elemental analyser coupled to an isotope ratio mass spectrometer (ANCA SL & 20-20, Europa Scientific, Crewe, UK). This method of measuring enzyme adsorption has recently been described by Kumar and Wyman [43]. As the cellulase mixture of Celluclast 1.5 L and Novozym 188 contains a proportion of non-binding enzymes, enzyme adsorption will never reach 100% of the added amount. To be able to subtract the nitrogen content of the liquid of the spun-down samples, the nitrogen content of the aqueous phase was measured with the Kjeldahl method.

Results and discussion

Compositional and substrate effects

Filter paper was used as a model substrate. As seen in Fig. 2A, filter paper hydrolysis displayed the characteristic profiles with a very high initial rate of conversion that decreases considerably after only six to eight hours. When the conversion was displayed as a function of initial solids content, the characteristic downward curve was obvious (Fig. 2B). Again, the relationship is near-linear with a decrease from 56.5% conversion at 5% initial solids content to 22.8% conversion at 25% initial solids content, both after 24h of hydrolysis at large laboratory scale (see explanation of "small" and "large" laboratory scale in the Methods section). The 5% solids conversions shown in Fig. 2B are slightly higher than the linear curve. This observation is not in accordance with previous results or hydrolysis at different scales and is possibly a measurement artefact [4,44]. Thus, the results show that lignin or other phenolics are not involved in the solids effect.

The filter paper used in the experiments for the present paper contained approximately 15% hemicellulose in the form of 14% mannan and 1% arabinan. However, experiments with hydrolysis of Whatman filter paper (98% cellulose) (not shown) and hydrolysis of α-cellulose also displayed the same trend at increasing solids loadings [21]. This clearly indicates that

hemicellulose-derived sugars/inhibitors are not the cause of the solids effect, either.

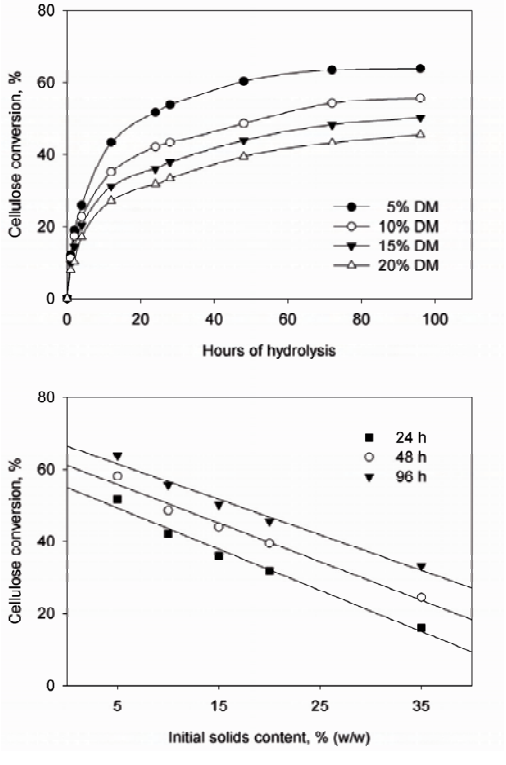

Fig. 2. Hydrolysis of filter paper at large laboratory scale with 5, 10, 15, 20 and 35% initial solids content (w/w) and an enzyme dosage of 10 FPU per gram dry matter (DM). A: Hydrolysis profiles for 5, 10, 15 and 20% DM as a function of time. B: Cellulose conversion as a function of initial solids concentration.

Product inhibition

To investigate the role of product inhibition in high solids enzymatic hydrolysis, various amounts of sugar was added to a hydrolysis of filter paper. An example of such an experiment (at large laboratory scale) is seen in Fig. 3. With 50 g/L glucose added, the rate of hydrolysis during the first few hours was significantly reduced compared to the reference, in particular for the 5% solids hydrolysis where the initial phase of fast conversion was completely absent. As there is a constant enzyme dosage per gram of solids in the experiments, the ratio between glucose and enzyme is much higher at 5% than 20% solids (for the hydrolyses with 50 g/L glucose added) and the stronger inhibition is thus not surprising. Although eight hours often makes up a small part of the whole hydrolysis time, the fast rate of hydrolysis in the first phase is responsible for conversion of a major part of the substrate. Interestingly, after approximately eight hours, the rate of hydrolysis (at each solids content) is nearly identical despite the significant difference in glucose level. This indicates that one of two things is happening. Either there are others and stronger factors inhibiting the hydrolysis after the first phase, thereby "masking" the product inhibition – or there is a certain glucose level threshold, above which the enzymes are inhibited to a similar extent and thus resulting in a similar conversion rate. However, if the latter is the case and product inhibition is the major factor responsible for the solids effect, then one would not expect a linear relationship between solids level and conversion.

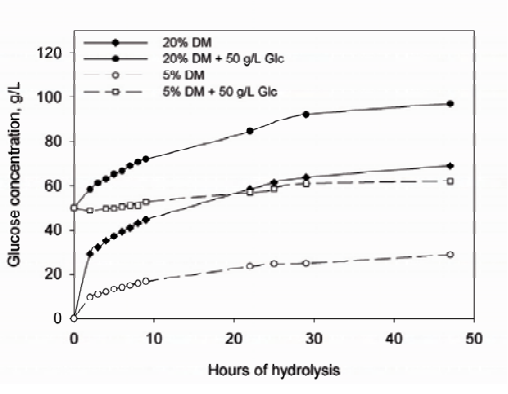

Fig. 3. Hydrolysis of filter paper at large laboratory scale with 5% (punctuated line) and 20% (solid line) initial solids content (w/w) and an enzyme dosage of 10 FPU per gram dry matter (DM). Before addition of enzyme, 50 g/L glucose was added to the substrate (open symbols). The references with no sugar addition are depicted with solid symbols. After eight hours the rate of the conversions (slope of curve) for each solids content is almost identical.

It is worth noticing; that it is not only the concentration of the inhibitor that is important but that the inhibitor-to-enzyme ratio is equally so. This means that when running hydrolysis at different solids contents but with constant enzyme to substrate levels, the degrees of inhibition should theoretically be identical. Xiao and co-workers showed that in hydrolysis of a cellobiose solution, addition of 20, 50 and 100 g/L of glucose to 2, 5 and 10% cellobiose (w/v) resulted in *β*-glucosidase inhibition of 53, 51 and 48%, respectively. The almost identical degree of inhibition at different sugar concentrations proves that the inhibitor-to-enzyme ratio is essential in product inhibition [32]. This shows that inhibition of *β*-glucosidase is not the main cause of the

solids effect. However, indirectly the cellulose-binding cellobiohydrolases are even stronger inhibited by glucose. The high glucose concentration leads to an accumulation of cellobiose, which acts as a particularly strong inhibitor of cellobiohydrolases [33].

Surprisingly, cellobiose concentrations in our experiments have generally been low. Normally, even at high solids concentrations and 80% conversion, less than 10% of the converted material is found as cellobiose (not shown). For comparison, during experiments with lower proportions of β-glucosidase, inhibition caused cellobiose proportions of over 35% of the converted material while still retaining a certain degree of hydrolysis (not shown).

SSF is normally used to offset the well-known effects of glucose and cellobiose inhibition but interestingly the solids effect has also been observed under those conditions [12,17,19]. Ethanol is also known to act as an inhibitor on cellulases (although less severe an inhibitor than cellobiose) [31,45], indicating that other factors may influence the conversion under these conditions.

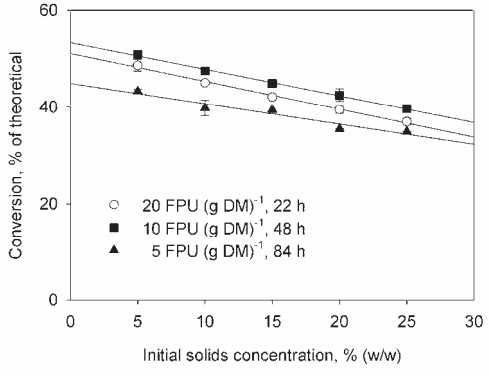

Fig. 4. Filter paper was hydrolysed at small laboratory scale to approximately the same extent by using three different enzyme loadings and lengths of hydrolysis time: 20 FPU per gram dry matter (DM), for 22 hours, 10 FPU per gram dry matter, for 48 hours and 5 FPU per gram dry matter for 84 hours. Points are averages of three observations. No significant difference in slope of the curves at the different enzyme loadings was observed.

To test if product inhibition was the sole cause of the solids effect a new experiment was carried out. Filter paper was hydrolysed to an extent of approximately 45% but at three different enzyme loadings and lengths of time: 20 FPU (g DM)$^{-1}$ for 22 hours, 10 FPU (g DM)$^{-1}$ for 48 hours and 5 FPU (g DM)$^{-1}$ for 84 hours. As seen in Fig. 4, the slopes of the three curves are near identical. If product inhibition alone was the cause, one would expect the hydrolysis with the lowest enzyme-to-substrate ratio to display the strongest degree of inhibition and thus a steeper curve. In other words, it is not possible to by-pass the solids effect by using higher enzyme dosages, at least not within the normal range of dosages. This is an important consideration when trying to alleviate the solids effect.

