Norwegian University
of Life Sciences

Master's Thesis 2018 60 ECTS

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Enzymatic Conversion of Cotton Textiles

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Master's degree in Chemistry
KBM

Acknowledgements

The present work was carried out at the Department of Chemistry, Biotechnology and Food Science at the Norwegian University of Life Sciences with Dr. Gustav Vaaje-Kolstad, Dr. Lasse Fredriksen and John-Kristian Jameson as my supervisors.

I would like to express my deep gratitude to Dr Gustav Vaaje-Kolstad for accepting the concept of my master's thesis and helping to evolve it into a complete study. I am also deeply thankful to dr. Vincent Eijsink for giving me this opportunity. Dr. Lasse Fredriksen has been supporting me more than anyone. By posing *and* answering my questions frequently, a true researcher's guidance, which I highly appreciate. My third supervisor, John-Kristian Jameson has shared his valuable knowledge with me, which has been particularly helpful through difficult days in the laboratory. My gratitude must also be expressed for experiencing the helpfulness and the impressive expertise on the field from Dr. Piotr Chylenski.

My thankfulness also goes to the whole PEP-group for help, support and for the good time I have had throughout the year.

Ås, May 15th 2018

Abstract

Cotton textiles are mass-produced worldwide, but little is done to recirculate this material that is rich in cellulose. Indeed, cellulosic textiles represent a potential resource for the production of glucose, that further can be converted into chemicals, biofuels, materials, food and food and feed purposes. In this study, investigation of saccharification of denim fabric cellulose pretreated by ball milling showed that reduction in particle size significantly increased the digestibility of the substrate with two commercially available enzyme cocktails, Celluclast® and Cellic® CTec2, the latter containing lytic polysaccharide monooxygenases (LPMOs). Samples of ball milled denim fabric with substrate concentrations of 20 % (w/v dry matter content) resulted in 78 % of the cotton textile into glucose after 96 hours incubation with 4 mg Cellic® CTec2 per g glucan. At 5 % substrate concentration, the respective conversion yields for ball milled white (undyed) and blue (indigo dyed) denim were over 90 % after 24 hours. Activity assays demonstrated that the fungal LPMO *Ta*LPMO9A produced several oxidized oligosaccharides from both denim fabrics studied when pretreated by ball milling. The applied pretreatment in this study seemed to increase the availability for LPMOs. When ascorbic acid was added as an external electron donor to reactions with Cellic® CTec2, no enhancement of cotton textiles hydrolysis was observed. By performing assays with and without ascorbic acid and by comparison with Celluclast®, the LPMO content of Cellic® CTec2 was assessed to not be of significant importance for the efficient degradation of ball milled cotton textiles. In conclusion, the present work shows that cotton textiles can be efficiently converted to glucose by simple mechanical pretreatment followed by enzymatic hydrolysis using conditions applicable to commonly industrial processes.

Further research is needed to investigate the efficiency of the method in a biorefinery scale and to assess the financial feasibility of the method developed in this master's thesis.

Sammendrag

Bomullstekstiler er masseprodusert i hele verden, men lite gjøres for å resirkulere dette cellulose-rike materialet. Cellulosebaserte tekstiler representerer en potensiell ressurs for produksjon av glukose, som videre kan konverteres til kjemikalier, biodrivstoff, materialer samt mat- og fôr-relaterte produkter. I denne studien viste undersøkelser av hydrolyse av cellulose fra dongeri-tekstil forbehandlet med ballmølling at reduksjon av partikkelstørrelse i betydelig grad økte tilgjengeligheten av substratet for to kommersielt tilgjengelige varianter enzym-cocktail, Celluclast® and Cellic® CTec2, hvorav sistnevnte inneholder lytisk polysakkarid-monooksygenaser (LPMOer). Prøver av ballmøllet dongeri-tekstil med substrat-konsentrasjon 20 % (vekt/volum tørrstoff) resulterte i at 78 % av bomullstekstilet ble konvertert til glukose etter 96 timer inkubering med 4 mg Cellic® CTec2 per gram glukan. Ved 5 % substratkonsentrasjon, ble utbytte for ballmøllet hvit (ufarget) og blå (indgio-farget) dongeri over 90 % etter 24 timer. Aktivitet-analyse viste at den fungale LPMOen, *Ta*LPMO9A produserte flere oksiderte oligosakkarider fra begge dongeri-tekstilene dersom de var ballmøllet først. Den benyttede forbehandlingen øker tilsynelatende tilgjengeligheten av substratene for LPMOer. Ved tilsetning av askorbinsyre som elektrondonor til reaksjoner med Cellic® CTec2, ble ikke økning av hydrolyse av bomullstekstiler observert. Ved reaksjoner med og uten askorbinsyre, samt sammenligning med Celluclast®, ble LPMO-innholdet i Cellic® CTec2 vurdert til å ikke være av signifikant betydning for effektiv nedbrytning av ballmøllet bomullstekstiler. Arbeidet som her blir presentert viser at bomullstekstiler effektivt kan konverteres til glukose ved enkel mekanisk forbehandling etterfulgt av enzymatisk hydrolyse under betingelser som er kompatible med vanlige industrielle prosesser.

Videre forskning er nødvendig for å undersøke effektiviteten av metoden på bioraffineri-skala samt vurdere finansiell gjennomførbarhet av metoden som ble utviklet i denne masteroppgaven

Abbreviations

DP	Degree of polymerisation
BBD	Ballmilled BD
BD	Blue cotton from denim fabric
BG	β-glucosidase
BWD	Ballmilled WD
CBH	Cellobiohydrolase
CBM	Carbohydrate binding module
DF	Dilution factor
DM	Dry matter content
DPA	Days post-anthesis
EG	Endoglucanase
GH	Glycoside hydrolase
Glc4gem(Glc_{DP-1})	Oligosaccharide with a geminal diol functional group at C-4
Glc4K(Glc_{DP-1})	Oligosaccharide with a ketoaldose functional groups at C-4
(Glc_{DP-1})Glc1A	Oligosaccharide with an aldonic acid functional group at C-1
HPAEC	High-performance anion exchange chromatography
HPLC	High-performance liquid chromatography
hrs	Hours
IEC	Ion exclusion chromatography
IEX	Ioon exchange chromatography
K	Partition coefficient
LPMO	Lytic polysaccharide monooxygenase
MALDI-TOF	Matrix assisted laser desorption ionization - time of flight
NMMO	N-methyl morpholine oxide
PAD	Pulsed amperometric detection
PASC	Phosphoric acid swollen cellulose
PES	Polyethersulfone
SRS	Sugar recovery standard
t	time
tR	Retention time
VR	Retention volume
w/v	Weight/volume
w/w	Weight/weight
WD	White cotton from denim fabric

Contents

1. Introduction

1.1. Biomass as an energy source

Cellulosic biomass is a potential resource for production of glucose that further can be used to produce energy, chemicals and materials. As an important part of replacing fossil fuels with renewable alternatives, there is a demand for renewable sources than can be processed into biofuels and chemicals, and various sources of biomass with high amounts of cellulose have therefore been in focus for finding methods of efficient sugar production. Biofuel production is commonly classified according to the applied resource used. Biomass with high content of carbohydrates that demands none or little pretreatment before the fermentation into ethanol define the first-generation biofuels. Most of the crops that fit into this category are edible sugar- or starch-based plants. The second-generation biofuels are produced from by-products and residues from agricultural and material industry and municipal waste. Conversion of this type of biomass to sugars is usually performed in several steps, where pretreatment increases the availability of the polymers of interest before enzymatic hydrolysis reactions degrade the polymers into fermentable sugars.

The chemical composition of the relevant substrates varies depending on the source. Cellulose is the most abundant of all organic polymers (Klemm et al. 2005). In most cases the main component of biomass is a network of cellulose fibres in combination with hemicellulose and lignin. In order to increase the availability and digestibility of cellulose from a biomass source, there has been major focus on pretreatment of the material and on optimizing the enzymatic degradation. For various substrates, methods are adjusted differently for achieving a high conversion. By using biomass for bioenergy and biochemical production, biorefineries could ideally contribute to reducing fossil CO_2 emissions, securing the energy supply and consuming as small amounts of water, agricultural area and other resources as possible (Cherubini 2010).

1.2. Cotton

Cotton textiles are mass-produced and highly consumed worldwide. In 2017, the global production of cotton reached 29,3 million tonnes according to the National Cotton Council of America. Reports from the US and Norway indicate that 64,5 % and 80 % of used textiles end up in landfills in the two countries, respectively (EPA 2016; Urdahl 2011). Cotton textiles discarded from homes and retailers are in many cases reused or reprocessed, usually by reselling or recycling into carpets, wipes, insulation and upholstery filling for furniture. Some clothing companies substitute virgin cotton fibres in the spinning or the weaving process of cotton textile manufacturing. These recycling methods consequently reduce the fibre quality, being the strength and length of the fibres, and is not majorly in use. Alternatively, or

at a later point of time, the textiles are disposed to landfills. Biomass in landfills, involving cellulosic textiles, contribute to the growth of different types of microorganisms that hydrolyse the biomass, convert the products further and perform methanogenesis. The final products are described unified as landfill gas and contains approximately 50 % methane and 45 % carbon dioxide (Themelis & Ulloa 2007). Several methods are applied for methane capture in landfills that allow methanogenesis, in order to reduce the emission of this greenhouse gas and to utilize the potential of methane (e.g. for burning to yield energy) rather than allowing the gas to be released in the atmosphere. Despite the efforts, reports show low capture capacity in most landfills (Themelis & Ulloa 2007; Timoney 2010). Another solution to the greenhouse gas emission issue is to avoid biomass deposit in landfills, applied by Norway (Forurensningsloven 1981) among other countries, where incineration currently is the main alternative (Schmidt 2016). Cotton production requires significant amounts of energy, water, chemicals and agricultural area. Reuse of the textiles or recycling of the fibres, further described as recycling, is assessed by several investigators to be ecologically and economically beneficial, while incineration of cotton recovers only a small portion of the energy originally required for the production (Schmidt 2016). Therefore, new ways of recycling cotton waste as a resource are needed.

1.2.1. Cotton fibres

The plant species that provide cotton belong to the genus *Gossypium*, and the species *G. hirsutum* represents the majority of cotton production (Wakelyn et al. 2006). Cotton grows in several cell wall layers that have different function and chemical composition, shown in the schematic illustration in Figure 1.2.1. The cuticle is the outer, protective layer, containing waxes, pectins, proteins, ions and non-cellulosic polysaccharides (Gordon & Hsieh 2006). The earliest stage of cellulose synthesis in cotton occurs in the primary wall development, 13-17 days after blooming (DPA) for *G. hirsutum*. The primary wall synthesises nearly 20 % cellulose (Lee et al. 2015), along with pectins, proteins, ions and non-cellulosic polymers. The winding layer (18-22 DPA) is an intermediate layer between the primary and secondary wall and contains approximately 20 %, and at the final stage of the secondary wall synthesis (45 DPA), over 90 % of the dry weight is cellulose (Haigler et al. 2012; Lee et al. 2015). At this stage the boll of the plant opens naturally (Figure 1.2) and

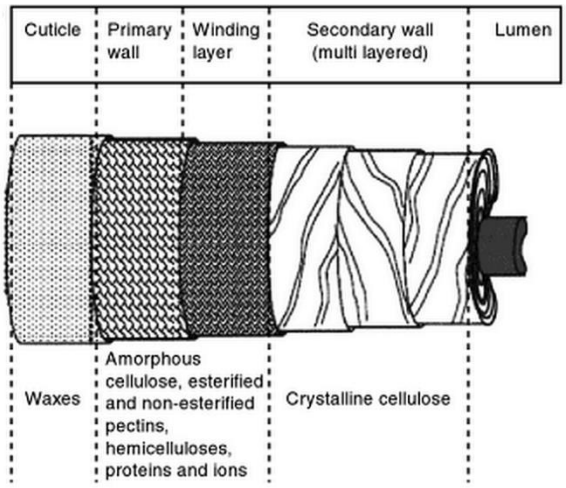


Figure 1.2.1. Cotton morphology. Cotton fibres are growing as several cell wall layers elongating around each seed. Cellulose content is low during the early growth stage represented by the primary wall and the winding layer and increases rapidly when the secondary wall layers develop and dries.

dries quickly to a water content of 3-5 % (Lee et al. 2015), ready for dispersing the cotton plant seeds. Botanically, cotton fibres are extensions of the cotton seed epidermis (Haigler et al. 2012), developed for secretion of metabolic compounds, exchange of water and carbon dioxide with the environment and protection (Beck 2010). The fibres are harvested as open bolls, and several processing steps are required in order to remove seeds and cuticle. This process is described in detail in section 1.3.

Picture 1.2. Mature cotton boll. When the epidermal fibres are fully developed, the white lint dries, and the fibres protect the seed when naturally dispersed. Cotton harvesting for textile productions occurs between drying and seed dispersion.

1.2.2. Structure and properties of cellulose

The biomass that remains in the fibres after processing is called *lint* and represents the fibrous material that is used for textile production. Lint mainly contains the long cellulose fibres from the secondary wall (Gordon & Hsieh 2006). The following sections will give an overview of the structure of cotton by describing the current knowledge on the structure of cellulose, the strands' superstructure in elementary fibrils and the packing of the elementary fibrils into microfibrils.

Cellulose is composed of the hexose D-glucopyranose (anhydrous glucose) covalently linked by β-1,4-glycosidic bonds. A glycosidic bond has an estimated half-life of nearly 5 million years when uncatalyzed, given 25°C and pH=7-14 (Wolfenden et al. 1998). The numbering of the glucose carbon atoms is assigned according to the IUPAC system, counting from the carbon of the chain with a functional group, the ether group. Because every other glucose unit is rotated by 180°, cellobiose is the repeating unit in cellulose, shown in Figure 1.2.2. On the C1-end of cellulose polymers, the anomeric

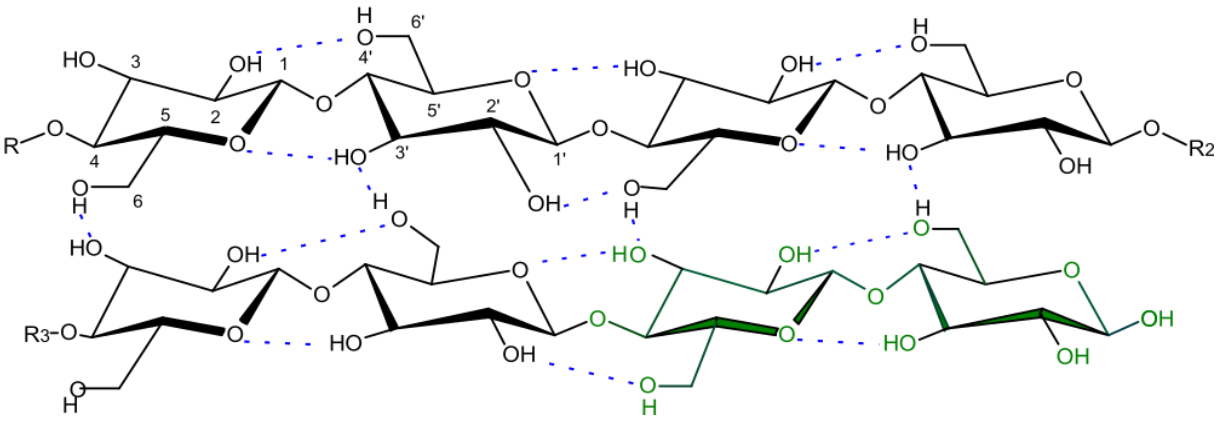


Figure 1.2.2.1:. Cellulose structure. Molecular structure of cellulose showing intra- and inter-molecular hydrogen bonds (blue). Cellobiose (green) is a dimer of β-D-glucopyranose and is the repeating unit in cellulose. (Albersheim et al. 2010; Baptista 2013; Pinkert et al. 2009)

hydroxyl constitutes a reducing end where closed keto-hexose and the open aldo-hexose are in equilibrium. The other end of the polymer (C4) is non-reducing. As illustrated in Figure 1.2.2, hydrogen bonds exist internally in the polymer chains, but also exist between chains, creating an intramolecular linkage between individual cellulose strands. When approximately 36 cellulose strands are agglomerated, the resulting bundle is called *nanocellulose*, or *elementary fibrils*, which are further packed in *microfibrils* with a diameter of 5-50 nm and a length of several micrometres (Sofla et al. 2016).