In conclusion, product inhibition at increased solids concentrations was found to be a significant and potentially determining factor for the solids effect. However, the linearity over a large range of solids contents of our experiments does not fit with the current models for product inhibition.

Water concentration

Oleyl alcohol was used to replace water in order to investigate the water to enzyme/solids ratio while keeping the viscosity rather similar. The reasoning behind these experiments is that by substituting part of the water, it is possible to run a hydrolysis with an altered water-to-enzyme ratio but with a more or less constant viscosity of the slurry. If it is a lack of water that is causing the solids effect, then the hydrolysis conversion where a certain amount of the water has been replaced should be lower, presumably at the level of the corresponding solids level (taking only the aqueous phase in consideration).

In Fig. 5, a quarter of the water (buffer) in an enzymatic hydrolysis of 20% solids filter paper has been substituted. At this level of substitution, the actual solids concentration in relation to water has therefore been increased from 20 to 25%. After 40 hours of hydrolysis, 5.6% less glucose compared to the reference (without oleyl alcohol addition) was released. However, the increase from 20% to 25% solids usually leads a decrease in conversion of over 12%. Thus, the decrease in conversion did not correspond directly to the lowered water content.

However, the sugar concentration is not the only parameter that has been changed. Oleyl alcohol may act as a mixing agent, fully or partially replacing the effect of water in assisting mass transfer, even if neither enzymes nor sugars can be solubilised in oleyl alcohol. As previously discussed, the interconnection of factors affecting the yield is very characteristic of lignocellulose hydrolysis, complicating the identification of limiting factors.

There is no doubt that water plays a number of important roles in enzymatic hydrolysis, and that these roles become even more crucial in systems with no free water. As cellulases can only break down cellulose when adsorbed onto the material, efficient mass transfer of enzymes is likely to increase conversion.

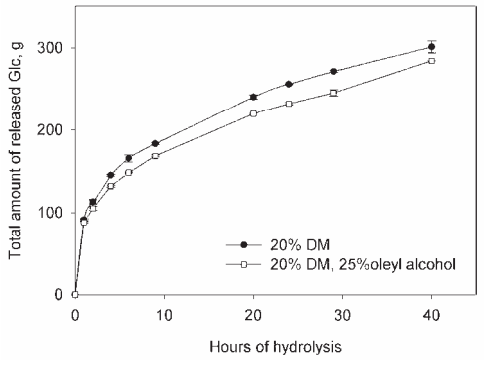

Fig. 5. Hydrolysis of filter paper at large laboratory scale with 20% initial solids content (w/w) and an enzyme dosage of 10 FPU per gram dry matter (DM). where 25% of the water was replaced with the inert oil, oleyl alcohol. This corresponds to an increase of biomass to water ratio of 25%. The yield was found to be less than the reference, but not as low as a 25% increase in solids normally results in. Points are averages of two experiments.

Also, diffusion of released sugars away from the catalytic sites will theoretically prevent local product inhibition. Mechanical stirring may also directly change the size distribution or larger particles. Unfortunately, our understanding of these mechanistic interactions is limited and also depends on the cell wall structure of the substrate. It is likely that such factors affect the degree of conversion at very high solids loadings, essentially causing a drop-off in yield over a certain solids loading. However, as already discussed the observed solids effect is also seen at loadings as low as 2-5% solids and thus, mass transfer at neither the macroscopic nor the molecular level can be responsible for the solids effect.

In conclusion, water itself as a substrate or diffusing agent in enzymatic hydrolysis does not appear to be the limiting factor responsible for the solids effect, nor is substrate inhibition involved.

Cellulase adsorption

Based on previous reports on inhibition of enzyme adsorption, it was investigated if the increased sugar concentration at high solids concentration could cause the solids effect in this manner. As seen in Fig. 6, there is a near-linear correlation between initial solids content and amount of adsorbed enzyme (percentage of nitrogen adsorbed on solids of total nitrogen added). After 24 hours of hydrolysis of 5% solids filter paper, approximately 40% of the added enzyme was adsorbed onto the remaining solids. The adsorption decreases with increasing solids content and at 30% solids content, only approximately 17% of the added enzyme is adsorbed, despite significantly more solids remaining than at lower solids contents. Even more interesting, there is a statistically significant correlation between the decrease in conversion and the decrease in enzyme adsorption. In other words, it appears that the increasing concentrations of glucose and cellobiose in high-solids hydrolysis result in inhibition of adsorption of the enzymes. As adsorption is a requirement for hydrolysis of the insoluble substrate, this in return results in lower conversion at increasing solids concentrations.

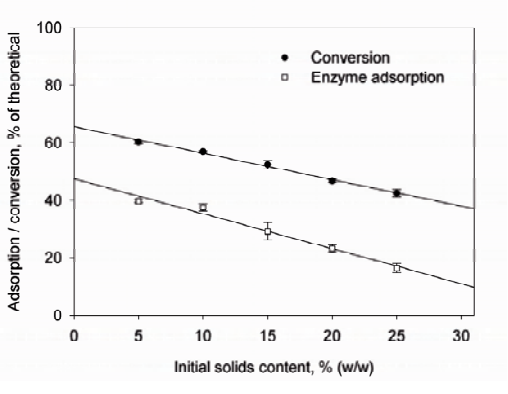

Fig. 6. Upper graph shows the decreasing conversion in enzymatic conversion of filter paper at increasing solids loading (20 FPU per gram dry matter (DM), 24 hours hydrolysis at small laboratory scale). Points are averages of three observations. The lower graph shows the adsorption of enzyme on the solid fraction based on total nitrogen content, also as a function of initial solids content. Values are averages of three observations and have been corrected for varying amounts of remaining solids.

Based on an experiment with a fixed cellobiose concentration, Kumar and Wyman argue that binding inhibition can be reversed using high substrate concentrations [43]. However, working with a fixed inhibitor concentration over a range of solids concentrations does not reflect the actual conditions since high solids loadings will invariably lead to higher product concentrations. At any degree of conversion, the ratio between substrate and inhibitor (product) in hydrolysis will be constant no matter the initial solids concentration. Xiao and co-workers also observed reduced impact of products on inhibition at higher solids loadings but again it was measured against a constant inhibitor concentration [32]. Based on our experiments we do not believe that increased solids

concentrations can reverse binding inhibition, rather the opposite.

It can be argued that the phenomenon described above is a variant of product inhibition. In both competitive and non-competitive inhibition the catalytic site is affected, which is not necessarily the case with inhibition of adsorption. Although β-glucosidase does not bind to the substrate and thus is not affected in this way, the binding inhibition of endoglucanases and cellobiohydrolases can possibly explain the low cellobiose-levels under conditions where the hydrolysis is obviously inhibited.

It is not known to what extent inhibition of adsorption is responsible for the solids effect or if it can be partially avoided through SSF. It has previously been shown that adsorption inhibition could not explain the decrease in cellulase activity [48]. In attempt to learn more about the nature of the inhibition, we used the data of the experiment in Fig. 2 to investigate the relation between the rate of reaction and glucose concentration. We found no direct relationship (not shown) - possibly due to the fact that different proportions of the substrate remained, i.e. when 50% of the substrate has been converted, the remainder is more difficult to hydrolyse.

It is likely that the cellulose binding domains (CBD) of the cellulases are affected by glucose and cellobiose. Binding of cellulases and clarification of the role of CBDs is an important topic in cellulosic biomass conversion, and has been the topic of numerous studies. Being able to alter the CBD to make it less susceptible to high concentration of products may contribute to making high yields at high solids concentrations a reality.

Conclusion

The extent of enzymatic conversion of cellulosic biomass was investigated at varying solids concentrations. The conversion decreased at increasing solids concentration in a linear fashion, an effect that appears to be a generic or intrinsic feature of lignocellulose conversion. This decrease partially off-sets the significant advantages of working at high solids concentrations. It was found that the solids effect was not caused by lignin content or hemicellulose-derived inhibitors. Mixing of the insoluble substrate did not appear to be causing the effect either.

The increased concentration of glucose and cellobiose at high solids concentration are likely to cause product inhibition even when the enzyme-to-inhibitor ratio is constant. However, the solids effect has also been observed in SSF where much less sugar is present.

It was found that at increasing solids concentrations, the proportion of adsorbed cellulase decreased. There was a statistically significant correlation between this adsorption inhibition and the decreasing yields at increasing substrate concentrations. Thus, the solids effect can be explained by inhibition of the binding of the cellulases. The exact extent and mechanism of the adsorption inhibition is still unknown. It is possible that improvement of cellulase CBDs can lead to enzymes that are more resistant to high sugar concentrations and thus higher conversions at high solids concentrations, significantly improving the viability of lignocellulosic biomass conversion.