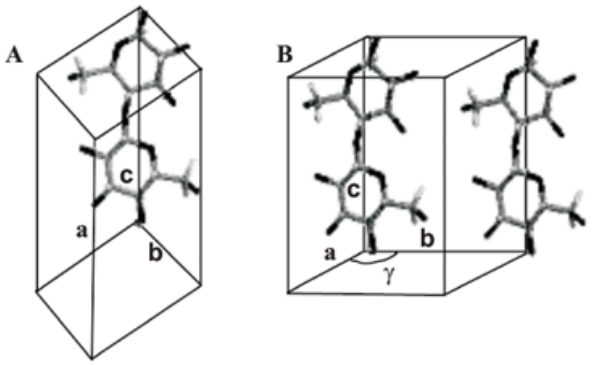


Figure 1.2.2.2: Schematic representation of cellulose I_α (A) and I_β (B). Native crystalline cellulose (type I) has a triclinic (A) or monoclinic (B) structure, resulting in conformational differences (Koyama et al. 1997). Copyright (2006) The National Academy of Sciences of the United States of America.

The network of hydrogen bonds is the structural cause of crystallinity in cellulose fibres. Cellulose crystallinity is a parameter for the orientation of the strands, which determines the degree of order in the packaging of strands in the fibres. Regions with low ordered orientation of the strands are described as amorphous (Kljun et al. 2011). Crystalline structures of native cellulose are classified as I_α and I_β (allomorphs). Native cotton fibres are 88,0 - 96,5 % I_α-cellulose. (Gordon & Hsieh 2006) The coordination angles in I_α-cellulose (Figure 1.2.2.2A), result in a shorter *intra*-chain hydrogen bond between the hydroxyl hydrogen on C2' and the hydroxyl oxygen on C6, relative to I_β-cellulose, which is the dominant type in higher plants (Festucci-Buselli et al. 2007). This results in denser packaging of the microfibrils. Furthermore, the allomorph I_α is less heat stable and can be converted into I_β by heating (J. Hardy & Sarko 1996). In cotton fibres, the degree of polymerisation is 800 to 10 000 glucose units long (Klemm et al. 2005). The polymer length of the cellulose strands in cotton textiles depends on the treatment of the fibres.

Furthermore, the cellulose crystal type (polymorph) can change by certain treatment methods. It is known that native cellulose can undergo intermolecular hydrogen bond breakage causing changes the crystal structure from type I into type II. The two processes that can cause this change are regeneration, where the fibres are re-precipitated after solubilization in a solvent, and mercerization, where the fibres are swelled by alkaline solutions (discussed further in section 1.3.3) (O'Sullivan 1997). Polymorph II is known to be thermodynamically more stable than polymorph I because the strands are anti-parallel, i.e. the reducing and non-reducing end point in alternate directions in the chain (Festucci-Buselli et al. 2007; Kljun et al. 2011). The crystal structure is monoclinic as for type I_β, while *inter*-chain hydrogen bonds between hydroxy groups on C3 and C6 (Figure 1.2.2.1) are replaced by *inter*-chain hydrogen bonds between the hydroxy groups on C2 and C6 in cellulose II (Festucci-Buselli et al. 2007). Amorphous regions are generally not believed to occur in cellulose II (O'Sullivan 1997). The digestibility of cellulose I and II by cellulases is introduced in section 1.4.3.

1.3. Cotton textiles and denim

The cotton textile industry requires certain standards regarding the lint fibre quality, and breeding of cotton has improved the fibre strength and fibre length since the 1980's (Smith & Cothren 1999). A common and representative example of cotton fabrics is denim, for which a high quality of cotton fibres is required. Since denim fabric represent the main substrate used in the present MSc project, a short overview of the processing steps that have an impact on structure and composition of the cotton cellulose will be described. A schematic flow chart illustrating the relevant processes is shown in Figure 1.3.

1.3.1. Cleaning, combing and spinning

Initially, the lint is cleaned and separated from the seeds in a process called ginning. The processes involved are moisture adjustment, seed-fibre separation and removal of particulate impurities. The remaining fibres are 95 % cellulose (Lewin & Pearce 1998). The next step is carding and combing, which are techniques of physical combing of the fibres that ensures separation of the fibres, appropriate fibre length and fibre orientation (Paul 2015). Further processing of the fibres into denim might vary slightly, although the essential steps seem to be consistent among manufacturers. When the yarn is spun, the twist, thickness, regularity and weight of the final thread is determined. There are several types of

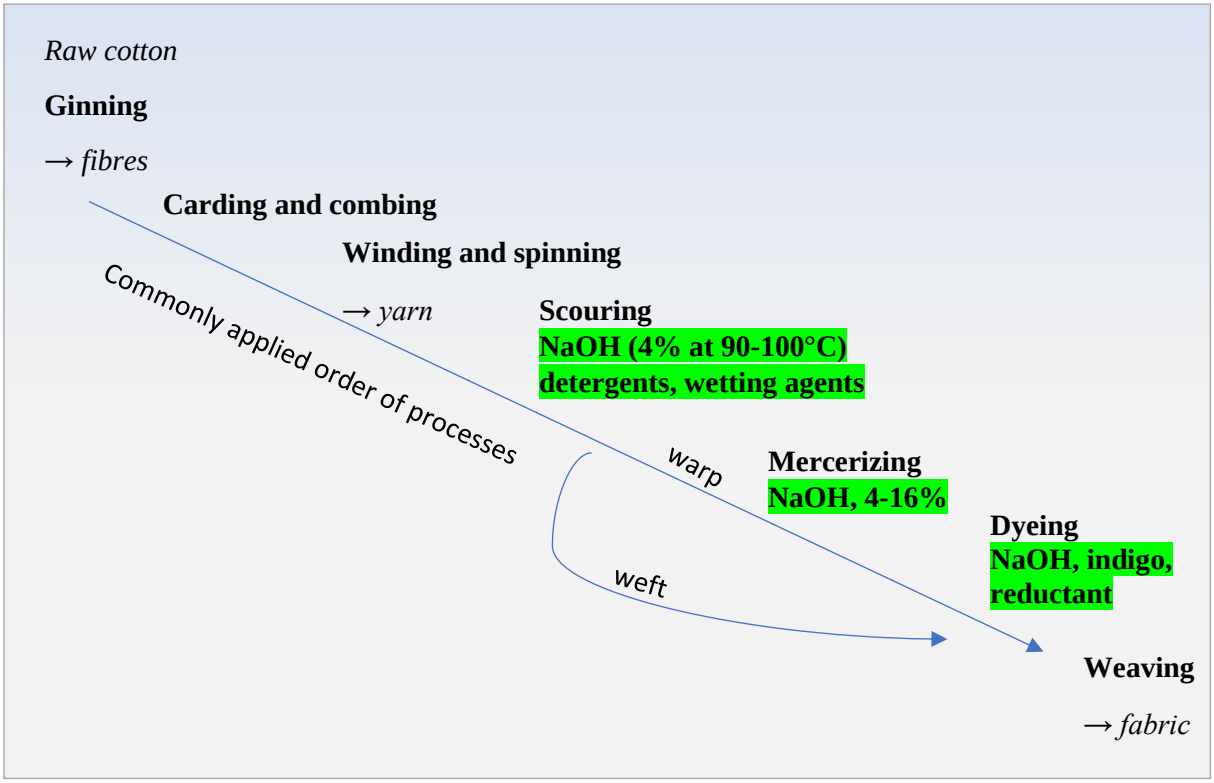


Figure 1.3. Flow chart of denim processing steps that influence cellulosic composition, structure and superstructure. The manufacturing of denim from raw cotton involves many steps, where details and order of processes vary. This schematic flow chart's purpose is to give an overview of processes cotton fibres generally go through in denim production that cause compositional and/or structural changes.

spinning methods used for denim manufacturing, and properties that might vary according to the spinning are volume and absorption capacity of the resulting threads, among others. Denim threads are winded, to finally yield a length between 97 to 180 cm (Clariant 2012) and spun to yarn. The final twist in spun yarn influences the elasticity and the resistance of the yarn. When determining the twist of the yarn, it is first divided into warp and weft, which have different roles in weaving of the fabric. Warp are threads that establish the frame of the fabric during the weaving, and they need to be resistant because of high tension during the process. Weft yarn are threads interlaced crosswise to the warp, filling the space between the established frame of the warp. In denim fabric, yarn with high strength might be of interest for some garments, while high elasticity is the priority for others. If most fibres of the yarn have the same orientation, adsorption capacity decreases, as well as denser packing of the fibres (Clariant 2012).

1.3.2. Scouring

In denim production, scouring is carried out before bleaching and dyeing of the yarn (warp and weft). For removal of waxes and other unwanted compounds from the yarn, the scouring process is traditionally applied and still predominant, although recently developed enzymatic scouring methods exist. Alkaline treatment containing approximately 1 M (4 % w/w) sodium hydroxide at 90-100°C result in saponification that solubilize lipids from the cuticle and primary wall (Chakraborty & Ledwani 2017). Also wetting agents and detergents are applied to solubilize and emulsify other impurities. The threads are rinsed well, and in most cases bleached with hydrogen peroxide or sodium hypochlorite which destroys natural colour compounds.

1.3.3. Mercerization

Mercerization is a process used for obtaining higher colour quality of dyed textiles and improving the appearance of the fibres. An aqueous solution of sodium hydroxide is used to swell the cellulose fibres at cold temperatures. The native crystal structure is first dissolved to amorphous cellulose by intermediate derivation to cellulose xanthate without inter-chain hydrogen bonding, whereas upon the removal of the swelling agent the cellulose adapts the structure of cellulose II (O'Sullivan 1997). Information about the amount of fibres undergoing this crystallinity change is not available, yet it is known to vary according to yarn differences. Different quality of the yarn makes it necessary to adjust the alkalinity concentration, although the concentration should generally give a liquid density of 6-22° Bè (Clariant 2012), which corresponds to 4 - 16 % (w/w) NaOH (Oxychem 2018). The yarn is in contact with the alkali for 30 to 60 seconds (Clariant 2012). Afterwards, the threads go through thorough rinsing for removing all alkali.

1.3.4. Dyeing and weaving

For dyed denim, the yarns are arranged in groups of rope-formation or flat sheets for dyeing. Either thiourea dioxide, $(NH)(NH_2)CSO_2H$ (*s*), or sodium hydrosulphite, $NaSO_2SO_2Na$(*s*), is applied as reductant in the dye vat where the yarn rope or sheet are coloured. Dilute sodium hydroxide is present in the vat in order to increase the pH of the vat enough to allow reduction to occur. After the fibres have absorbed the dye, oxidation of the fabric is conducted in order to ensure permanent colour. This is commonly done by allowing the fibres air access for a sufficient amount of time (Clariant 2012).

Sizing agents are utilised in the weaving of denim fabrics. The objectives are protecting the yarn from friction, increasing resistance to traction, separate the warp from agglomerating, increase tensile strength and prevent dust cluster formation. Chemicals applied as sizing agents are completely removed from the fabric after the weaving process.

Figure 1.3.4.2. Denim fabric. Warp (blue) and weft (white) are yarn spun from cotton, pretreated with and without dyeing, respectively, and woven to denim fabric (Clariant 2012).

1.4. Saccharification of cotton

1.4.1. Cleavage of o-glycosidic bonds

Several strategies can be used to degrade cellulose into cello-oligomers and glucose. Cleavage of glyosidic bonds by acid hydrolysis involves nucleophilic substitution and the addition of H_2O to the glucopyranose units on the anomeric side of the cleavage (Figure 1.4.1.1). Firstly, a water molecule is protonated by the acid to form a hydronium ion (H^+), which protonates the oxygen in the glycosidic bond (Lelekakis et al. 2014). The deprotonated hydronium attacks the anomeric carbon in a nucleophilic substitution, and the hydroxyl on the C4 created in the first step is the leaving group (Bochkov et al. 2016). This caus**es** the cleavage of the C-O-bond and leaves both products hydrated. The net consumption in the reaction is one water molecule, while the hydronium concentration remains unchanged. Temperature and pH play major roles in the reaction kinetics. At high temperatures and a sufficiently low pH, all glycosidic bonds will be cleaved (Sluiter 2012).

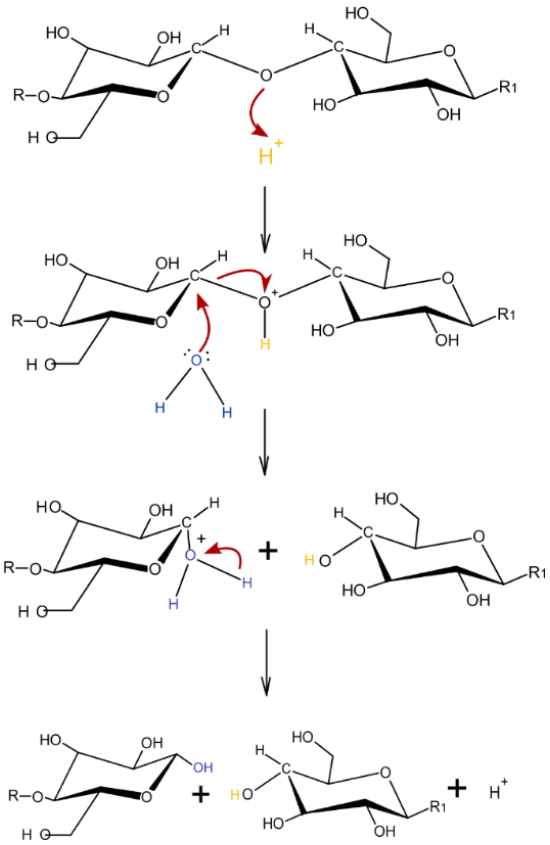


Figure 1.4.1.1: The mechanism of acid hydrolysis of cellulose. Degradation of cellulose by acid is initiated by protonation of the oxygen in the glycosidic bond. The formation of a protonated intermediate is followed by a nucleophilic attack by H_2O on the anomeric carbon. Cellulase hydrolysis proceeds through the same reaction principles.

Cellulose can also be degraded enzymatically through the action of cellulolytic enzymes such as cellulases and LPMOs. Cellulases are astoundingly efficient catalysts, able to increase the rate of glycosidic bond hydrolysis by a factor up to 10^{17} (Wolfenden et al. 1998). The chemistry of cellulases, also referred to as glycoside hydrolases (GH), involves two main mechanisms (Figure 1.4.1.2). The principle of the reaction is in any case the acid catalysis. When the reaction is catalysed by a cellulase, the protonation of the oxygen is provided by an acidic residue. In one of the mechanisms, both the protonation of the oxygen and nucleophilic attack on the anomeric carbon are initiated by acidic residues from the cellulase. A substrate-enzyme intermediate is formed with the nucleophilic residue. A water molecule is activated by the deprotonated catalytic acid residue and attacks the anomeric carbon which causes the cleavage of the substrate-enzyme bond. In the other mechanism, two catalytic residues act as acid and base, respectively. The base residue activates a water molecule which does the nucleophilic attack on the anomeric carbon, and the acid residue protonates the non-reducing end. The two mechanisms give retention or inversion of the anomeric configuration, resulting in a remained equatorial or an inverted axial hydroxyl group, respectively (Davies & Henrissat 1995; Payne et al. 2015).

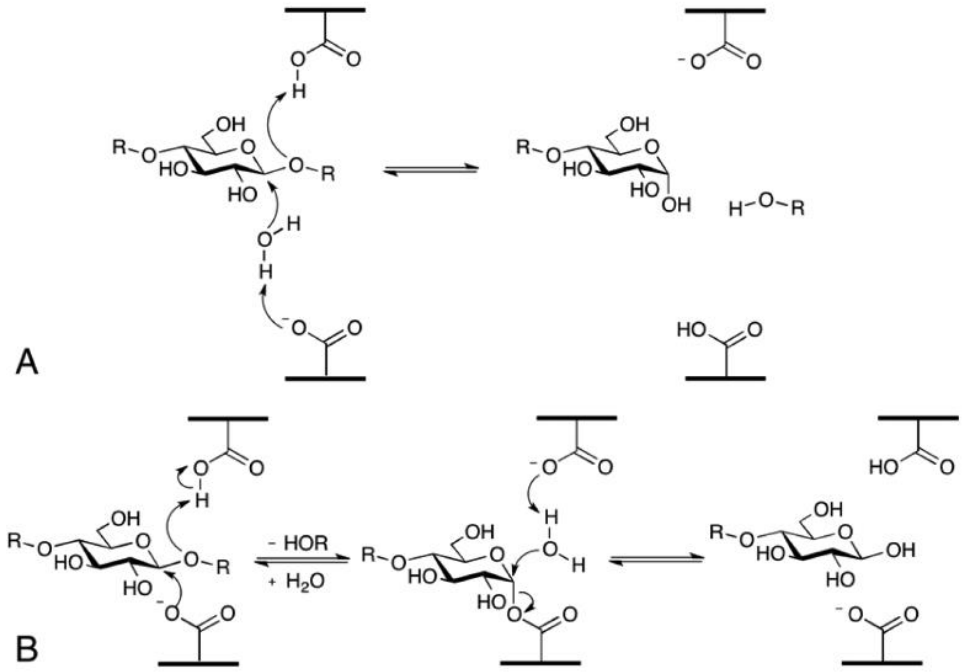


Figure 1.4.1.2. The mechanism of Cellulases. Both mechanisms are initiated by deprotonation of an acidic residue (top). In the inverting GH mechanism (A), a catalytic residues act as a base (bottom), which activates a water molecule that does the nucleophilic attack on the anomeric carbon, before the acid residue protonates the non-reducing end(Payne et al. 2015). The retaining GH mechanism (B) form a substrate-enzyme intermediate with the nucleophilic residue (bottom). A water molecule is activated by the deprotonated catalytic acid residue and attacks the anomeric carbon which causes the cleavage of the substrate-enzyme bond. The figure is obtained from Payne et al. (2015).

1.4.2. Cellulose degrading enzymes

Cellulose is estimated to be the most abundant polymer on Earth (Klemm et al. 2005) and is known to be a feedstock for a variety of microorganisms. Cellulolytic microorganisms exist within the domains of fungi, bacteria and protozoa found in ecosystems such as the sea, fresh water sediments, soil, compost as well as the digestive tracts of ruminants and termites (Cragg et al. 2015). For efficient cellulase production, fungi are preferred, as they are more versatile and inherit better penetration ability, allthough one fungus does not generally produce all the cellulases necessary for effective biomass hydrolysis. (Srivastava et al. 2018)

One of the most important fungi for enzymes applied for cellulose degradation is *Trichoderma reesei*, well known for secreting large amounts of cellulolytic enzymes (Ivanova et al. 2017). The capacity of T. *reseei* (recently re-classified to *Hypocrea jecorina*) to degrade cellulose was firstly discovered by the U.S. Army during the second world war, degrading cotton fabric of their troops' tents. (Reese 1976). With the wild strain called QM6a as a starting point, strains have been developed progressively by modern genetic engineering.

For an efficient enzymatic degradation of cellulose, three classes of cellulases are commonly used. Figure 1.4.2.1 shows an overview of synergistic cellulases acting on cellulosic biomass. Endo-1,4- β-glucanases (EG) catalyse the hydrolysis of glycosidic bonds at random internal positions along the cellulose chain. Amorphous parts of the fibres might be cleaved into shorter polymers and later be further depolymerized into cellobiose and glucose. For cleaving off the terminal glucopyranose units, the relevant enzymes are exo-1,4- β-glucanases, which also are known as cellobiohydrolases (CBH) since their dominant product is **cellobio**se. Figure 1.4.2.1 illustrates CBHs acting on non-reducing (CBH2) and reducing ends (CBH1). The reaction catalysed by EGs and CBHs is show in Equation 1.4.2.1

The main product from endo- and exo-cleavage is cellobiose, released from reducing and non-reducing ends of the polymers. A glucose-producing family of enzymes, namely the β-glucosidases (BG), catalyse the hydrolysis of cellobiose to glucose, as well as cleaving off glucopyranose units at the non-reducing ends of oligosaccharides. The reaction catalysed by BGs is shown in Equation 1.4.2.2 and Figure 1.4.2.1 illustrates how BGs cleave cellobiose provided by other enzymes functioning as synergy partners.

Equation 1.4.2.1.

$$(C_5H_{10}O_5)_n + H_2O \rightarrow (C_5H_{10}O_5)_{n-2} + C_{12}H_{22}O_{11}$$

Equation 1.4.2.2.

$$C_{12}H_{22}O_{11} + H_2O \rightarrow 2C_6H_{12}O_6$$

Most known cellulases consist of one catalytic domain and one carbohydrate-binding module (CBM), although there are examples on efficient cellulases without the CBM (Várnai et al. 2014). The CBM is non-catalytic and binds either amorphous or crystalline cellulose, illustrated in Figure 1.4.2.1 as domains on CBH.

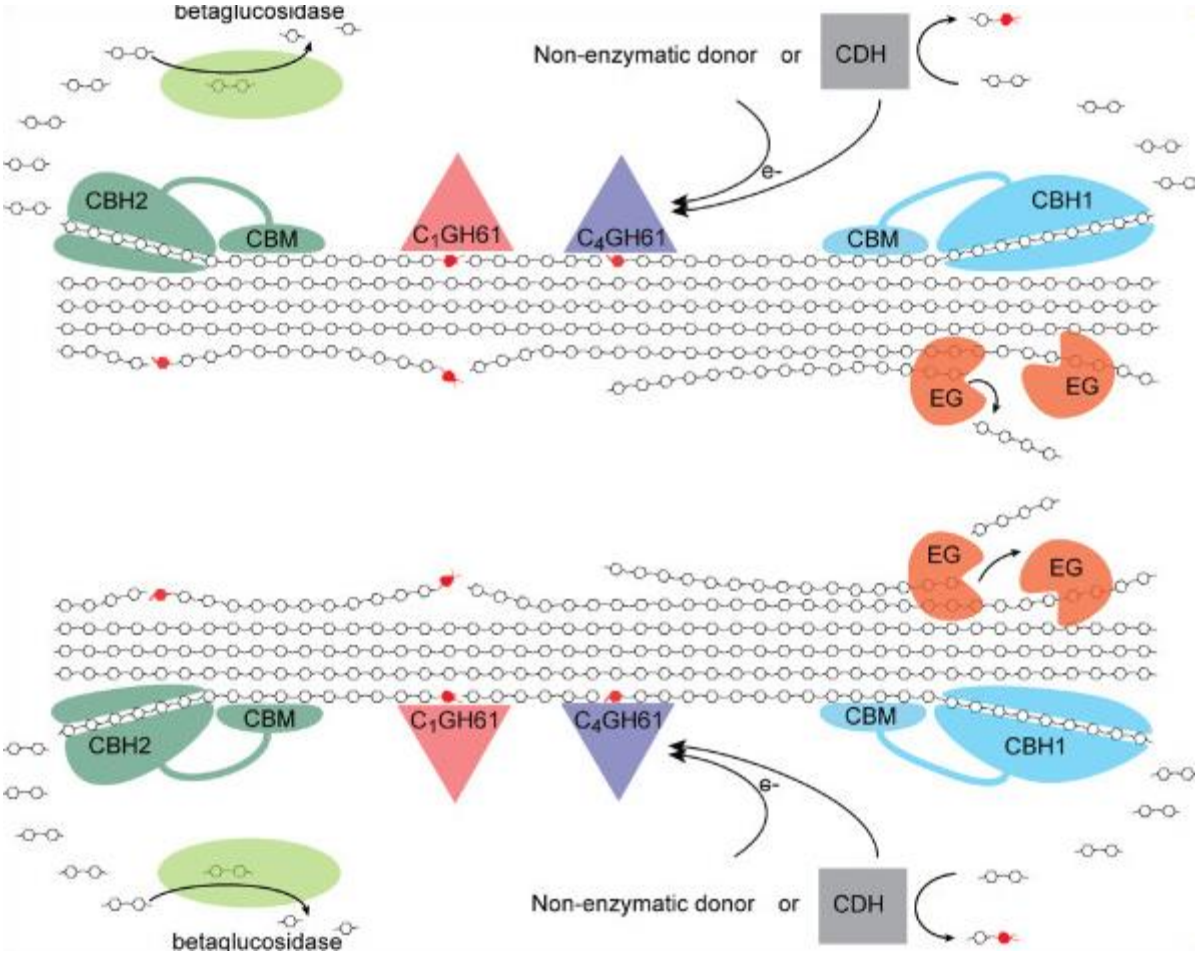


Figure 1.4.2.1. An overview of the classic model of enzymatic cellulose degradation. Conversion of a crystalline microfibril of cellulose into glucose by glycosidic hydrolases and LPMOs (here entitled according to their previous family, GH61) synergistically degrading at the location of their respective specificity. Figure obtained from Horn et al. (2012).

A recent addition to the group of enzymes depolymerizing cellulose is the family known as the lytic polysaccharide monooxygenases, LPMOs (Phillips et al. 2011; Quinlan et al. 2011; Vaaje-Kolstad et al. 2010). Certain LPMOs were previously classified in the family of glycoside hydrolases and are illustrated in Figure 1.4.2.1 as GH61. The redox-active enzymes catalyse oxidative cleavage of glycosidic bonds and are believed to primarily act on crystalline areas of the substrate (Forsberg et al. 2018; Villares et al. 2017). While cellulases have been commercially available for over 30 years, and are utilized in the industry for producing paper, textiles, food and detergents among other, intense research for new utilisation is still ongoing within many sectors. Optimising methods for degradation of biomass from different sources is subject to investigation by commercial actors as well as stakeholders working for more ecological friendly energy production (Kuhad et al. 2011).

Since the discovery of LPMOs in 2010 (Vaaje-Kolstad et al. 2010), new knowledge about the enzyme class and about individual LPMOs continuously lead to increased understanding of mechanism and

effect in their appliance. Their flat substrate-binding surface allows them to interact with the likewise flat surface of the crystalline polysaccharides where they use an oxidative mechanism (the net reaction is showed in Figure 1.4.2.2) dependent on copper ions, an electron donor and dissolved dioxygen as a co-substrate (Horn et al. 2012). When used in laboratory experiments the electron supply for LPMO is commonly provided by addition of ascorbic acid to LPMO reactions, which donates electrons from the hydroxyl groups on the 3,4-dihydroxyfuranone in order to reduce the copper ion and thereby allow activation of dioxygen. Intriguingly, recent studies suggest that H_2O_2 is the preferred co-substrate of LPMOs (Bissaro et al. 2017). Furthermore, details on the radical-involving mechanisms and intermediates formed by the catalyst are not fully understood, and several pathways are suggested (Chylenski 2017).

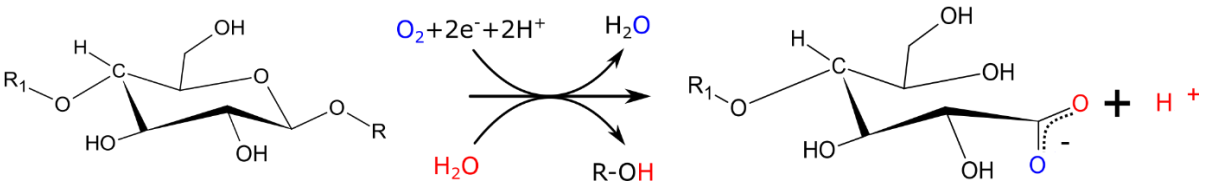


Figure 1.4.2.2: Scheme for the enzymatic reaction catalysed by LPMOs. The figure shows the net reaction of C1-oxidation of cellulose and reduction of molecular oxygen cleaves a glycosidic bond. Molecular oxygen and water provides an oxygen each to the anomeric carbon. Several detailed mechanism pathways have been suggested, differencing in enzyme-substrate-intermediates, radical formation and preferred co-substrate. Mechanism obtained from Horn et al. 2012.

While products from a cleavage catalysed by cellulases remain mono-, oligo- or poly-saccharides with one hydroxyl or carbonyl at the anomeric C1 and a hydroxyl on C4, the products from oxidative cleavage have other functional groups (Figure 1.4.2.3). If C1 is oxidized, lactones and aldonic acids are produced and exist in an equilibrium. Likewise, oxidation at C4 gives keto aldoses and geminal diols in an equilibrium. For the C1-oxidized products, the equilibrium if shifted to the right at a neutral pH, while the C4-oxidized products will both be present in considerable amounts. (Chylenski et al. 2017b; Villares et al. 2017). Lactones are not discussed further in this study.

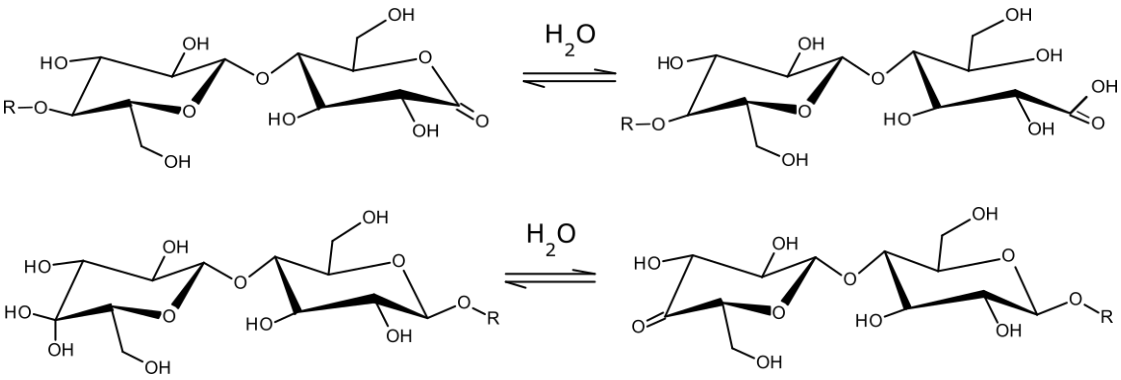


Figure 1.4.2.3. Products of LPMO-catalysed cellulose cleavage. The C1-oxidation of cellulose produces a lactone and an aldonic acid in equilibrium, while the C4-oxidation gives a geminal diol and a keto aldose in equilibrium (Loose et al. 2014).