Acknowledgements

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APPENDIX

APPENDIX

Related publications:

VI Felby, C., Thygesen, L.G., Kristensen, J.B., Jørgensen, H., Elder, T.:
Cellulose-water interactions during enzymatic hydrolysis as studied by time domain NMR
Cellulose 2008, 15(5):703-710.

VII Elder. T., Kristensen, J.B., Thygesen, L.G., Jørgensen, H.:
Hydrothermal pretreatment of wheat straw. Temperature effects
Submitted for publication.

Cellulose (2008) 15:703–710
DOI 10.1007/s10570-008-9222-8

Cellulose–water interactions during enzymatic hydrolysis as studied by time domain NMR

Claus Felby · Lisbeth G. Thygesen · Jan B. Kristensen · Henning Jørgensen · Thomas Elder

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Abstract The different states and locations of water within the cellulose matrix can be studied by the use of time domain low field NMR. In this work we show how the state and location of water associated with cellulose in filter paper fibers are affected by enzymatic hydrolysis. Three locations of water were identified in the filter paper; (1) bound water associated with the microfibril surfaces and (2) water in the cell wall or cellulose matrix and (3) capillary water in the lumens and between fibers. The different mechanisms of cellulase enzymes can be seen in their effect on the cellulose–water interactions and the synergistic effects between endo- and exo enzymes can be easily detected by time domain NMR. An interesting observation is that it is possible to link the state and location of water within the cellulose fiber with structural changes upon enzymatic hydrolysis.

Keywords Cellulose · Hydrolysis · Enzymes · Time domain NMR

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Introduction

Enzymatic hydrolysis of cellulose has become an important research area due to the potential use of cellulosic biomass as feedstock for fermentation into ethanol.

The enzymatic breakdown of cellulose to fermentable sugars is done by enzymatic hydrolysis of the glucosidic bonds. The reaction is thus a two-substrate reaction involving both cellulose and water. While there has been considerable interest in the cellulose–enzyme interactions as well as on the cellulose composition, limited attention has been paid to the role of water in the process.

When water is sorbed to cellulose in a plant cell it has properties which are highly different from the properties of bulk water (Kollmann and Côté 1968).

Within the plant cell wall matrix, water is subjected to a number of interactions caused by the chemical and physical composition of the cell wall. Thus the structure and composition of the cell wall produce different states and locations of water, all of which may be important for our understanding of the interactions between cellulose and enzymes.

In the following a general and somewhat simplified description of the state and location of water in lignocellulose is given.

In the range from molecular to micro-scale, the lignocellulosic matrix has several structures that affect the state of water. On a molecular scale the prime source of interaction is the polar groups,

dominated by the hydroxyl groups, which readily form hydrogen bonds with water. At the structural nano-scale level on the surface of the cellulose microfibrils, water is packed in ordered layers or clusters reflecting the crystalline structure of the cellulose. This packed water is here denoted primary bound water, and the density of this water may be as high as 2.5 g/cm^3 (Matthews et al. 2006).

At the cell wall level, sorbed water is located in a porous structure with confined spaces where the water is bound. The mechanisms of bonding are either by capillary forces, by hydrogen bonds to hydroxyl groups on hemicellulose and lignin or by hydrogen bonds to other water molecules already bound to cellulose, hemicellulose and lignin. This type of water is commonly classified as secondary bound water. One also encounters the classification freezing and non-freezing water for the secondary and primary bound water, respectively (Hartley et al. 1992).

Below the fiber saturation point of approximately 25–30% moisture, the major part of the water will be present as primary or secondary bound water within the cell wall Combining the primary and secondary pools of water gives an average density of water in the cell wall of approximately 1.2 g/cm^3. Above the fiber saturation point water fills the cell lumens until full saturation in the area of 60–70% moisture content.

Since water exists in several different states within the cell wall matrix, several issues may be raised in relation to enzymatic hydrolysis.

What is the importance of primary bound water with a density of 2.5 g/cm^3 on top of the cellulose fibrils where the enzymes are active? Do the enzymes affect the primary bound water? Furthermore, the enzyme action may change the state and location of secondary bound water within the cell wall matrix. If so, will it be possible to monitor the effect of enzymatic breakdown on the cell wall matrix structure through the state and location of water?

To answer these questions, time-domain nuclear magnetic resonance (TD-NMR) is a promising technique, in which the relaxation times of the hydrogen nuclei can be used to asses the different states of water in lignocellulose. Two different types of relaxation times can be obtained; spin–lattice (T_1) and spin–spin (T_2). For practical reasons, and because this approach has given useful information, studies of water within solid substrates such as wood, pulp and paper have mostly relied on spin–spin relaxation, and the present work is no exception. The T_2 relaxation time of hydrogen nuclei depends both on how free the hydrogen nuclei is to move, i.e. which molecular environment it is part of and the physical state of that solid or liquid environment. Generally, tight bonding and small compartments shorten the spin–spin relaxation time of hydrogen nuclei.

In some of the earliest work reported on the use of TD-NMR to study cellulose–water interactions, Froix and Nelson (1975), measured both T_1 and T_2 relaxation times for cotton linters in a range of 0–25% moisture content. Four different states of water were identified: Primary bound water on the cellulose crystal, and two types of secondary bound water associated with the cellulose structure as well as bulk water.

Menon et al. (1987) did a comprehensive study on water in wood, finding three pools of water which they assigned to the cell wall, the ray and tracheid lumens and the earlywood tracheid lumens. Also by choosing different tree species, they found that species had an effect on the T_2 values. Species with smaller cell lumens had shorter T_2 values for the lumen water.

Araujo et al. (1993) examined the location of water in white spruce softwood by TD-NMR identifying bound water, lumen water in late wood cells and lumen water in early wood cells. The latter having the largest lumens and thus the longest relaxation time.

Antique paper was examined by Blümich et al. (2003). They assessed paper as a bi-component material made from cellulose and water, and found that T_2 values were correlated to the condition i.e. level of breakdown of the paper.

Elder et al. (2006) used TD-NMR to study water in hardwood chars. They found different distributions between bound and free water as a function of moisture content as well as effects of changing temperatures and pore sizes. In addition, a clear effect of species on T_2 relaxation time as well as an effect of moisture and temperature was observed.

In a study of fungal attack on commercial paper, Capitani et al. (1998) described that by adding a cellulase extract from *Aspergillus niger*, a fast response in the T_2 relaxation times upon addition of the enzymes was found. This response was assigned to changing water pools in what was labeled as amorphous regions of cellulose.

The bulk of the cited work was done at low moisture contents as compared to the conditions that would be applied in a commercial process for conversion of cellulose to fermentable sugars. Such moisture levels will be in the area of 60–80%, thus significantly above the fiber saturation point and presumably with most lumens water-filled.

In the present work we applied TD NMR to examine the states and locations of water in a cellulose–water system subjected to hydrolysis by endo and exo-cellulases as well as a complete cellulase system. Filter paper was chosen as the model substrate as it consists almost of pure cellulose, but still has an intact cellulose matrix and cell wall structure.

Materials

Whatman No. 1 filter paper. Enzymes; purified *Tricoderma longibrachiatum* endoglucanase (EG) EC 3.2.1.4 and cellubiohydrolase (CBHI) EC 3.2.1.91 both from Megazyme, Ireland. Cellulase mixture: commercial product Celluclast 1.5 L from Novozymes A/S, Denmark. The EG and CBH preparations were formulated with 3.2 M ammonium sulphate. The exact formulation of the cellulase mixture is not accessible, but a main component is glycerol.

Experimental

Cellulase treatment

About 0.9 g of filter paper was cut in pieces of approximately 4×4 mm^2. The filter paper was placed in the NMR sample tube and 1.8 ml deionized water with or without enzyme added. The enzyme was mixed with water on a weight basis of 1 mg of protein for each treatment. Mixing of filter paper and water with or without enzyme was done by adsorption only. Temperature during the treatment was identical to the NMR operating temperature 40 °C. The pH of the filter paper–water mixture was 4.9.

HPLC of released sugars

For verification of enzyme activity the content of cellobiose, and glucose was quantified on a Dionex Summit HPLC system equipped with a Shimadzu RI-detector. The separation was performed in a Phenomenex Rezex RHM column at 80 °C with 5 mM H_2SO_4 as eluent at a flow rate of 0.6 ml min^{-1}. Samples were filtered through a 0.45 μm filter and diluted with eluent before analysis on the HPLC.

NMR measurement

NMR analyses were done using a Bruker mq20-Minispec, with a 0.47 Tesla permanent magnet (20 MHz proton resonance frequency), operating at 40 °C. The transverse (T_2) relaxation times were determined using the Carr–Purcell–Meiboom–Gill (CPMG) sequence. About 3,000 echoes were collected with a pulse separation of 0.05 ms, the acquisition of 32 scans and a 5 s recycle delay. The magnetization decay curves were analyzed using mono-exponential and bi-exponential fitting routines to determine discreet values for T_2. The Laplace transformation method CONTIN, as described by Provencher (1982) was used to determine relaxation time distributions. This method is only one of a number of different ways to assess CPMG relaxation curves, and one should keep in mind that different models might fit a relaxation curve equally well from a mathematical point of view (Whittall and Mackay 1989). Here, we have chosen to use CONTIN and discrete exponential fitting, and to focus on differences between sample types.