The recalcitrance and crystallinity of most cellulose-containing biomass is a major challenge for degradation. LPMO activity has been shown to contribute positively in digestion reactions where high degree of crystallinity causes low digestion by cellulases (Chylenski et al. 2017b; Forsberg et al. 2011). Several commercial enzyme cocktails produced for conversion of polymeric biomass into monosaccharides therefore contain one or several LPMOs. It has been estimated that Cellic® CTec2, a commercially available cellulolytic enzyme cocktail (Novozymes Inc.) contains approximately 15 % LPMOs (Müller et al. 2015). Costs is another challenge commonly agreed to be an important threshold for applying biomass degradation in production of energy and chemicals. (Obeng et al. 2017)

1.4.3. Pretreatment

The use of various pretreatment methods has been shown to significantly enhance the enzymatic degradation of cellulose. Lignocellulosic biomass is plant biomass rich in cellulose, non-cellulosic β(1,4)-linked polysaccharides of D-xylose, D-mannose and D-glucose (hemicellulose) and lignin. Lignin are heterogeneous polymers of cross-linked phenyl-propane, with a three-dimensional polymer structure different from the cross-linked fibre structure of hemicellulose form and the sheet-like fibre structure cellulose form. Commonly used pretreatment methods for lignocellulose include dilute acid, ammonia fibre explosion (Mathew et al. 2016), steam explosion (Rana et al. 2012) and sulphite pulping (Chylenski 2017). The most important role of pretreatment of lignocellulose in the process of enzymatic depolymerization is reducing recalcitrance of the biomass by removing the barriers of hemicellulose and lignin in addition to reducing the crystallinity of the cellulose.

The degree of crystallinity is of major importance for the ability of cellulases to initiate digestion of cellulosic biomass. Biomass containing crystalline cellulose in absence of hemicellulose and lignin is known to give an initial rate of the enzymatic hydrolysis closely associated with the crystallinity index.

The tight packing of cellulose strands in crystalline cellulose physically blocks the cellulase resulting in availability only to the surface (Sun et al. 2016). Amorphous cellulose, however, is less densely packed, increasing the access for cellulases. Research on enzymatic conversion of cotton textiles has mainly relied upon chemical pretreatment for decreasing the crystallinity. Solvent systems that dissolve crystalline cellulose into amorphous cellulose without derivatisation or degradation are *N*,*N*-dimethylacetamide in lithium chloride (DMAc/LiCl), trifluoroacetic acid in methylene chloride (TFA in DCM), calcium thiocyanate in water, *N*-methyl morpholine oxide (NMMO) in water and ammonium thiocyanate (Lewin & Pearce 2007). While DMAc/LiCl and TFA/DCM are known to be toxic, NMMO is not. Certain ionic liquids efficiently dissolve cellulose. Ionic liquids are organic salts with melting points around room temperature, and some of them contain strong hydrogen-bond acceptors monovalent anions. For extraction, absorption, degradation and synthesis they are regarded as environmental friendly solvent because of low volatility compared to alternatively applied organic solvents. However, potential contamination of soils, sediments, surface and ground water is a concern many researchers currently investigate and that remains unclear (Amde et al. 2015).

The most industrially applied cellulose solvent is NMMO, which can disrupt the hydrogen bonds between and within the cellulose polymers, by the high electron density from a strongly polar tertiary amino oxide group (Kanga 2016). Cellulose fibres for production of lyocell, a textile of regenerated cellulose extracted from wood, are spun from aqueous solutions of NMMO. The past decades, research has revealed that also chemical recycling of cellulosic textiles can be done with the use of NMMO. From dissolved pulp new threads are spun and textiles without decreased quality can be created (Negulescu 1998). The method of dissolving crystalline cellulose into amorphous cellulose has also been applied for pre-treatment prior to saccharification.

The mercerization process used for denim production, changes the crystal structure in a different way than the discussed solvents (for details, see section 1.2.2 and 1.3.3), and this change is reported to enhance enzymatic digestion (Kljun et al. 2011; Peciulyte et al. 2015). Severe challenges of using NaOH as pretreatment method, however, are the costs of recycling or neutralizing alkaline chemical waste and the corrosion it causes on the equipment (Hasanzadeh et al. 2018).

An acid assessed to be non-hazardous as well as efficient for swelling cotton cellulose is phosphoric acid. It has been demonstrated that enzymatic digestibility is enhanced by swelling the fibres in concentrated phosphoric acid (Jeihanipour & Taherzadeh 2009) as well as fibre swelling in 83 % w/w phosphoric acid (Zhang, J. et al. 2010). The swelling occurs by a two-step process, initiated by the acid and the cellulose hydroxyl groups reaction by esterification, forming cellulose-phosphate. The following step is a competitive interaction between hydrogen bonds between water and cellulose, and inter-chain hydrogen bonds within cellulose. Acid catalysed hydrolysis of glycosidic bonds will occur at elevated temperature, and as a pretreatment method the dissolution temperature is kept under 50°C (Zhang et al.

2009). The economic feasibility of using phosphoric acid depends on whether the acid is recovered and reused.

1.4.3.1. Comminution

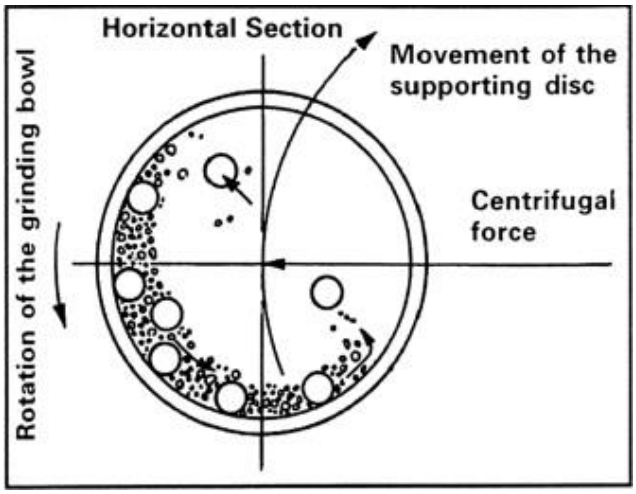


Figure 1.4.3. Schematic illustration of grinding action in a planetary ball mill. Grinding in a planetary ball mill occurs at high dynamic energies due to the superimposed rotational movements of the chamber and the supporting disc. Obtained from (Zhang et al. 2008).

Published research on enzymatic saccharification of cotton textiles with mechanical pretreatment does not show yields equal to those of chemical pretreatment. At the other hand, the number of attempts on this field is relatively limited. Among mechanical pretreatment, comminution is an important principle. Comminution is a reduction of average particle size in a solid material. Dry ball milling, wet ball milling, vibratory ball milling and compression milling are different applied comminution techniques. Planetary ball milling is a type of ball milling (wet and dry), which is based on the principle of superimposed rotational movements. Because the rotation of the grinding chamber has an opposite direction relative to the rotation of the platform on which it stands (supporting disc), the speed of the balls differs from the speed of the chamber, making the balls achieve high dynamic energy. This provides colloidal grinding and high centrifugal forces resulting in high pulverization energy (Aliofkhazraei 2015; Li 2016). Planetary ball milling is applied under wet and dry conditions, depending on the substrate and the objective of the milling.

Ball milling is widely used for mechanical metal alloying and production of various nano-particles (Hotta et al. 2007). Cellulose has attained attention as a potential nanocomposite material, for applications in e.g. technology and filtration, and one way to produce cellulose fibres of nano level particle size is ball milling. Ball milling of crystalline cellulose has been observed to produce nanofibrils with simple operation and relatively low costs (Sofla et al. 2016; Zhang, L. Y. et al. 2010). Ball milling has been applied with successful conversion yields as replacement for chemical pretreatment of enzymatic degradation of crystalline chitin (Nakagawa et al. 2011) and conversion of cellulose from steam-treated lignocellulose (Suckling et al. 2017)

The current use of chemicals contributes to biorefineries costs in degradation of lignocellulose. While mechanical pretreatment is a potential replacement for chemical pretreatment in lignocellulose

degradation, it is reviewed to be a too energy demanding technique that may be cost efficient only if combined with other pretreatment methods (Barakat et al. 2013). However, cotton does not contain lignin or significant amounts of hemicellulose, and the energy consumption per conversion yield might be lower than for lignocellulose.

1.5. Substrate related factors that might impact the enzymatic conversion of cotton textiles

1.5.1. Increased dry matter

Increasing the dry matter content in enzymatic hydrolysis is a bottleneck for biorefineries producing ethanol from lignocellulosic biomass. The exact reasons for inhibition of enzymatic hydrolysis at high substrate loadings depends mostly on the substrate. Lately, it was reported that the inhibition of glucose and cellobiose appears to be the main cause for lignin- and hemicellulose-free substrates (Kristensen et al. 2009). Mixing of the reactions might in some reactions be hindered from high dry matter content (Georgieva et al. 2008), although it is thought to be a less important limiting factor than the inhibition by reaction products (Jørgensen et al. 2007), especially cellobiose, inhibiting catalysation from CBH and EG (Kristensen et al. 2009; Tolan, Jeffrey S. 2002). The rate of enzymatic conversion in viscous solutions are known to be limited mainly by the mass transfer rate, i.e. the mobility and diffusion rate of the enzymes in the reaction solution (Battista et al. 2018).

1.5.2. Indigo dyed textiles

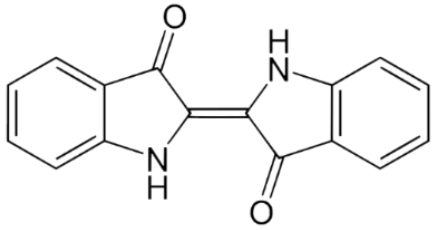


Figure 1.6. The structure of an indigo molecule. The two secondary amine groups make indigo a base that might interact with acidic amino acids in a cellulase.

Campos and co-workers have previously shown interactions between the dye indigo and cellulase enzymes and demonstrated affinity between the molecules (Campos et al. 2000). They found differences in affinity according to the number of acidic residues in the cellulases, promoting affinity to indigo. The indigo molecule has two secondary amine groups, weakly alkaline. This affinity might influence enzymatic digestion. Denim fabric production has variations that might give differences in the effect of ball milling and the digestibility of enzymes in saccharification. For instance, denim that is going to be coloured need to have a sufficient adsorption capacity, enhanced by mercerization. This process is usually not applied on white denim.

1.6. Quantification with high-performance liquid chromatography (HPLC)

The principle of chromatography is based on separation of analytes between an immobile (stationary) and a mobile phase. The mobile phase in HP**L**C is liquid, while the stationary phase is in the majority of appliances solid. The mixture of analytes is introduced into the mobile phase, which carries them through the system at a given flow. As the mobile phase passes through the stationary phase, the analytes partition between the two phases. Different interaction with the resin beds in the stationary phase result in different migration rates through the system. The difference in this interaction is usually defined as retention volume (V_R) of an analyte, which directly correlate to the retention time (t_R), i.e. the time of analyte detection upon emerging from the stationary phase. The following equation describes how the total volume of mobile phase (V_R) depends on the dead space of the system the flow runs through, the volume of mobile phase per time (flow) while the analyte is held immobile, the volume of the stationary phase (V_S) and the partition coefficient (K): $V_R = V_M + KV_S$ (Miller 2005). The partition coefficient (K), being the distribution of the analyte between stationary and mobile phase, is the parameter effectively separating the analytes. In ion exclusion chromatography (IEC), K generally depends on the pK_a of the analytes (often applied for organic acid separation), although it cannot be considered as an isolated principle in separation. Other principles considered to contribute to separation in IEC are: hydrophobic adsorption on the resin network, the effect of functional group screening in the analysed sample, normal phase retention, van der Waals, polar interactions of the sample compound with the support as well as size exclusion (Głód 1997).

1.7. Aim of study

Efficient saccharification of cotton cellulose with low ecological footprints was the overall aim of this study. A white denim fabric was used as the main substrate, and the enzymatic digestibility was investigated before and after ball milling, at different enzyme loadings and applying different commercial cellulase cocktails with and without LPMOs. LPMO activity was then investigated by two approaches: assays with conversion by an LPMO-rich enzyme cocktail in presence and absence of ascorbic acid, and reactions with LPMOs as the sole enzyme activity. Comparative experiments to assess whether the digestibility of indigo dyed denim, a majorly produced cotton textile, differ from the undyed textile were carried out. Finally, the optimised method was tried at increased dry matter concentrations.

2. Materials

2.1. Chemicals

Table 2.1. Chemicals used throughout this study are presented along with the relevant supplier.

Chemical	**Supplier**
Acetic acid 99.8 %	VWR
Ascorbic acid ($C_6H_8O_6$)	Sigma Aldrich
Cellobiose ($C_{12}H_{22}O_{11}$) (Glc_2)	Megazyme
Cellohexaose ($C_{36}H_{62}O_{31}$) (Glc_6)	Megazyme
Cellopentaose ($C_{30}H_{52}O_{26}$) (Glc_5)	Megazyme
Cellotetraose ($C_{24}H_{42}O_{21}$) (Glc_4)	Megazyme
Cellotriose ($C_{18}H_{32}O_{16}$) (Glc_3)	Megazyme
Glucose ($C_6H_{12}O_6$) (Glc)	AnalaR NORMAPUR
Sodium acetate, anhydrous (CH_3COONa)	Sigma Aldrich
Sodium hydroxide (NaOH) solution, 50%	FLUKA Honeywell
Sulfuric acid 95-98%	FLUKA Honeywell
Tris Base, ULTROL® Grade ($C_4H_{11}NO_3$)	Sigma Aldrich

2.2. Substrates and enzymes

Table 2.2.1. Substrates used throughout this study are presented along with the relevant supplier.

Substrate	**Abbreviation**	**Supplier**
Avicel® PH-101	Avicel	Sigma Aldrich
Cotton from blue denim fabric, *Soft washed denim 10oz*	BD	Stoff og stil
Cotton from white used denim jeans	WD	Second hand shop
Phosphoric acid swollen cellulose	PASC	Produced from Avicel® PH-101

Table 2.2.2. Enzymes and cocktails used throughout this study are presented along with the relevant supplier.

Enzyme and enzyme cocktails	**Supplier**
Cellic® CTec2	Novozymes, Denmark
Celluclast®	Novozymes, Denmark
TaLPMO9A 80 µM	Produced in the laboratory by Dr. Dejan Petrovic

2.3. Laboratory equipment, apparatus and materials

Table 2.3. Apparatus, equipment and instruments used throughout this study are presented along with the relevant supplier.

Apparatus, equipment and instruments	**Supplier**
Autoclave 120°C	CertoClav
Automated pipettes	Thermo Scientific
Beaker	VWR
Büchner flask	Pyrex
Centrifuge Tubes Cellstar® 15 mL and 50 mL	Greiner
Centrifuge	Eppendorf
Crucibles, porcelain	
Cuvettes	Eppendorf
Desiccator	Duran
Drying cabinet 106 °C	Memmert
Eppendorf 2,0 mL	Axygen Scientific
Filter crucibles, 15 mL ROBU-GLAS filters porosity 4	Pyrex
Filter plates, 0,45 µm Durapore®	Thermo scientific
Filtropur membrane, 0.2 µm Polyethersulfone (PES)	VWR
Filtropur membrane, 0.45 µm PES	VWR
Filtropur syringe filtration, 0.45 µm PES	Sarstedt
Funnel with 2 rubber conical gaskets	
Freezer, -20°C	Bosch
Glass stir rods	
Heat block	Grant
HPAEC system, ™ ICS-3000	Dionex ™
CarboPac PA1 2 × 250 mm analytical column	

CarboPac PA1 2 × 50 mm guard column	
HPLC system,	Dionex ™
HPLC analytical column, Rezex RFQ Fast Acid H+ (8%) 7.8×50 mm	Phenomenex
HPLC analytical column, Rezex RFQ Fast Acid H+ (8%) 7.8×100 mm	Phenomenex
HPLC vials	VWR
Liquid dispenser 50 mL	Brand
MALDI-TOF	Daltonics, Germany
Milli-Q® Direct Water	Merck Millipore
Magnet, Teflon Stirring Bar	SP Science ware
Magnetic Stirrer, RCT Basic	IKA
Measuring cylinger	VWR
Muffle furnace 575 °C	
PCR Tubes, 0.2 mL	Axygen
pH-meter SI400	Sentron
Pipette Refill Tips	Thermo Scientific and VWR
Plates, multiscreen®$_{HTS}$ with 96 wells	Thermo scientific
Pressure tubes 50 mL with sealing (tubes resistant to 120°C)	Kimax
Refrigerator, 4°C	Bosch
Retsch PM100 ball mill	Retsch
Retsch yttrium stabilized oxide chamber of nominal volume 500 mL	Retsch
Retch ceramic balls with mass 0,40 ± 0,01 g	Retsch
Scale with 0.1 mg accuracy	Sartorius, Germany
Scissors with sharp blades	Fiskars
Sonics Vibra-Cell™ Ultrasonic Processor	Sonics & Materials, Inc.
Spectrophotometer, Multiscan™ FC Microplate Photometer	Thermo Scientific
Syringe Filtration Unit	Sarstedt
Target plate MTP 384 ground steel	Daltonics, Germany
Thermomixer™ C	Eppendorf
Vacuum pump	VWR
Volumetric flask 1.0 L	Duran
Volumetric flask, 2.0 L	Duran
Vortex, MS2 Minishaker	IKA
Water bath, 30 °C	Julabo

3. Methods

3.1. Buffers and dilutions

Diluted sulphuric acid (72% w/w)

Materials and chemicals: 96% (w/w) H_2SO_4 (*l*)
dH_2O
Graduated cylinder

Utilizing a graduated cylinder, 133 ml H_2SO_4 (96 % w/w) was diluted in 82 ml dH_2O. The dilution was slowly conducted, and the solution was allowed to cool in room temperature.

Sodium acetate buffer, pH 5.0

Materials and chemicals: $NaCH_3COO$ (*s*)
dH_2O
CH_3COOH (*l*)
Volumetric flask 1.0 L
0.45 PES membrane
Apparatus: Vacuum pump
Scale (accuracy 0.1 g)

Hydrolysis experiments with enzyme cocktails were carried out with a 50 mM sodium acetate buffer with pH 5.0. A stock solution of 0.1 M buffer was prepared by dissolving 136.1 g sodium acetate in dH_2O and adjusting the pH to 5.0 with approximately 50 mL of acetic acid (100 % w/w). The buffer volume was adjusted with dH_2O in a volumetric flask to a final volume of 1,0 L. The buffer was filtrated under sterile conditions using a 0.45 µm PES membrane vacuum filtration system and stored at room temperature. It was subsequently diluted to 50 mM and stored at 4°C.

Tris-HCl buffer, pH 8.0

Materials and chemicals: $C_4H_{11}NO_3$ (*s*)
dH_2O
HCl (*l*)

	Volumetric flask 1.0 L
Apparatus:	Autoclave
	Scale (accuracy 0.1 g)

The Tris-HCl buffer utilized for the experiment with *Ta*LPMO9A was prepared by dissolving 121.1 g of Tris base ($C_4H_{11}NO_3$) in 800 mL dH_2O. Hydrochloric acid (HCl) was used to adjust the pH to 8.0, before the final volume was adjusted by dH_2O to 1.0 L in a volumetric flask. The buffer was autoclaved for 20 minutes at 121°C and stored at room temperature. The buffer was subsequently adjusted to pH 6.5 and diluted to 70 mM. The dilution was stored at 4°C.

Ascorbic acid

Materials and chemicals:	$C_6H_8O_6$ (*s*)
	dH_2O
	Volumetric flask 1.0 L
Apparatus:	Autoclave
	Scale (accuracy 0.1 mg)

The 100 mM ascorbic acid stock for enzymatic hydrolysis experiments was made by dissolving 1.8 mg ascorbic acid in dH_2O in a volumetric flask to a final volume of 100 mL. Aliquots were stored in 0.2 mL in PCR tubes at -20°C.

Eluent of 5 mM sulphuric acid

Materials and chemicals:	96% (w/w) H_2SO_4 (*l*)
	dH_2O
	Graduated cylinder
	Volumetric flask, 2.0 L

The eluent utilized as mobile phase in ion exclusion chromatography was prepared shortly within chromatography. Firstly, 372 µL acid was pipetted into a beaker containing dH_2O. Secondly, the content of the beaker was poured into a volumetric flask. Thirdly, the flask was filled with dH_2O to 2.0 L.

Eluent of 1 M $NaCH_3COO$ in 0.1 M NaOH

Materials and chemicals:	NaOH (*l*)
	$NaCH_3COO$ (*s*)
	dH_2O
	Scale (accuracy 0.1 g)
	Volumetric flask, 1.0 L
	0.2 µm PES membrane
	ICS eluent bottle
Apparatus:	Vacuum pump
	Ultrasonic Processor
	Pump with N_2 flow (*g*) (included in the HPLC system)

For ion exchange chromatography, the eluents utilized were freshly prepared. 82.0 g $NaCH_3COO$ was dissolved in dH_2O, adjusting the volume to 1.0 L in a volumetric flask and filtrating it through a 0.2 µm PES membrane. The buffer was degassed for 20 minutes and the air in the headspace was exchanged with N_2 before 5.2 mL NaOH was pipetted into the bottle. The bottle was closed well and carefully shaken to mix the buffer.

Eluent of 0.1 M NaOH

Materials and chemicals:	NaOH (*l*)
	dH_2O
	Volumetric flask, 2.0 L
	ICS eluent bottle
Apparatus:	Ultrasonic Processor
	Pump with N_2 flow (*g*)

In a volumetric flask, 2.0 L of dH_2O was filled directly after filtration by MilliQ®. The bottle was covered loosely with a lid and degassed for 20 minutes and the air in the headspace was exchanged with N_2 before 10.4 mL NaOH was pipetted into the bottle. The bottle was closed well and carefully shaken to mix the buffer.

Eluent of dH_2O

Materials:	dH_2O
	ICS eluent bottle
Apparatus:	Ultrasonic Processor
	Pump with N_2 flow (*g*)

2 L of dH_2O was filled into the eluent bottle directly after filtration by MilliQ®. The bottle was covered loosely with a lid and degassed for 20 minutes and the air in the headspace was exchanged with N_2.

3.2. Compositional analysis

Avicel PH 101, scissor cut white denim fabric (WD), scissor cut blue denim fabric (BD), ball milled WD (BWD) and ball milled BD (BBD) where characterized by a compositional analysis. A protocol developed at the U.S Department of Energy was used to accurately quantify water, ash and cellulose (Sluiter, Hames et al., 2008). As cotton is lignin- and hemicellulose-free, the procedure was adapted for quantification of cellulose, oligo- and polysaccharides referred to as glucan. Glucan was quantified for Avicel, WD and BD, not for ball milled substrates.

Materials and chemicals:	72% (w/w) H_2SO_4 (*l*)
	dH_2O
	Pressure tubes (glass tubes solid enough for autoclaving when tightly sealed, 50 mL)
	Glass stir rods
	Glass filter crucibles 15 mL
	Büchner flask and a funnel fitting the filters
Apparatus:	Scale (accuracy 0.1 mg)
	Water bath 30 °C
	Autoclave 120 °C
	Liquid dispenser (>50mL)
	Vacuum pump
	Dry oven of 106 °C
	Desiccator
	Muffle furnace (575 °C)

3.2.1. Dry matter content

Triplicates of samples of approximately 300 mg dry matter were weighed into pre-weighed (after incineration) and tared porcelain crucibles for dry mass and ash determination. All samples were dried for 24 hours at 106°C and cooled in a desiccator for 20 minutes before the dry mass was registered. Dry matter content was calculated according to Formula 3.2.2.1. The same samples were then incinerated for 24 hours at 575°C, allowed to cool in a desiccator for 1 hour, and the remaining mass was determined. Ash content was calculated according to Formula 3.2.2.2. Dry mass and ash quantification were both carried out by monitoring 1 hour further drying/incineration to assure complete processes. The dry matter content was used for calculations in glucan quantification and the set-up of enzymatic hydrolysis experiments.

Formula 3.2.1.1:

$$DM = \frac{dm_{bs} - dm_b}{m_s} \cdot 100\%$$

dm : registered mass after drying $_s$: sample
m : registered mass before drying $_b$: beaker

Formula 3.2.1.2:

$$Ash = \frac{im_{bs} - im_b}{m_s} \cdot 100\%$$

im : registered mass after incineration $_b$: crucible
m : registered mass before drying and incineration

3.2.2. Quantification of glucan

In the first step, the samples were incubated at 30 °C for 1 hour with 72 % (w/w) sulfuric acid to break the hydrogen bonds and solubilize the crystalline structure of cellulose. In the second step, the acid was diluted to 4 % (w/v) by addition of dH_2O followed by temperature increase to 120 °C. Glucan that is already cleaved in the first step are depolymerized to monosaccharides. Further degradation of glucose into hydroxy-methyl-furfural ($C_6H_6O_3$) might occur in reactions with acidic conditions and high temperature over time. Sugar recovery standards (SRS) were utilized to account for the possible loss of glucose. Products of acid hydrolysis were diluted 1:5 and subsequently separated by ion exclusion chromatography.

For cellulose quantification, about 150 mg sample was weighed into pressure tubes. The mass was accurately registered by using a scale with 0,1 mg precision. 72 % (w/w) H_2SO_4 aliquots of 1,5 mL were added to each pressure tube, yielding a concentration of 3.97 % (w/w). Glass stir rods were used to mix the sample and sulphuric acid solutions thoroughly. A water bath set to 30°C was used for 60 minutes

incubation. During incubation in the water bath the samples were stirred every 5 minutes with glass rods to ensure acid distribution onto all sample particles. A liquid dispenser was then used to add 42 mL dH_2O to the samples, diluting the acid concentration to 4 % (w/w). In order to prepare SRS, a 30 g/L stock solution of high purity D-glucose was diluted to 3 g/L. 10 mL of the solution was pipetted into three pressure tubes and added 174 µL H_2SO_4. The pressure tubes containing the cellulose samples and SRS's were tightly sealed and autoclaved at 121°C for 60 minutes. All pressure tubes were allowed to cool in room temperature before they were vortexed for 10 seconds and filtered through glass filter crucibles set up with a funnel and Büchner flask underneath, air tightened on both sides by rubber conical gaskets and connected to a vacuum pump. Centrifuge tubes were used to catch the filtrate. Glucose was separated by ion exclusion chromatography with the analytical column Rezex RFQ Fast Acid (8%) (see section 3.6.3 for detailed description of this protocol) and quantified from undiluted samples using calibration standards within the range of 0,3-30,0 g/L. Calculations were made applying Formula 3.2.2.

Formula 3.2.2:

$$Glucan\ content(\%\ of\ total\ solids) = \frac{[Glc_{HPLC}](g/L) \cdot DF \cdot V_s(L) \cdot \frac{Mm_{an.Glc}}{Mm_{Glc}}}{R \cdot TS} \cdot 100$$

V_s: Volume sample $[Glc_{HPLC}]$: anhydrous glucose quantified by HPLC

$$Total\ solids = TS = \frac{m_s(g) \cdot DM(\%)}{100} \qquad R = \frac{[glc_{recovered}](g/L)}{[glc_{diluted}](g/L)}$$

3.3. Ball milling of denim substrates

Materials: Sharp scissors
Sieving equipment, mesh size 0.8 mm
White and blue denim fabric

Apparatus: Retsch PM100 ball mill
500 mL chamber and ceramic balls

Scissors and a Retsch PM100 ball mill were used to pre-treat cotton from white and blue denim fabric prior to enzymatic hydrolysis. The substrates WD and BD were cut to 25-50 mm pieces by scissors before ball milling. A Retsch PM100 was used, with an yttrium stabilized oxide chamber of nominal volume 500 mL. The chamber was filled with 5,0 g sample and 342 g ceramic balls. The milling was carried out by 10 minutes effective intervals at 350 rpm and 15 minutes

Picture 3.3: Denim powder. Photography of substrate BWD (upper left) and BBD (lower right) used in hydrolysis experiments.

break intervals. The breaks were important for avoiding elevated temperature due to high dynamic energy in a closed system. The duration of efficient milling was 1 hour and 10 minutes (total duration of 2h 40 min) for WD. BD needed a longer milling time in order to obtain a similar powdering to WD and was therefore ball milled for 1 hour and 50 minutes (total duration 4 h 20 min). The substrates were milled in 5 batches, thereafter cooled and sifted through a 0,8 mm sift. The particles passing through the sieve were mixed well and stored in centrifuge tubes at 4°C. Very small fractions of particles were remaining in the sieve.

3.4. Particle size distribution measurement

Materials: Ball milled white denim and ball milled blue denim

Apparatus: Malvern Mastersizer 3000

Ball milled denim samples were analysed by Malvern Mastersizer 3000 in order to determine the mean particle size. A particle refractive index of 1,58 was used, as axial refractive indices of different cotton types are measured to be 1,575 to 1,580 by Meredith (1946). Three table spoons of sample, (approximately 10 g) were inserted into the instrument, which distributes the sample gradually into two lasers of red and blue colour. A sensor across the chamber registers the signals. The feeding of sample is repeated until the measurements fits into a statistical model with a low weighted residual.

The size of a particle is given as the volume of a sphere. The equivalent sphere theory is applied for measuring particles of various shapes by Malvern Mastersizer 3000 and its software. A sphere has mean diameter given by $\frac{\sum d^2}{n}$, where n is the number of particles. The weight of a sphere is given by the formula $\frac{4}{3}\pi r^3.p$ which can be combined with the mean diameter formula to give a formula we can use for determining the mean diameter on the basis of weight, by the formula $\frac{\sum d^3}{n}$. Moment Means are introduced to the formula to release it from the dependency on the number of particles in the calculations. Surface area moment mean (mathematically written D[3,2]) and volume moment mean (D[4,3]), are the moment means used for particle size measurement in this experiment, and they are calculated by $D[3,2] = \frac{\sum d^3}{\sum d^2}$ and $D[4,3] = \frac{\sum d^4}{\sum d^3}$. These two formulas describe around which central point of the frequency the surface area (for D[3,2]) and the volume (for D[4,3]) distributions rotate. In order to use a mean value to explain possible differences in digestibility of the cotton substrates, both D[3,2] and D[4,3] give valuable information, as they describe the distribution of surface and of volume or mass. The surface area moment mean can further be used for comparing specific surface area of the materials, although the density is required in those calculations.