The NMR measurement started 15 min after addition of water with or without enzymes to the filter paper ($t = 0$). Measurements were done at 0, 15, 30, 45, 60, 90, 120 and 360 min. All NMR measurements were repeated on three set of enzyme treatments.

Conditioning of filter paper

For assignment of water pools, samples of filter paper with different moisture contents (5%, 25% and 66%) were prepared. Air dry 5% moisture content was measured as received. Samples at the fiber saturation point (approximately 25% moisture) were prepared by conditioning the filter paper in a dessicator over deionized water for 10 days. Saturated samples (66% moisture) were prepared by adding 2 g of deionized water to 1 g of filter paper. All samples were placed

in NMR tubes and measured according to the description above.

Results and discussion

Prior to assessing the effect of enzymatic hydrolysis, the effect of different moisture levels upon the state and location of water in the filter paper was studied. The fibers in filter paper are derived from kraft pulped softwoods and though they are composed almost purely of cellulose, the overall cell structure (cell wall and lumen) remains intact. In comparison to non-pulped lignocellulosic fibers, however, the filter paper will have a higher porosity and lack of pore-structures due to the breakdown of lignin and pectin components during the pulping process.

The assignments of the observed peaks are based on the observations shown in Fig. 1. All measurements were done in triplicate, and the observed peaks and changes were consistently seen in all measurements. It can be seen that at 5% moisture, water with a short relaxation time of less than 1 ms dominates and only trace amounts of water with longer relaxation times can be seen. At 5% moisture level it is generally recognized that only bound water is present on the cellulose, thus the 1 ms peak is assigned to primary bound water.

At 25% moisture two peaks at 0.7 and 3 ms, respectively, can be seen. This moisture level is just below the fiber saturation point, i.e. no or little water is present in the lumens. Therefore the peak at 3 ms is assigned to less tightly associated secondary bound water situated in the cell wall structure. At the highest level of 66% moisture content, the peaks assigned to bound and cell wall water are clearly visible, but also to be seen is a large peak at 110 ms due to lumen and inter-fiber water bound by capillary forces. While this water pool could be assigned to bulk water, pure water exhibits a T_2 as high as 3 s (results not shown). As a consequence, the 110 ms peak is identified as water bound by capillary forces in the lumen of the cellulose fibers and denoted lumen water. Unbound bulk water as such is not present in the system even at 66% moisture content. Note that the relaxation times for the cell wall and lumen pools are increased as the water is adsorbed and swells the cellulose structure.

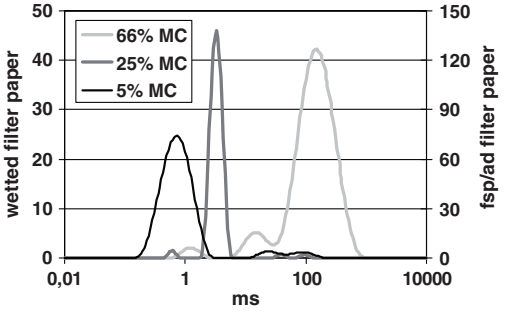

Fig. 1 Comparison of water pools in filter paper found by time domain NMR at three moisture contents (MC). 5% air dried (ad), 25% fiber saturation point (fsp) and 66% wetted. The signal strength is proportional to the water content and for clarity different vertical scales are used

Our assignments of water to three different locations are different to the assignment done by Araujo et al. (1993) on water in white spruce. They state that only bound- and lumen water can be seen, attributing peaks around 10 and 100 ms to water in differently sized cell lumens.

In this work there is a reasonably good agreement with the relaxation times found by Araujo et al. However, we show that up to the fiber saturation point where no or little lumen water is present, there are two distinct peaks; one is bound water and the other around 3 ms must be from water in the porous cell wall. When the moisture content is increased to 66%, the cell wall swells and the relaxation times increase, but we still observe a distinct intermediate peak between the primary bound water and lumen water. Similar observations at different moisture levels on early- and late wood cells from softwoods confirm our assignments. The distinction between primary bound water, cell wall secondary bound water and lumen water in plant cells detected by TD-NMR, therefore appears to be generic (Thygesen et al. under preparation).

We therefore conclude that three different pools and two different states of water can be seen in the filter paper at 66% moisture content; primary bound water tightly associated with the cellulose fibrils, cell wall water—secondary bound by capillary forces or hydrogen bonds in the cell wall, and lumen water—secondary bound capillary water in the cell lumen or between fibers.

With these assignments in mind, we now turn to the measurements on filter paper. For the control samples (water only) it can be seen that the fiber

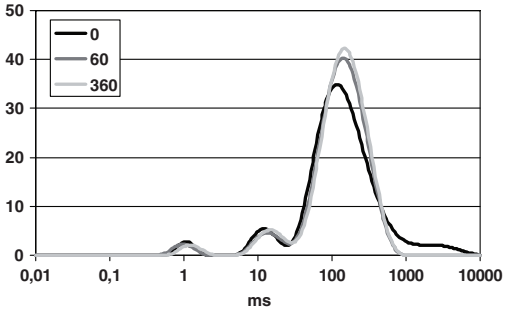

Fig. 2 Control treatment deionised water only. Time domain NMR recorded at 0, 60 and 360 min. For clarity only the measurements at 0, 60 and 360 min are shown

lumen water peak becomes narrower with time as the cellulose adsorbs the water. This is interpreted as an increase in porosity, when the sorbed water swells the cellulose structure and increases the capillary bonding of the lumen water, see Fig. 2.

To ensure the enzymes where active, glucose production was checked by HPLC confirming an increase in glucose throughout the test period. For all three enzymes 0.4–0.7% of the cellulose was hydrolyzed to glucose or cellobiose. At such a low level of enzymatic breakdown it can be assumed that the fiber structure is fully intact. For all enzymes tested, controls with heat inactivated enzyme were performed. The inactivated controls containing the full enzyme preparations were identical to the water only control (results not shown), and we conclude that under the applied conditions, there is no effect of the enzyme formulations or the protein itself on the T_2 values.

Upon the addition of endoglucanase to the filter paper, the T_2 distributions are changed compared to those of the controls (Fig. 3a). The main effect can be seen on the T_2 for the lumen water which when displayed on a linear scale (Fig. 3b) has a more narrow distribution i.e. a stronger adsorption of capillary bound water in the lumen region. This observation can be explained by the mechanism of the enzyme, randomly cleaving the cellulose chains inside the fibrils. This introduces cavities and micropores inside the cellulose structure, increasing the ability of the water to interact with the cellulose as seen on Fig. 3a. This interpretation is also confirmed by observations of Dourado et al. (1999) who found that cellulase treatment of cellulose increased the water holding capacity.

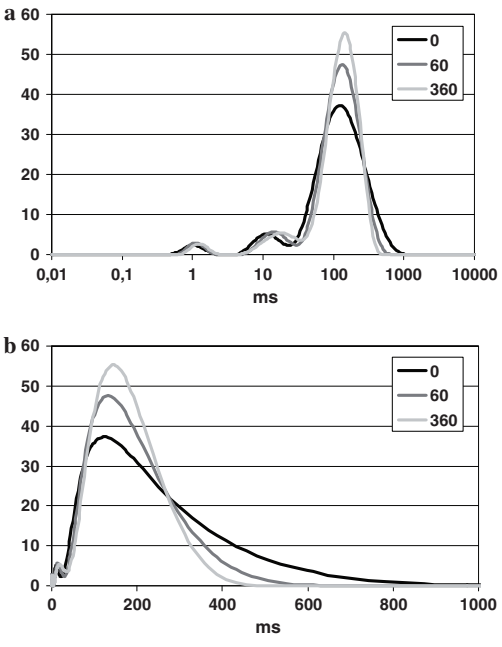

Fig. 3 Time domain NMR spectra of endoglucanase treated filter paper from 0 to 360 min. (**a**) logarithmic scale showing all three water pools; primary bound water, cell wall water and lumen water. (**b**) Linear scale showing how the lumen water is more strongly adsorbed as the enzyme reaction proceeds

It can also be seen how the EG increases the relaxation time of the water associated with the cell wall (Fig. 4). The longer relaxation time can be interpreted as a loosening or opening up of the structure at the earliest point of cellulose breakdown. Thus, the initial action of the EG not only splits the cellulose chains, but may also introduce water into the cellulose structure by the formation of cavities and micro pores.

Contrary to the EG, the cellobiohydrolase (CBH) has no detectable effects on the water pools under the applied conditions. Both the T_2 distributions and the relaxation times found from the peak values are identical to the control (Fig. 5). The activity of the enzyme was confirmed by the release of glucose, but the exo mechanism which cleaves the cellulose from the ends does not cause any structural changes that would affect the state or location of the water.

The final enzyme tested was the commercial Celluclast 1.5 L enzyme preparation from Novozymes. This enzyme mixture is derived from

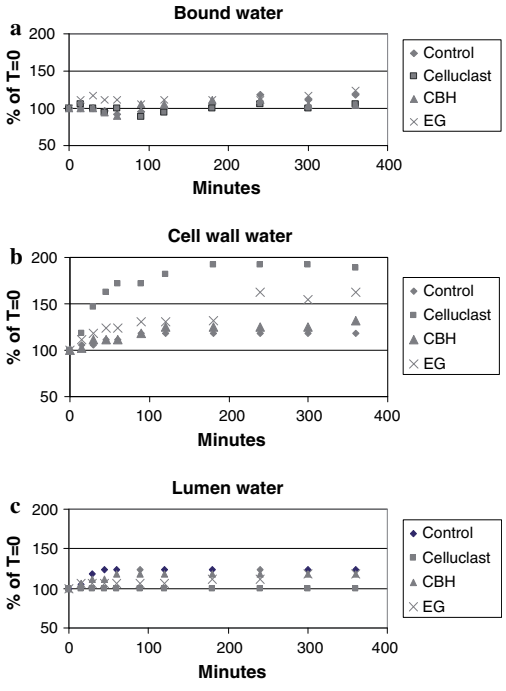

Fig. 4 Development in relaxation times for the different treatments as found for primary bound-, cell wall- and lumen water. The values reported are average for three independent measurements. The vertical scale is the relative change of the T_2 value compared to the relaxation time at $t = 0$

Trichoderma reesei and contains a number of endo- and exo-acting cellulases. Celluclast does not have any significant beta-glucosidase activity, which may cause product inhibition from a build up of cellobiose. However, this was not considered to be relevant for the current study since only the initial phase of the cellulose breakdown was the focus of the work.

The addition of Celluclast has by far the most pronounced effect on the state and location of water (Fig. 6a). The lumen water peak is even narrower compared to the endoglucanase from *T. longibrachiatum*, the cell wall water peak is apparently split into two peaks, and a magnified view (Fig. 6b) of the primary bound water peak shows that it is also narrowed, similar to the lumen water peak. The reason for the change in the primary bound water peak is not known.

The increased relaxation time for the main part of the cell wall water associated with the 25 ms peak indicates a significant loosening or fragmentation of the whole cell wall matrix. Interestingly, this is only associated with a limited release of glucose and cellobiose, and what we observe may be described as enzymatic "drilling" as proposed by Dourado et al. (1999). Thus enzymatic drilling is associated with a

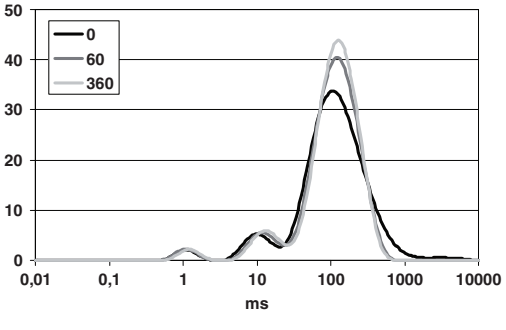

Fig. 5 Time domain NMR spectra of cellobiohydrolase treated filter paper from 0 to 360 min. The distribution and character of water pools are identical to control

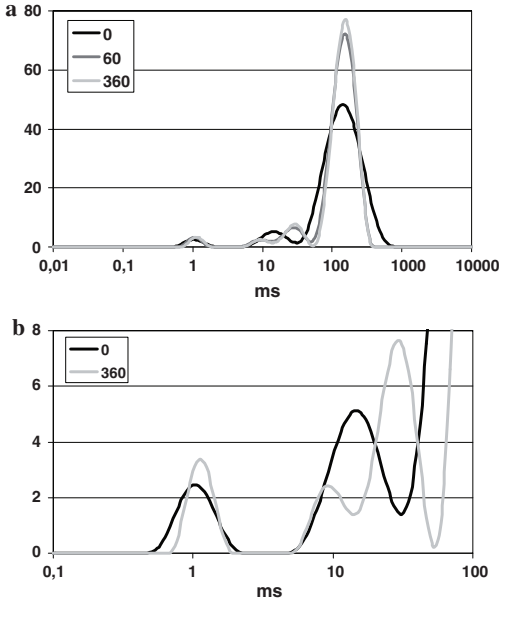

Fig. 6 Time domain NMR spectra of Celluclast 1.5 L treated filter paper from 0 to 360 min. (**a**) Both lumen and matrix water are affected by the cellulase system. (**b**) Expanded view of the cell wall water showing how two different pools of cell water develops

loosening of the cellulose matrix structure creating cavities and micropores, but still maintaining the overall structural characteristics. The remaining cell wall water peak at 9 ms can be interpreted as belonging to non-accessible or recalcitrant structures in the cell wall structure, however, more studies on the time development is necessary in order to elucidate this.

Based on the time dependant development of the cellulose–water interactions and the lack of effect using heat inactivated enzyme preparations i.e. containing both enzymes and stabilizers, we have assigned the observations to the catalytic effect of the enzyme. However, it must be considered whether the changes in the cellulose–water interactions can be caused by the adsorption of cellulose binding domains (CBD) onto the cellulose structure rather than the hydrolytic breakdown. Both the EG and CBH are isolated from *Tricoderma longibrachiatum* and both enzymes have catalytic cores with an CBD attached. The fact that the EG has an time dependant effect upon the cellulose–water interaction and the CBH has none, excludes major effects from the adsorption of the CBD's. The changes in the cellulose–water interactions must be assigned to the catalytic activity of the enzymes. This does not rule out a possible role of CBD's and accessory proteins in the interactions of cellulose and water, but the interpretation of the results in this work should be assigned to the enzymatic hydrolysis.

Considering the development of the relaxation times from 0 to 360 min for all three enzyme preparations (Fig. 4), little or no effect can be seen on the primary bound water. Most likely, higher enzyme protein loadings than the applied approximately 0.1% are required to reveal possible effects on primary bound water. For cell wall water, a clear effect of increasing relaxation times i.e. a degradation and thereby looser cellulose matrix structure can be seen both for Celluclast 1.5 L and EG.

The lumen water shows similar behavior for the control, EG and CBH with slightly increasing relaxation times caused by the swelling of the cellulose cell wall structure, whereas Celluclast 1.5 L stays constant. The latter can be explained by that even though Celluclast 1.5 L causes the most pronounced changes on the cellulose matrix and the cellulose–water interactions, it also increases the porosity and water bonding capacity of the cell wall, which counteracts the effect of swelling and loosening of the cell wall.

The relaxation time behavior found in this work are different from that reported by Capitani et al. (1998), who reported a shortening of T_2 relaxation times upon cellulase addition. Shorter relaxation time does not appear logical, as the breakdown of cellulose should result in a less organized structure and thus longer relaxation times. This discrepancy to the present work is most likely caused by the fact that Capitani et al. (1998) used commercial office paper with a high content of clays such as kaolin.

The results from this TD-NMR study of water during enzymatic hydrolysis are interpreted in terms of its effect on the cellulose matrix structure as illustrated in Fig. 7. What is surprising to the authors are not the observed mechanisms or structures, but the fact that under the applied conditions with no stirring and a relatively low enzyme dosage, the combined action of an endo- and exo-glucanase system caused substantial changes in the cell wall matrix, as observed on the cellulose–water interactions. These changes most likely occur at the molecular level and are at best only marginally detectable by chromatographic or microscopic methods, but their effect on cellulose structure and the cellulose–water interactions are clearly seen by time TD-NMR. The term "enzymatic drilling" is thus a good description of the initial cellulase action, and we believe that it is of prime importance for the overall performance of industrial enzyme preparations for cellulose hydrolysis.

In this work we have used filter paper for simplicity. This substrate is of course not identical to the lignocellulosic substrates to be used in e.g. a commercial cellulose to ethanol process. However, our previous experience on thermally pretreated

Fig. 7 Illustration of the enzyme action upon the structure of the cellulose matrix for endoglucanase (EG), cellubiohydrolase (CBH) and the cellulase mixture Celluclast 1.5 L

wheat straw (Kristensen et al. 2006; Jorgensen et al. 2007), show that the basic factors regulating the enzyme hydrolysis are quite identical to a pure cellulose substrate. The results presented in this work may with some caution be extrapolated to lignocellulosic substrates as well.

Conclusions

The results show that TD NMR can provide detailed information on cellulose–water interactions during enzymatic hydrolysis. During the initial enzymatic hydrolysis of cellulose, the action of the enzyme system is a breakdown and loosening of the cellulose introducing more water into the structure and providing better access for the enzymes. In particular, the cell wall matrix is affected by a combined cellulase system, even under conditions where no stirring is applied. The use of TD-NMR is a promising technique for further elucidation and understanding of the enzyme–cellulose–water system and its interactions.