3.5. Bradford protein quantification

Materials and chemicals: Plastic cuvettes
Celluclast® (Novozymes Inc.)
dH_2O
Coomassie Brilliant Blue reagent
Bovine serum albumin (BSA)

Apparatus: Spectrophotometer

One of the enzyme cocktails used for cellulose hydrolysis (see section 3.6.2, the cocktail Celluclast® from Novozymes Inc.) had an unknown protein concentration. The Bradford Method, a colorimetric protein assay was used to measure the protein concentration in Celluclast®. All proteins in the cocktail are assumed to be active enzymes. The principle of the assay is light absorbance of Coomassie Brilliant Blue in acidic solutions, and the change of absorbance maximum from 465 to 595 nm when it binds to protein (Bradford 1976). Only basic amino acids bind to the reagent compound; hence the method only measures the content of proteins that inherit alkaline residues. The calibration curve was pre-calibrated with bovine serum albumin (BSA) calibration standards diluted in dH_2O. The sample was analysed in triplicate.

Celluclast® was diluted 1:10 000 in dH_2O by mixing 10 µL Celluclast® with 790 µL dH_2O, before adding 200 µL of the Coomassie Brilliant Blue reagent. Samples were' vortexed for 3 seconds. After 5 minutes static incubation at ambient room temperature, the sample was pipetted into a plastic cuvette and analysed by measuring absorption spectrophotometrically at 595 nm. The concentration of the protein in the sample was estimated based on a standard curve ranging from 0.25 µg/mL to 10 µg/mL.

3.6. Enzymatic conversion of cotton textile to glucose

Conversion of cellulose from cotton textile was investigated by using the enzyme cocktails Cellic® CTec2 with a protein concentration of 83 g/L and Celluclast® with a protein concentration 40 g/L. Cotton samples investigated were scissor cut white denim fabric (WD), scissor cut blue denim fabric (BD), ball milled WD (BWD) and ball milled BD (BBD). As a reference substrate, the laboratory standard Avicel was analysed along with the denim substrates. Avicel is described by the producer as micro crystalline particles of approximately 50 µm particle size, chemically produced from cotton linters. In order to avoid unaccounted changes in substrate concentration, calibration standards went

through the same dilution steps as samples in enzymatic hydrolysis experiments and are throughout chapter 3.5 treated as the samples. According to the substrates' respective total solids contents, an ideal mass corresponding to 25 mg total solids in the reactions was aimed for to obtain 5 % DM and the deviations in weighing were calculated for each tube as a correction factor. A buffer volume adjusted to total solids of each replicate was added. The reactions total volume in a tube containing 25,0 mg total solids was 0,50 mL. Sample volume was included in total volume, where 1 mg was calculated as 1 µL. The addition of enzyme solution was adjusted for each sample using the correction factor from deviations in weighing.

3.6.1. Experiments with Cellic® CTec2

Apparatus: Thermomixer with 2.0 mL thermoblock
Scale (accuracy 0.1 mg)
Vortex shaker
Heat block (100°C)
Centrifuge
Vacuum pump

Materials. Textile substrates (WD, BWD, BBD)
Avicel
50 mM Sodium acetate buffer, pH 5.0
100 mM ascorbic acid
Cellic® CTec2 (Novozymes Inc.)
Eppendorf tubes 2.0 mL
Ice
Filter plates 0,45 µm membrane

3.6.1.1. Loading analysis

The loading 2 mg, 4 mg, 8 mg, 12 mg, 16 mg and 20 mg enzyme per g cellulose were analysed for BWD. Cellic® CTec2 was diluted in buffer to 1,8 g/L and mixed well before and regularly during preparation of samples. This enzyme solution represented the stock solution used further in this experiment. Approximately 27 mg BWD was weighed into 2 mL eppendorf tubes. The correction factor from deviations in weighing was used to determine appropriate volumes of enzyme and buffer for each sample. A buffer volume adjusted to give a final concentration of 50 g/L total solids of each replicate was added. Aliquots of 10 µL ascorbic acid stock was added to all samples, which gave a final

concentration of 2 mM. The samples were pre-heated to 50 °C in a thermomixer. The respective volumes of the enzyme stock solution were added, where 25 µL multiplied with the correction factor represented 2 mg enzyme per g glucan, giving an enzyme concentration of 0.09 g/L. Likewise, 50, 100, 150, 200, 250 µL gave 4, 8, 12, 16 and 20 mg enzyme loading and an enzyme concentration range up to 0.9 g/L. (All details are given in Table S-1 in the Appendix) The samples were mixed by 5 seconds vortexing. All samples were incubated at 50 °C and 1000 rpm shaking for 48 hours. The reactions were stopped by heating the samples to 100 °C for 15 minutes on a heat block. The samples were cooled on ice for 2 minutes, vortexed for 5 seconds, centrifuged at 4 000 rpm for 30 seconds and filtered through a 0,2 µm filter plate before the products were analysed by HPLC (see section 3.6.3 for details). Sample set-up is showed with details in Table S-1.

3.6.1.2. Time course analysis with cotton substrates and Avicel

Triplicates of Avicel, WD and BWD were used in a time course hydrolysis experiment with the enzyme loading seen to achieve the most efficient conversion in the loading analysis. Section 5.3.1 explains how loadings were selected. Reactions were set up and incubated with similar conditions to the loading analysis. Duration of hydrolysis was 4 hours, 24 hours, 48 hours and 96 hours. BBD and BWD were later used in an identical experiment.

Approximately 27 mg BWD, 25.6 mg WD, 26.0 mg BBD and 25.8 mg Avicel was weighed into 2 mL eppendorf tubes to obtain 25 mg total solids. Adjusted buffer volumes were added, and ascorbic acid stock to a final concentration of 2 mM. The samples were pre-heated to 50 °C in a thermomixer. The respective volumes of the enzyme stock solution were added, 100 µL multiplied with the correction factor represented 4 mg enzyme per g glucan, giving an enzyme concentration of 0.18 g/L. (All details are given in Appendix S-3.). In order to use the same enzyme dilution for all samples, dry matter contents of reactions were 4,93 % for Avicel and 5,00 % for textile samples, a difference that is considered in calculations of conversion yield. The samples were incubated at 50 °C and 1000 rpm shaking for 48 hours. The reactions were stopped at 100 °C and filtrated before the products were analysed by HPLC (see section 3.6.3 for details). Sample set-up is showed with details in Table S-2.

3.6.1.3. Time course analysis at increased dry matter content

In order to investigate whether or not the conversion of ball milled cotton increased or decreased significantly with higher dry matter content of reactions, an experiment with increased dry matter content of BWD was carried out with Cellic® CTec2. The analysis was carried out by increasing the substrate concentration to 100 g/L and 200 g/L dry matter, by weighing in BWD masses corresponding to 50 mg and 100 mg total solids, respectively. To maintain the enzyme loading, the volumes of the

enzyme stock solution was increased by the same factor as the dry matter. Sodium acetate buffer was added to a final volume of approximately 0.5 mL, adjusted with the correction factor from weighing deviations. The time course analysis' duration was 96 hours. The reactions were stopped at 100 °C and filtrated before the products were analysed by HPLC (see section 3.6.3 for details). Sample set-up is showed with details in Table S-3.

3.6.2. Loading analysis and time course analysis with Celluclast®

Materials and chemicals.	Textile substrates (WD, BWD)
	Avicel
	50 mM sodium acetate buffer, pH 5.0
	Celluclast® (Novozymes Inc.)
	Eppendorf tubes 2.0 mL
	Ice
	Filter plates 0,45 µm membrane
Apparatus:	Thermomixer with 2.0 mL thermoblock
	Scale (accuracy 0.1 mg)
	Vortex shaker
	Heat block (100°C)
	Centrifuge
	Vacuum pump

Loading of Celluclast® on BWD was investigated at 2 mg, 4 mg, 8 mg, 12 mg, 16 mg and 20 mg enzyme per g glucan. Celluclast® was diluted in buffer to 1,8 g/L and vortexed for 10 seconds. Samples were prepared with 5 % dry matter in reactions and incubated at 50°C for 48 hours (for details, see 3.6.1.1. and S-4)

Celluclast® was used in a time course hydrolysis experiment with the enzyme loading seen to achieve the most efficient conversion in the loading analysis. Section 5.3.1 explains how loadings were selected. Reactions with WD, BWD and Avicel were set up and incubated with similar conditions to the loading analysis. Duration of hydrolysis was 4 hours, 24 hours, 48 hours and 96 hours. Celluclast® was diluted in buffer to 0.91 g/L and vortexed for 10 seconds. Approximately 27 mg BWD, 25.6 mg WD and 25.8 mg Avicel was weighed into 2 mL eppendorf tubes and adjusted buffer volumes were added. The samples were pre-heated to 50 °C in a thermomixer. The respective volumes of the enzyme stock solution were added, 200 µL multiplied with the correction factor represented 4 mg enzyme per g glucan, giving an enzyme concentration of 0.37 g/L. The samples were incubated at 50 °C and 1000 rpm shaking

for 48 hours. The reactions were stopped at 100 °C and filtrated before the products were analysed by HPLC (see section 3.6.3 for details). Sample set-up is showed with details in Table S-5.

3.6.3. Determining the enzymatic conversion yield by HPLC (IEC)

Materials: HPLC vials

dH_2O

Glass test tubes

Eluent of 5 mM H_2SO_4

Apparatus: Dionex HPLC system (Thermo Fisher Scientific, USA)

Rezex RFQ Fast Acid (8%) analytical column with dimensions

(1) 7.8×50 mm internal diameter

(2) 7.8×100 mm internal diameter

Chromeleon 7.0 software

Vortex shaker

Analysis of the reaction products and quantification of glucose from the majority of cellulose degradation assays was performed using ion exclusion chromatography (IEC) (some reactions were analysed using IEX; see section 3.7.3 for details). Calibration standards and samples were diluted 50 times in test tubes by mixing 20 µl with 980 µl of water. The dilutions were vortexed for 10 seconds and stored in HPLC-vials until products were analysed by IEC. All samples from experiments with Cellic® CTec2 and Celluclast® were immediately diluted and transferred into HPLC vials after filtration. The analysis was conducted within 24 hours stored at 4°C, or at a later time point, then stored at -20°C. An injection volume of 8 µL of samples and calibration standards was inserted to the system by the autosampler.

Products were separated by Rezex RFQ Fast Acid (8%) analytical columns (1) and (2) with 5 mM sulphuric acid at 1) 1.0 ml/min flow rate and 85°C column temperature or 2) 0.6 ml/min flow rate and 65 °C column temperature. Column (2) was used in all experiments including Avicel, because products of this substrate included xylose, having approximately the same retention volume to glucose when applying the shorter column. The stationary phase of Rezex RFQ is composed of polymers containing the functional groups sulfonated polystyrene divinylbenzene (Phenomenex 2017). The sphere around the sulphate groups is negatively charged, repelling species with negative charges while allowing neutral species and partially charged species (Transgenomic 2007). This causes the separation of compounds according to their pKa (Tanaka & Haddad 2000). Glucose and cellobiose are weak polyprotic acids with several pKa values. The hydroxyl with the lowest proton affinity is on the anomeric carbon, with pKa ~ 12 (Brown 1994). Cellobiose has a slightly less proton attaining anomeric carbon, slightly lowering the

pKa. However. all hydroxyl groups of cellobiose and glucose were protonated in 5 mM sulphuric acid (pH 2), and they passed through the column with retention times 1.6 and 2 minutes, respectively. The different retention volumes (V_r) of glucose and cellobiose is thought to be caused by the size exclusion principle which is known to be an additional occurring principle in IEC (Głód 1997). The space surrounding the resin beds of the stationary phase is neither interacting with, repelling or excluding glucose, while cellobiose is excluded to to a larger size. Chromatograms from separation of glucose and cellobiose calibration standards (a) and from a sample (b) are shown in Figure 3.6.3

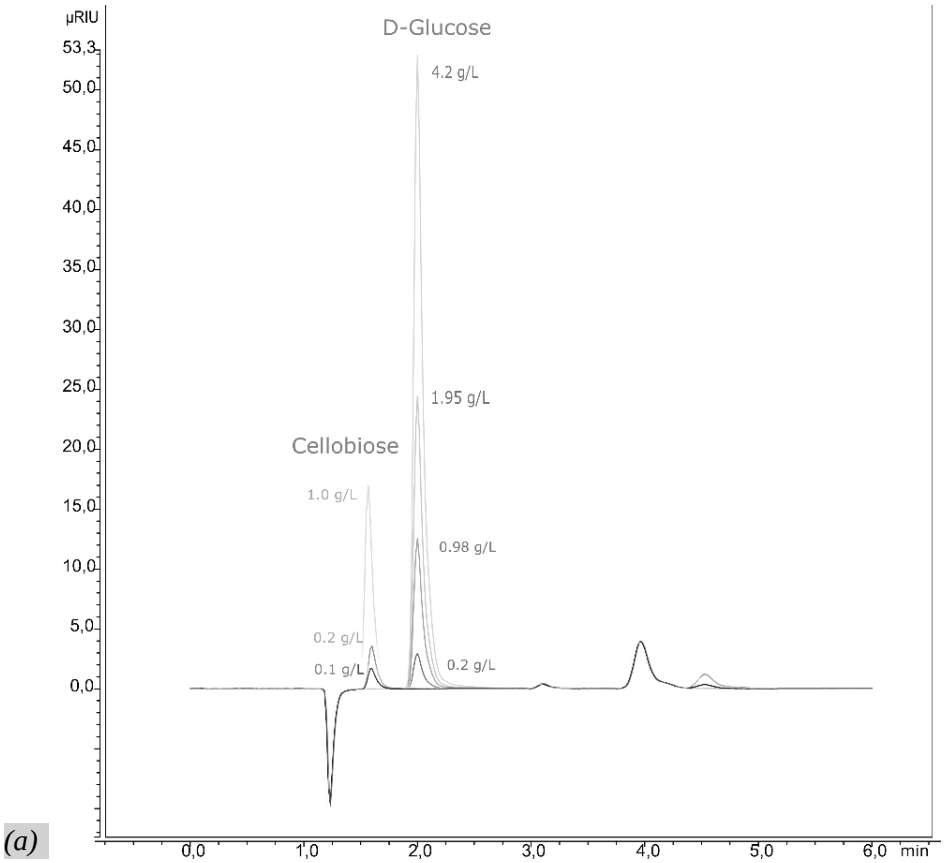
Concentrations of glucose and cellobiose were calculated based on a calibration curve made from 4 concentrations of D-(+)-glucose and D-(+)-cellobiose (weighed in and diluted identically to the samples, containing sodium-acetate buffer and 2 mM ascorbic acid). The peak area integrals for peaks representing cellobiose and glucose were converted to concentration using the formula shown in Equation 3.6.3.1. The substrate conversion yield was then calculated according to Equation 3.6.3.2. The cellobiose content and glucose content of the enzymatic cocktail was subtracted from the amounts in the samples after conversion, in order to avoid over-estimation of products due to the sugar content in the cocktail (only for Cellic® CTec2).

Equation 3.6.3.1:

$$[sugar](g/L) = \left(\frac{calibration\ standard\ signal\ (\mu RIU \cdot \mathrm{min})}{concentration\ of\ calibration\ standard\ (g/L)} \cdot sample\ signal\ (\mu RIU \cdot min) \right) \cdot dilution\ factor$$

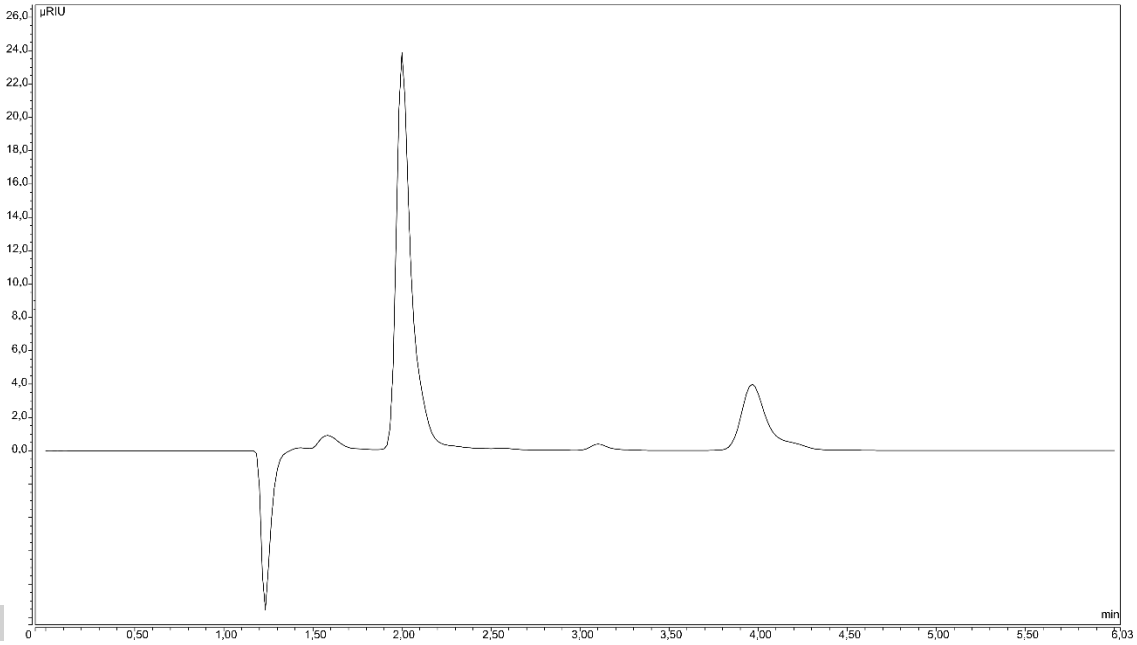
Equation 3.6.3.1

$$conversion\ (\%) = \frac{[glc](g/L) + \left(\frac{Mm_{Glc_2}}{Mm_{anh.Glc_2}}\right) \cdot [glc_2](g/L)}{\frac{Mm_{Glc}}{Mm_{anh.Glc}} \cdot glucan\ content\ (\%) \cdot dry\ matter\ content\ (g/L)} \cdot 100$$



(a)

Figure 3.6.3. Separation of cellobiose and glucose from calibration standards (a) and a sample (b). The figures show typical chromatograms obtained by IEC applying Rezex RFQ Fast Acid (8%) with dimensions 7.8×50 mm internal diameter at 0.6 ml/min flow rate and 65 °C column temperature. Calibration standards of D-(+)-cellobiose and D-(+)-glucose are shown in (a), while products from 10 % DM after 96 hrs incubation with 4 mg Cellic® CTec2 per g glucan are the analytes separated in the chromatogram in (b).



(b)

3.7. Assays with LPMOs as the sole enzyme activity

3.7.1. Reactions

Materials and chemicals.	Textile substrates (WD, BWD, BD, BWD)
	Avicel
	H_3PO_4-swollen cellulose PASC
	70 mM Tris-HCl buffer, pH 6.5
	100 mM ascorbic acid
	*Ta*LPMO9A
	Eppendorf tubes 2.0 mL
	Ice
Apparatus:	Thermomixer with 2.0 mL thermoblock
	Scale (accuracy 0.1 mg)
	Vortex shaker
	Heat block (100°C)
	Centrifuge

In order to investigate the role of lytic polysaccharide monooxygenases in enzymatic degradation of cotton substrates, reactions were set up with *Ta*LPMO9 to react alone without cellulases. This is an LPMO from the fungus *Thermoascus aurantiacus* that acts on crystalline cellulose. The cotton samples investigated were WD, BWD, BD and BBD. As reference substrates, Avicel® PH-101 and 12 % PASC were analysed along with the samples. Freezer stored stock solution of 100 mM ascorbic acid was used and a 1M Tris-HCl buffer with pH=6.5 was diluted to 70 mM. The 80 mM *Ta*LPMO9A solution was diluted in buffer to 10 µM.

Duplicates and controls of cotton substrates and reference substrates were weighed into 2 mL eppendorf tubes. The mass weighed in was adjusted according to the substrate's moisture content to achieve 2.5 mg total solids in the reaction, which is a concentration of 10 g/L or 1 % dry matter. A volume responding to total solids of each replicate was added, giving total volumes around 250 µL. Substrate volume was included in total volume. The final concentration of BisTris was approximately 50 mM. Aliquots of 2,5 µL ascorbic acid was added to all samples for a final concentration of approximately 1 mM. *Ta*LPMO9A was added to final concentration of 1 µM and the samples were incubated at 45 °C and 1000 rpm shaking for 16 hours. The reactions were stopped by heating the samples to 100 °C for 15 minutes on a heat block. They were cooled on ice for 2 minutes, centrifuged at 4 000 rpm for ½ minute and filtered through 0.2 µm filter plates. Sample set-up is showed with details in Table S-6.

3.7.2. Identification of oxidized products by mass spectrometry

Materials and chemicals:	(Hair) dryer
	2,5-dihydroxybenzoic acid matrix
	Ground steel target plate with frame (Daltonics, Germany)
	Standard mixture containing glc, glc_2, glc_3, glc_4 and glc_5
Apparatus:	UltrafleXtreme MALDI-TOF (Daltonics, Germany)
	Flex control software
	Flex analysis software

Mass spectrometry with matrix assisted laser desorption ionization and time-of-flight detection (MALDI-TOF) was used for the identification of oxidized products from the reactions described in chapter 3.7.1. MALDI-TOF is commonly used for detection of large, involatile molecules like oligosaccharides. Time-of flight mass analysers detect the ions and sorts their mass by their arrival. The matrix is composed of an acidic compound easily ionized by the laser and capable of protonating the sample dissolved in the matrix. That way, the sample obtains a monovalent charge. The ions way to the detector depend on their size and weight, where the small ones travel faster. Time-of flight mass analysers detect the ions and sorts their mass by their arrival. Mass per charge ratio is calculated by time and length of the ions' travel as well as the kinetic energy.

Samples were transferred directly from filtrate to a target plate, where they were diluted 3 times in 2,5-dihydroxybenzoic acid matrix and dried by warm air for 2 minutes. The target plate was inserted into an UltrafleXtreme mass spectrometer from Daltonics GmbH equipped with a Nitrogen 337-nm laser beam. A standard containing native cello oligosaccharides was used for calibration. The applied intensity of the laser was 60 %. The software used for collecting spectra was FlexControl, and the spectra were analysed in Flexanalysis software.

From long cellulose fibres and shorter oligosaccharides, the oxidative cleavage catalysed by *Ta*LPMO9A is observed to involve oxidation of carbon number 1 and 4 around the glycosidic bonds (Petrović unpublished). Oxidized monomers, disaccharides and native cellooligosaccharides associated with such reactions are searched for in this analysis.

3.7.3. High performance anion exchange chromatography (HPAEC)

Materials and chemicals:	Eluent A: 0.1 M NaOH
	Eluent B: 1 M $NaCH_3COO$ in 0.1 M NaOH
	Eluent C: dH_2O
	Standard mixture containing glc, glc_2, glc_3, glc_4 and glc_5
	HPLC vials
Apparatus:	Dionex ICS3000 system (Thermo Fisher Scientific, USA)
	CarboPac PA1 2 × 250 mm analytical column
	CarboPac PA1 2 × 50 mm guard column
	Chromeleon 7.0 software

HPAEC was used to analyse product profiles and quantify gluconic acid from reactions with LPMOs and cotton textile substrates. A comparative analysis of oxidized products in WD, BWD, BD and BBD was conducted, by separating native oligosaccharides and oxidized oligosaccharides and comparing the abundance of their occurrence in the LPMO treated substrates.

All samples from section 3.7.1 were analysed on the Dionex ICS 3000 system (Dionex, Sunnyvale, CA, USA) with high performance anion exchange chromatography and pulsed amperometric detection (HPAEC-PAD). The separation was performed by a CarboPac PA1 2 × 250 mm analytical column with a CarboPac PA1 2 × 50 mm guard column. The eluents were prepared, and the instrument method was set up to perform a gradient elution profilie at a flow rate of 0.25 mL/min. Initial conditions were set to 100% of eluent A, followed by a linear gradient of eluent B which 10 min after sample injection reached 10% and 30 % 35 min after injection. This was followed by a 5-min exponential gradient to 100% eluent B, after which the column was reconditioned by running the initial conditions for 9 min. The stationary phase is composed of 10-µm diameter polystyrene/divinylbenzene substrate agglomerated with 350-nm Dionex MicroBead quaternary amine functionalized latex (Rohrer 2013). Quaternary ammonium is a permanently positively charged functional group that interact with oligosaccharides, as all hydroxy groups are deprotonated in 1 M NaOH (pH~13). By increasing the concentration of CH_3COO^- in the mobile phase, negative ions are provided to the positively charged resin beds and because of the higher affinity between the amino groups and the acetate ions, the oligosaccharides are forced out of the beds. The longer the polymerization of the sugars, the more acetate is needed to displace the deprotonated sugars. This causes an elution of sugars in an order with positive correlation between time and size. (Rohrer 2013; Westereng et al. 2013).

LPMOs cleave sugars into a native sugar and an oxidized sugar, for instance an oxidized dimer (Figure 3.7.3). In principle, the native sugars have lower V_r than the oxidized products and will elute first,

although there is an overlap of the retention time of native pentamer and cellobionic acid (GlcGlc1A). The explanation for the larger V_r of oxidized oligosaccharides than native oligosaccharides is not fully understood. Neither is the explanation for the difference in V_r for C1 and C4 oxidized products. However, indications of additional oxidations for geminal diols have been demonstrated, occurring at the non-reducing monosaccharide during high alkaline conditions in the analytical step (Westereng et al. 2016). Double oxidation and associated depolymerization and tautomerization was not indicated in C1 oxidized sugars. The additional oxidation causes a tautomerization of the geminal diol into a double oxidized linear monosaccharide cleaved off from the geminal diol produced in the LPMO reactions. The change of size and shape might cause larger V_r for C4 oxidized products, by a size allowance/exclusion principle (Westereng 2018). Further, the depolymerization of a geminal diol ($Glc4GemGlc_n$) implies that the oligosaccharide cleaved off by decomposition during the analytical step (Glc_n) is eluted separately as a native oligosaccharide.

Identification of native cello oligosaccharides was done by analysing a calibration standard consisting of cellobiose, cellotriose, cellotetraose and cellopentaose. In order to identify LPMO activity, a calibration standard of the C4-oxidized dimer (Glc4gemGlc/Glc4KGlc) was used at concentrations 0,12 g/L and 0,03 g/L. The oxidized standard was generated with *Nc*LPMO9C from cellopentaose by dr. Piotr Chylenski. Substrates of LPMO reactions may be cellulose with different degree of polymerisation. In order to quantify a compound by chromatography, accurate calibration standards are required. To compare the quantities of oxidized products in the substrates, one might look at C1- and C4-oxidized sugars of various length. However, the standards are of limited quantity because they are not commercially available and challenging to produce due to poor stability. Due to the limitation of available calibration standards, the only oxidized standards utilized is the oxidized dimer, assumed to be representative.

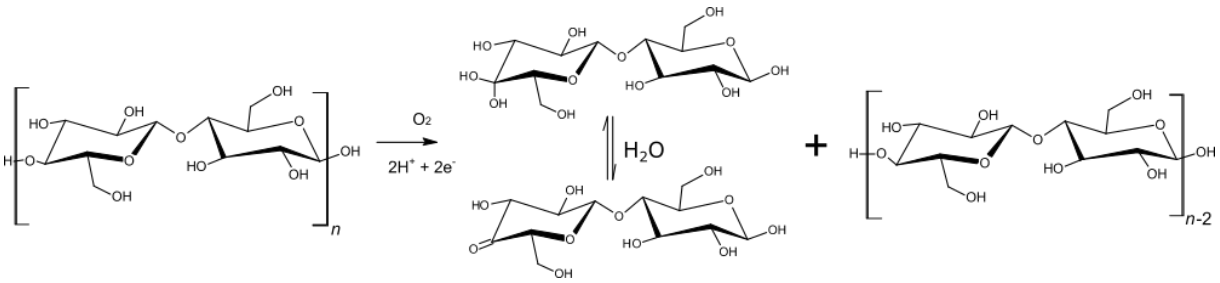


Figure 3.7.3. Oxidative cleavage. The figure shows the reaction scheme of C-4 oxidative cleavage of cellulose into oxidized cellobiose and a native oligosaccharide, based on information and illustrations from Loose and co-workers and O'Dell and co-workers (Loose et al. 2014; O'Dell et al. 2012)

The content of oxidized dimer was not quantified. Information about accurate quantities of the oxidized dimer lacks significance for the analysis, as there was done no attempt to degrade oxidized products the

content of oxidized dimer in the reactions are not comparable. Moreover, the applied analytical method is acknowledged for quantification of C4 oxidized sugars due to complexity caused by decomposition. The intensity of the peaks for the C-4-oxidized dimer in the respective substrates were compared, hence the study was comparative.

4. Results

4.1. Composition of substrates

Compositional analysis of solid substrates used for enzymatic hydrolysis is important in order to enable precise calculations of hydrolysis yields. In this study, the compositional analysis was performed in three steps. Firstly, determination of the total solids (dry matter) was carried out, and the dry matter content was further used for the calculations of the second and third steps. Second, the dried samples were combusted, and the inorganic material left after incineration (ash) was weighed. The ash content was calculated as a fraction of total sample weight and as a fraction of total solids in the sample. Third, quantification of cellulose by acid hydrolysis and chromatography was used to estimate cellulose as fractions of total weight and total solid weight. The gap in the mass balance that cannot be accounted for from the ash and cellulose estimations is named "others".

In table 4.1, the content of ash, cellulose and unidentified compounds are shown as fractions of total sample weight. The main component of the denim substrates is cellulose, representing 89 ± 2.4 % and 88 ± 1.5 % of the total sample weight for WD and BD, respectively. The water content in white denim fabric (WD) more than doubles after ball milling (i.e. increasing from 2,6 % to 7 %), whereas blue denim fabric (BD), only sees a marginal increase in hydration after this treatment (increasing from 4.0 to 4.6%). The unidentified compounds of the white cotton are 6 % and 1 % for WD and BWD, respectively, showing a significant difference after ball milling. For the blue cotton, 7 % of the total mass is unidentified.