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Hydrothermal Pretreatment of Wheat Straw. Temperature Effects.

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Abstract

Wheat straw samples were pretreated in the IBUS (Integrated Biomass Utilization System) process at temperatures of 160, 175, 185 and 195°C. With increasing temperature the xylan percentage decreased rapidly, with concomitant increases in cellulose and lignin percentages. The release of simple sugars upon enzymatic hydrolysis also increased with pretreatment temperature. Attenuated-total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) analyses were consistent with the wet chemical assays. Atomic force microscopy revealed changes between the untreated control and pretreated samples, and low field-time domain-nuclear magnetic resonance spectroscopy results indicated changes in relaxation time of bound water, even at the lowest pretreatment temperature.

Keywords: AFM, ATR-FTIR, IBUS, LF-TD-NMR, pretreatment, wheat straw

Introduction

In the conversion of lignocellulosic biomass to ethanol, the polysaccharides in the substrate are first hydrolyzed to simple sugars which are subsequently fermented by yeasts to ethanol. The hydrolysis process is enhanced by a pre-treatment step, making the polysaccharides more accessible to the enzymes currently used for their depolymerization. A recent review (Wyman et al., 2005) examined a number of pre-treatment methods including ammonia explosion, aqueous ammonia recycle, controlled pH, dilute acid and lime-based methods. Other techniques that have been proposed are wet-oxidation (Lissens et al., 2004) and hydrothermal treatment without the addition of chemicals (Laser et al., 2002, Negro et al., 2003).

In the current work, wheat straw has been pre-treated using the IBUS (Integrated Biomass Utilization System) pilot plant, a hydrothermal pre-treatment that has proven to be effective at preparing straw for enzymatic hydrolysis (Thomsen et al., 2006, Larsen et al., 2008). This method is designed to handle large particles (pieces of straw over 5 cm in length) and run at high dry matter levels (exceeding 30% w/w) (Thomsen et al., 2006). After the initial chopping, the straw is soaked in water, transported to the pretreatment reactor and heated by steam to 190-200˚C. Integrated with the pretreatment reactor is a washing and pressing step which produces a solid fraction of 30-40% dry matter. The liquid fraction, rich in salts and hemicellulose derived sugars, can be concentrated to produce feed molasses (Larsen et al., 2008). A pilot plant with a capacity of up to 1000 kg/h has been working since 2006, and it has been reported that the pre-treated straw can be enzymatically liquefied, saccharified and subsequently fermented into ethanol at initial dry matter levels of up to 40% w/w with resulting ethanol concentrations of up to 48 g/kg (Thomsen et al., 2006, Jørgensen et al., 2007).

This paper reports on the impact of the IBUS process over a temperature range from 160 to 195°C. Using the lowest temperature possible, while maintaining acceptable levels of conversion, will contribute to the energy efficiency of the pretreatment process. The resultant material, compared to an untreated control was assayed for composition and conversion efficiency, along with examinations by attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR), atomic force microscopy (AFM) and

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low-field, time-domain-nuclear magnetic resonance spectroscopy (LF-TD-NMR). Previous work, using scanning electron microscopy and atomic force microscopy has found that IBUS pretreated (195°C for 12 minutes), exhibits minimal structural changes in the cell wall, from which it is proposed that lignin redeposition onto the surfaces of the fibers is occurring (Kristensen et al., 2008). It was also reported from ATR-FTIR results that waxes and hemicelluloses were removed, and surface lignin was increased.

Fourier-transform infrared spectroscopy has been specifically used in the analysis of wheat straw pre-treated by wet alkaline oxidation (Schmidt et al., 2002) and steam explosion (Sun and Chen, 2008, Zhang et al., 2008). The results are consistent between pre-treatment methods, all indicating a decrease in the presence of ester linkages that occur between hemicelluloses and lignin.

The application of atomic force microscopy to the examination of straw has been reported in the literature by Yan et al. (2004) and Yu et al. (2005, 2008). Observations indicate a waxy layer on the outer surface of the straw, the removal of which reveals the presence of lignin (Yan et al., 2004). Other work (Yu et al., 2005; 2008) shows that the vessels within the vascular bundles and epidermal cells possess highly ordered cellulose microfibrils, while the microfibrils of parenchyma cells are randomly oriented.

The NMR methods reported in the current work, working in the time, rather than frequency, domain have been applied as a quality control tool in various manufacturing applications such as foods and cosmetics, textiles, and well-logging in oil exploration. In earlier work reported in the literature, this method has been used in the examination of wood, paper, and cellulose to elucidate the nature of water present (Flibotte et al., 1990, Araujo et al., 1992; 1994) in the material and how processing will alter the behavior of the water (Elder et al., 2006, Labbé et al., 2002, Felby et al., 2008, Thygesen and Elder, 2008). The experiments that are typically performed measure the spin-lattice (or longitudinal) relaxation time (T_1) and the spin–spin (or transverse) relaxation time (T_2). The former is a measure of the rate at which energy is transferred from the hydrogen nuclei to other types of energy in the surrounding atoms (the lattice), while the latter is concerned with the dissipation of spin energy to other magnetic nuclei.

Methods

Wheat straw was subjected to the IBUS process in a pilot-plant at Inbicon A/S, Skærbæk, Denmark. The straw was chopped into lengths of up to 5cm and pretreated at 160, 175, 185 and 195°C, for approximately 10 minutes at a water:straw ratio of 5:1, followed by pressing to a dry matter content of approximately 30% (Larsen et al., 2008). In addition, an untreated control sample was collected.

The composition of the straw produced at each temperature was determined using a two-step acid hydrolysis according to the procedure published by the National Renewable Energy Laboratory (NREL) (Sluiter, 2008). Before acid hydrolysis, the samples were dried at 45°C for one day and then ground. The dried samples (~300mg) were treated with 3ml of 72% H_2SO_4 and placed in a water bath at a temperature of 30° C for 60 minutes. The samples were then diluted with 84 ml of Milli-Q water to give a H_2SO_4 concentration of 2.5%. The samples were autoclaved for 1 h at 121°C. After cooling, the hydrolyzate was subsequently filtered, with collection of the insoluble residue. The residues were heated to 500°C to determine volatiles, and the remaining material is reported as Klason lignin. The filtrates were neutralised with $CaCO_3$ to pH 5.6, filtered through a 0.45 µm filter and diluted with eluent, followed by HPLC analyses. The monosaccharides (D-glucose, D-xylose and L-arabinose) released were quantified with a Dionex Summit HPLC system equipped with a Shimadzu RI-detector. The separation was performed with a Phenomenex Rezex RHM column at 80°C, with 5 mM H_2SO_4 as the eluent, at a flow rate of 0.6 ml min^{-1}. Monosaccharide levels were anhydro-corrected to determine the amounts of glucan, xylan and arabinan. Ash content of each straw sample was determined by heat treatment at 500°C for three hours.

To determine the affect of pretreatment temperature on conversion of the polysaccharides, pretreated straw was hydrolyzed using and enzyme loading of 5FPU/g dry matter of Celluclast and Novozyme 188 in a 5:1 ratio. Pretreated straw corresponding to 2.5 g dry matter was added to 50mM sodium-citrate buffer, pH 5.0, and enzyme to yield a total of 50g. The samples were hydrolyzed for 72 hours at 50°C, with shaking at 200 rpm and analyzed for monosaccharides as before. Conversion was calculated as the percentage of glucose released relative to the theoretically possible value, based on the compositional analysis.

ATR-FTIR spectra were collected using a Thermo-Nicolet Nexus 670 FTIR with a Golden Gate MKII single reflection ATR system. Spectra were collected in absorbance mode from 4000 to 650 cm^{-1}, at 4 cm^{-1} resolution, with 32 scans per spectrum. Spectra were

collected in triplicate and averaged. Background spectra were collected in air.

Atomic force microscopy was performed using a Veeco-Dimension 3100 scanning probe microscope with a Nanoscope IIIa controller. Images were acquired in tapping mode using a 1-10 ohm-cm phosporus (n) doped silicon tip with a nominal frequency of 150 kHz. Roughness calculations were performed on the height images in an attempt to quantify structural differences resulting from the thermal treatments.

Given the range of treatments to which the original straw was subjected, the samples were found to be variable in structure which can have a marked effect on the NMR results. In order to isolate this effect a second set of samples was ground on a Wiley mill fitted with a 20 mesh screen. The moisture content of all samples was adjusted by equilibration in a dessicator over deionized water for 10 days. The moisture content of each sample was determined in duplicate by oven drying sub-samples at 105°C.

The NMR experiments were performed with a Bruker-minispec mq20, with a 0.47 tesla permanent magnet, 20MHz proton resonance frequency, operating at 40° C. The transverse (T2) relaxation times were determined using the Carr–Purcell–Meiboom–Gill (CPMG) sequence with a 0.04 ms pulse separation, the collection of 1000 echoes, 32 scans and a 5 second recycle delay. CONTIN (Provencher, 1982), an inverse Laplace transform method, was used to determine distributions of T2 relaxation times from the decay curves.

Results

The composition and conversion levels of the straw are as shown in Table 1 and Figure 1, respectively.

Sample	% Klason lignin		% Glucan		% Xylan		% Arabinan		% Ash	
	Avg	Stddev	Avg	Stddev	Avg	Stddev	Avg	Stddev	Avg	Stddev
Raw straw	22.6	1.0	39.8	2.0	24.5	1.4	2.8	0.2	4.2	0.1
160°C washed	20.4	2.5	40.9	2.2	23.0	1.3	2.0	0.1	2.9	0.2
175°C washed	26.1	0.5	47.8	0.4	18.1	0.2	1.1	0.0	3.3	0.1
185°C washed	30.7	0.4	53.3	0.1	8.5	0.0	0.0	0.0	3.0	0.0
195°C washed	28.7	0.6	56.6	0.3	4.6	0.0	0.0	0.0	3.2	0.0

Table 1. Compositional analysis of wheat straw

It can be seen that there is an increase in the content of cellulose and lignin, coupled with a decrease in hemicellulose, as the pretreatment temperature increases. The convertibility of the material increases sharply as the pretreatment temperature is increased. It is also interesting to note that while relatively small changes in composition are observed at 160° C, in comparison to the untreated control, the conversion process exhibits considerable progress. These results indicate a marked increase in accessibility of the polysaccharides, even under the least severe conditions.

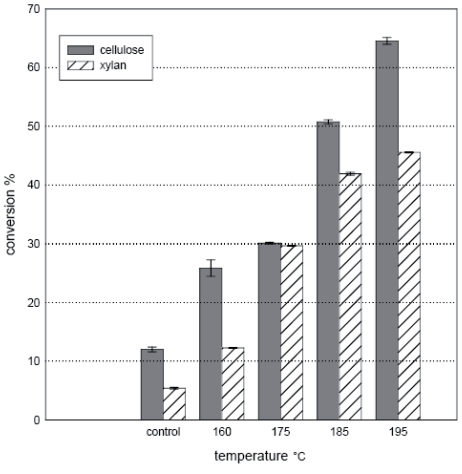

Figure 1. Conversion percentages of cellulose and xylan into simple sugars with temperature. Error bars show two times the standard deviation.

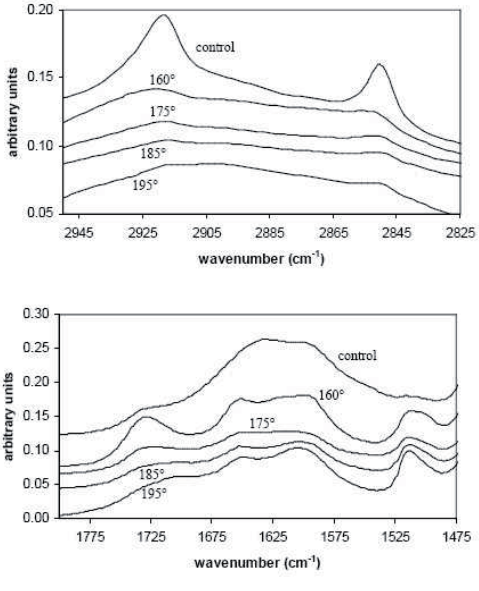

Figure 2. ATR-FTIR results for pretreated wheat straw.

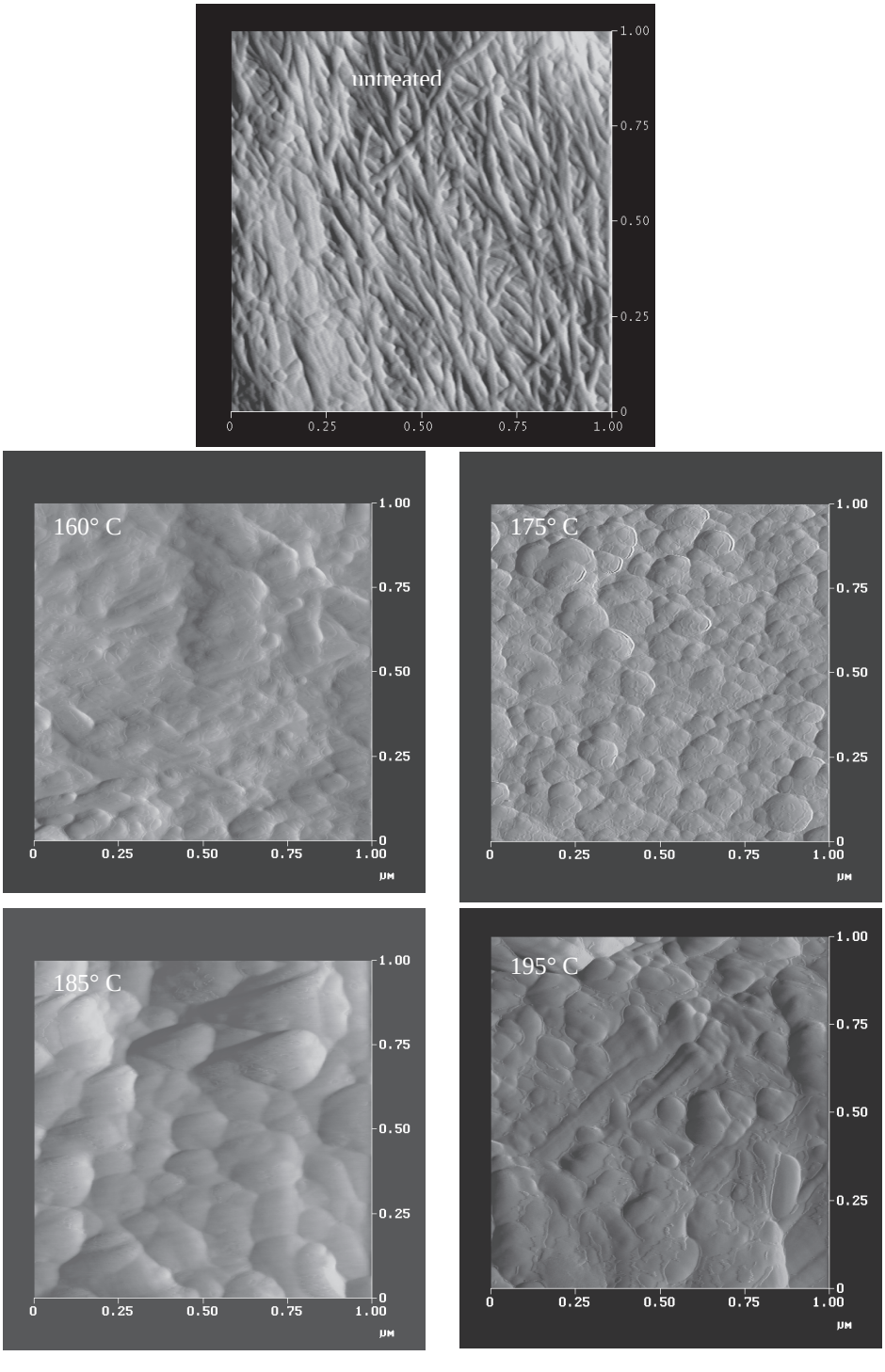

Figure 3. Atomic force microscopy, amplitude images, of pretreated straw.

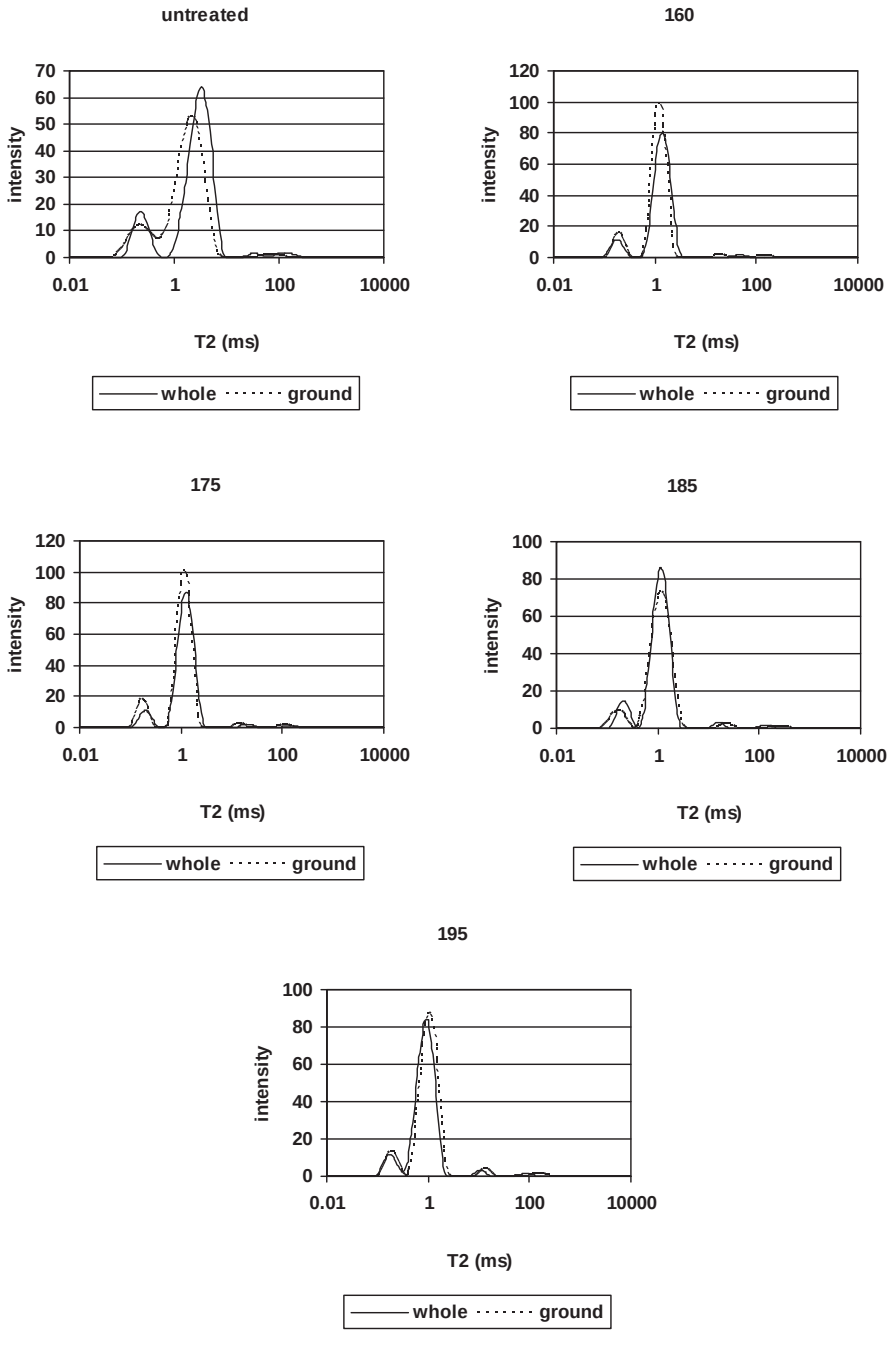

Figure 4. LF-TD-NMR relaxation time distributions of pretreated straw.

ATR-FTIR results are presented in Figure 2. Peaks at 2850 and 2920 cm^{-1} (Figure 0.6a) assigned to CH_2 stretches have been attributed to wax in the straw. It can be seen that these peaks gradually diminish with treatment temperature, indicating wax removal. Figure 0.6b, ranging from 1800 to 1450 cm^{-1}, contains peaks assigned to carbonyls in the hemicelluloses (1735 cm^{-1}) and to ring structures in lignin (1510 and 1595 cm^{-1}). The intensity of the peak at 1735 cm^{-1} increases from the control to 160°C, but the amount of hemicelluloses exhibits only minimal changes (Table 1). At temperatures above 160°C, the intensity of this peak decreases in concert with the reduction in hemicellulose percentage. Given the sensitivity of ATR-FTIR to surface composition, these observations could be due to relocalization of hemicelluloses to the surface. During the pretreatment, hemicelluloses are partially degraded into oligomers, which migrate from the cell wall and into solution. Larger oligomers of hemicellulose, or modified oligomers with decreased arabinosyl and O-acetyl substitution have higher affinity for adsorption to cellulose and will redeposit on the cell wall surface upon cooling, whereas small oligomers will remain in solution (Kabel et al., 2007).

The amplitude of the lignin peak at 1510cm^{-1} also changes systematically, increasing sharply with increased processing temperature, while the lignin percentage (Table 1) of the straw increases only slightly (22.6 for the control and 28.7% at 195°C). Again, these data may be interpreted as evidence of surface redeposition of lignin.

Amplitude images from atomic force microscopy are as shown in Figure 3. As previously reported (Kristensen et al., 2008), fibrils are readily apparent in untreated straw. With thermal treatment the surfaces take on a granular or nodular appearance, interpreted as redeposition of lignin. Especially at the temperatures of 185 and 195°C which contain only about 4-8% xylan, and since no fibrils are apparent, it is logical, as before, to conclude that that lignin is coating the surface of the material. Roughness determinations revealed no consistent differences between temperature treatments, indicating that the mobility of lignin occurs even at 160°C, consistent with the ATR-FTIR results

The T2 distributions from LF-TD-NMR are shown in Figure 4. These distributions are typical of other lignocellulosic material at the fiber saturation point, exhibiting a major peak at 1-3 ms and a minor peak at about 0.3 ms. For the current samples these peaks are generally well-resolved. The exceptions to this observation are the untreated samples, for which the ground samples are less well-resolved, perhaps indicative of an increase in the uniformity of the bound water environment due to grinding. Figure 5 shows the changes in relaxation time, taken at the maximum of the major peak, vs the dry basis moisture content. It can be seen that the moisture content varies considerably and systematically with temperature, such that the higher the treatment temperature the lower the moisture content. This is consistent with the compositional results which report a decrease in hemicellulose percentage (the most hygroscopic of the polymers) and an increase in the lignin percentage (the most hydrophobic of the polymers). In addition, this is in accord with the literature, which indicates a shortening of the main T2 relaxation time peak (1-3ms) as the moisture content decreases below fiber saturation point (Almeida et al., 2007). The relaxation times decrease sharply from the untreated controls to the pre-treated samples, the latter of which although relatively constant exhibit a general decrease with temperature. It can also be readily seen (Figure 4) that there is a difference in the relaxation time of the untreated whole vs. ground straw, while this difference is much smaller for the IBUS treated samples. Additionally, the T2 of the untreated, ground material is similar to the IBUS treated straw. Given that IBUS treatment causes partial disintegration of the material, this similarity in relaxation time may be due to physical compartmentalization of the water, resulting in reduced mobility in both samples.

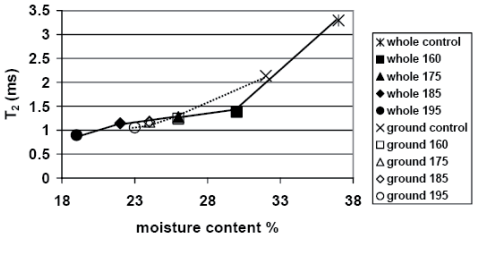

Figure 5. T2 vs. moisture content for pretreated straw.

The large reduction in both T2 and moisture content between the control and the sample treated at 160° C are in contrast to the summative analyses in which only slight changes were found. This may be interpreted in terms of changes in physical structure of the straw that would restrict mobility of the water, chemical modifications of the polymers that are not reflected in the total composition or, as proposed previously, a rearrangement of the polymers that would alter their accessibility towards water. The latter may be supported by the abrupt decrease in moisture content upon treatment. Furthermore, the decrease in

relaxation time as the temperature of treatment increases from 160 to 195° C may be related to either the decrease in moisture content, the more severe disintegration at higher temperatures, or indirectly to the removal of hemicellulose or redeposition of lignin.

Discussion

It is apparent from these observations that the pre-treatment of straw, prior to enzymatic hydrolysis is a complex process controlled by a number of interacting factors. It has been found that the level of conversion of the polymers to simple sugars is enhanced even at the mildest of the pre-treatment conditions examined. Under these conditions, however, there are minimal changes in overall chemical composition. These results would indicate the occurrence of structural changes or chemical modifications to the polymers that are not detected by summative analyses. This interpretation is supported by the ATR-FTIR results that show an increase in surface hemicelluloses, coupled with decreases in both moisture content and relaxation time as measured by LF-TD-NMR. Furthermore, the surface morphology of the pre-treated straw, as seen by atomic force microscopy, is markedly different from the control. Taken together these methods show changes in both location and chemistry of the cell wall constituents, but also illustrate the limitations of bulk analysis in studying the effects of pretreatments of biomaterials such as straw.

As the pre-treatment temperature increases, the conversion level increases in concert with slight increases in the cellulose and lignin percentages and a large decrease in the xylan percentage. The decrease in xylan is consistent with its thermal sensitivity, hygroscopicity, as reflected in the lowered moisture content of the straw, and ATR-FTIR results.

Across all hydrothermal treatments the total lignin content does not change markedly, while results from atomic force microscopy and ATR-FTIR indicate the presence of surface lignin with pre-treatment. This would indicate that lignin removal is not a pre-requisite for effective pre-treatment, but that modification is an important factor. Such modification may also be related to the observed loss of xylans, which have been shown to be covalently bonded to lignin. Xylan removal may therefore contribute to structural and chemical changes to the lignin, thus enhancing conversion of polysaccharides to fermentable sugars.

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