Table 4.1. *Compositional data for textile substrates, calculated as percentage of total sample weight.* The laboratory standard Avicel was analysed for cellulose and dry matter along with the samples, while ash content was not determined. Contents of ash and glucan are assumed to be consistent before and after milling and is therefore only determined for un-milled samples.

		(% of total sample weight)				
Material	Pretreatment	Glucan	Ash	Total solids	Water	Others
White cotton from denim	Scissor cut	89 ± 2,4	0,52 ± 0,05	97,4 ± 0,2	2,6 ± 0,2	6
	Ball milled	-	-	93 ± 2	7 ± 2	1
Blue cotton from denim	Scissor cut	88 ± 1,5	0,57 ± 0,04	96,0 ± 0,1	4,0 ± 0,1	7
	Ball milled	-	-	95,4 ± 0,2	4,6 ± 0,2	7
Avicel® PH-101	None	92,5 ± 0,49	-	96,8 ± 0,3	3,1 ± 0,3	-

Figure 4.1 illustrates the content of water, ash, cellulose and unidentified compounds shown as fractions of dry matter weight. The glucan content of the dry matter is 91.7 % in WD and 96.1 % in BWD. For BBD, glucan makes up 88.7 % of the dry matter. The unidentified compounds of the substrates are 6%, 1%, 7 % and 4 % of the dry matter content for BWD, WD, BBD and Avicel. The respective fractions of ash are 0,53 % for BWD and WD %, 0,59 % for BBD and not determined for Avicel.

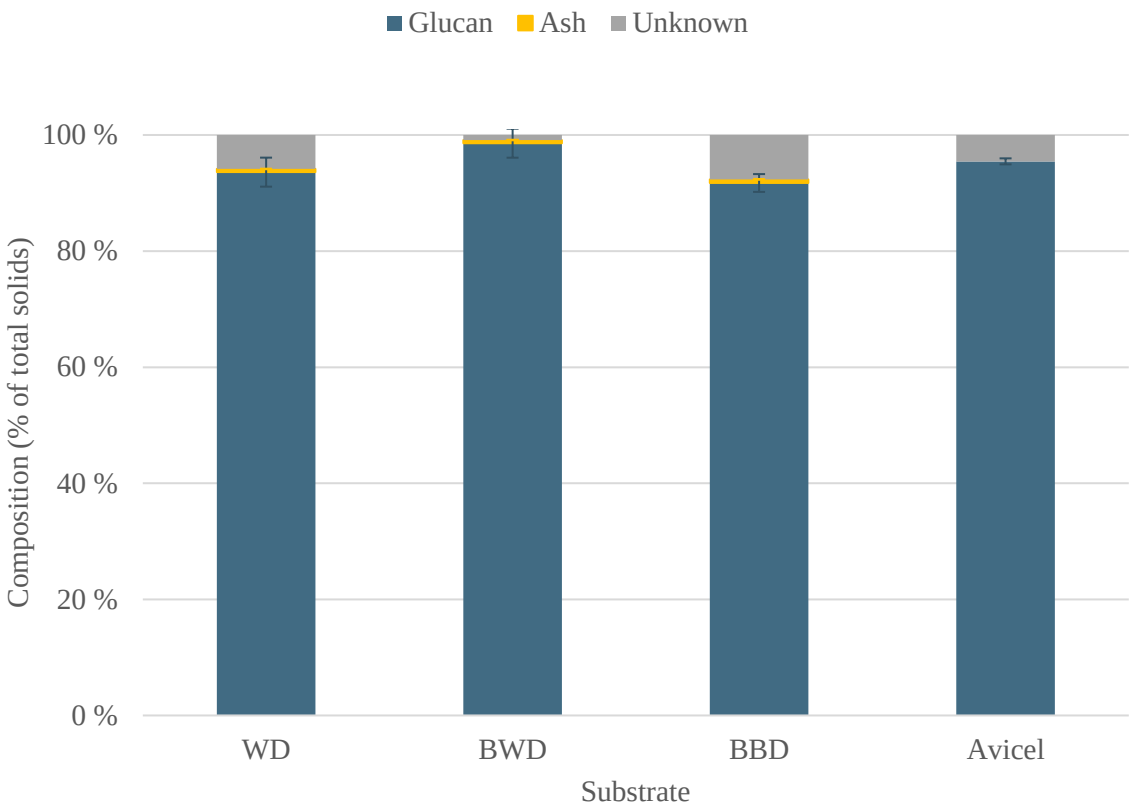


Figure 4.1. Compositional data for textile substrates calculated as percentage of total solids. Acid hydrolysis, drying and incineration were applied for determination of cellulose, total solids and ash, respectively, here shown as percentage of TS of substrates. The glucan content per TS after ball milling is not determined separately, only calculated as percentage of the new TS. Standard deviations are taken from calculated % as a fraction of total sample weight.

4.2. Particle size after ball milling

Ball milled samples were measured by Mastersizer 3000 in order to determine the particle size distribution. The refractive index of cotton depends on the location of the fibre where the refraction occurs. The axial index was utilized in this analysis, while the longitudinal index (1,52) was applied for controlling whether the results deviated. Particle size and surface area give valuable information about the availability of a substrate and might explain differences in digestibility of two ball milled samples.

The surface area moment mean and the volume/mass moment mean for blue cotton substrate and white cotton substrate are shown in Table 4.2, whereas the particle size distribution is illustrated in Figure 4.2.

Table 4.2. Moment mean values determined for ball milled substrates. Information about particle size distribution is given by surface area moment mean and volume moment mean.

	Ball milled cotton (µm)	
Moment mean	white denim	blue denim
D[3;2]	19,7	14,5
D[4;2]	160	164

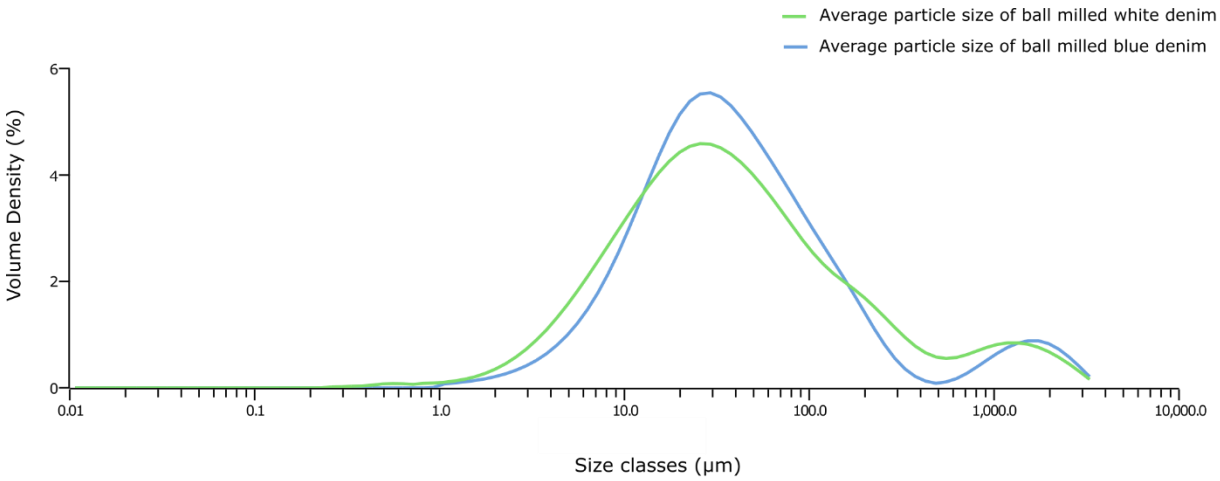


Figure 4.2. Particle size distribution of ball milled white and blue cotton. An overview of the size classes for which ball milled samples were determined.

4.3. Enzyme loading experiments

The primary aim of the hydrolysis experiments was to convert cellulose to glucose as efficiently as possible. One major factor of costs and efficiency is the enzyme loading, i.e. how much enzyme cocktail that is used in the textile conversion process. In order to find the optimal enzyme load for converting the substrate to soluble sugars after 48 hours (a common incubation time used by the industry), enzyme loading experiments were performed. The commercially available enzyme cocktail Cellic® CTec2 was used to convert samples of 5 % dry matter content for 48 hours, and the products were analysed by HPLC for separation and quantification of cellobiose and glucose. Calibration curves were made from a range of 4 sugar concentrations for glucose and cellobiose, respectively (for details, see tables S-1 and S-2 in the Appendix). Concentrations of soluble sugars obtained in the experiments were determined by HPLC and calculated using Equation 3.6.3.1. The conversion yield obtained was calculated using the formula presented in Equation 3.6.3.2 (section 3.6.3)

For ball milled white denim, conversion to glucose and cellobiose was 67 ± 1 % when the loading was 2 mg, while it increased to 88 ± 1.4 % when the loading was 4 mg and 93 ± 0.7 % when 8 mg (Figure 4.3.1). When increasing the enzyme loading further, conversion of the substrate to glucose and cellobiose reached 98 ± 1.2 % conversion for 12 mg and 16 mg loading, and 97 ± 0.8 % for 20 mg.

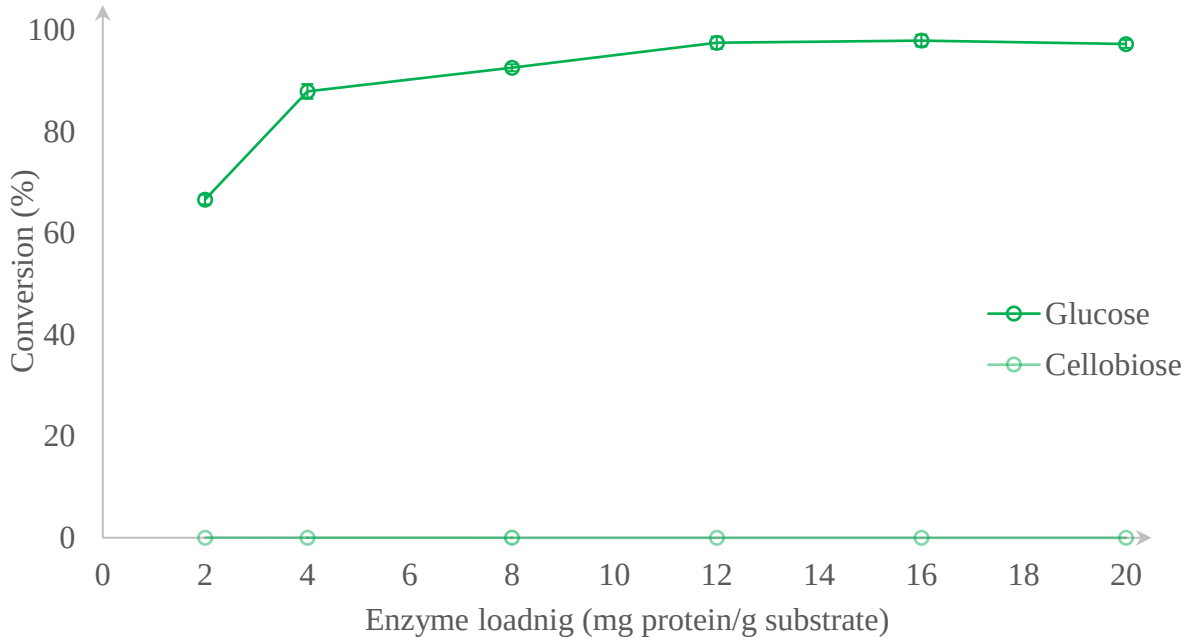


Figure 4.3.1. Loading analysis. Enzymatic conversion of ball milled white cotton substrate for 48 hours with 5 % dry matter content and 2 mM ascorbic acid. Various loadings of Cellic® CTec2 were applied and products were analysed by HPLC.

An enzyme loading analysis was also done with the Celluclast® enzyme cocktail, using the same conditions as with Cellic® Ctec2, in order to compare the response curves of the cocktails. (Figure 4.3.2.)

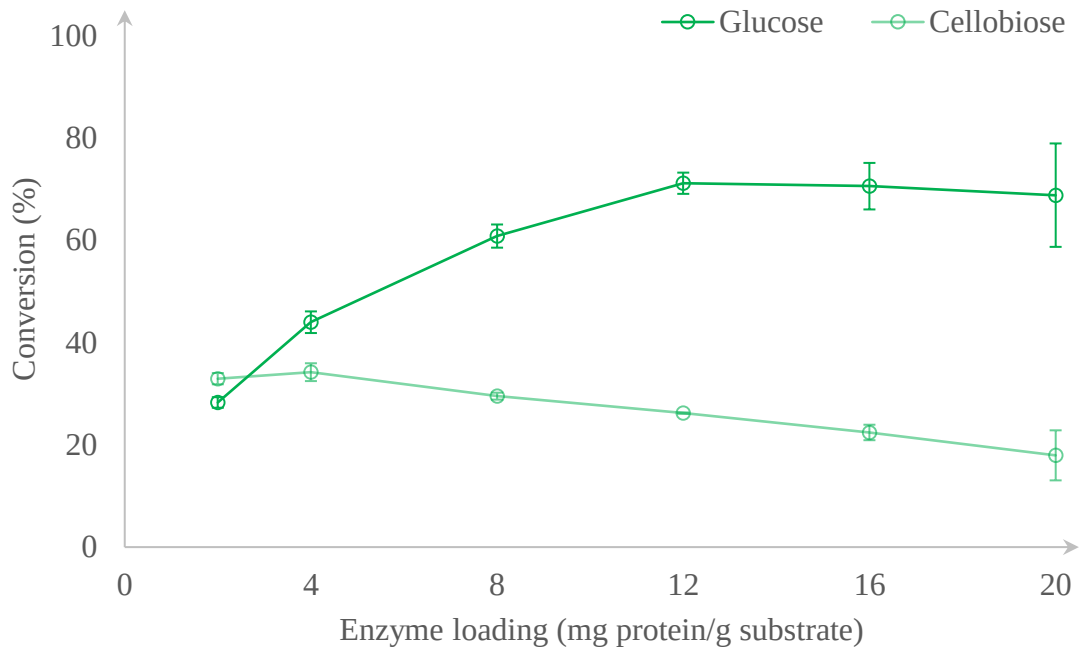


Figure 4.3.2. Loading analysis. Enzymatic conversion of ball milled white cotton substrate for 48 hours with 5 % dry matter content and 2 mM ascorbic acid. Various loadings of Celluclast were applied and products were analysed by HPLC.

In the Celluclast® enzyme loading analysis, an enzyme loading of 4 mg per g cellulose converted 44 ± 2 % of the cellulose into glucose, while 61 ± 2 % was converted when using 8 mg loading. The conversion into glucose was 71 ± 2 % when increasing to 12 mg loading and does not seem to increase further for higher loadings. In regards of total conversion (cellobiose and glucose), the conversion was 76 ± 4 % at 4 mg enzyme loading, 89 ± 3 % at 8 mg, and 96 ± 2 % when increasing to 12 mg. For 16 mg and 20 mg loading, the total conversion approximately reaches 100 %, standard deviations are 6 % and 14 %, respectively. The triplicates representing 16 mg loading had a even dispersion with 86 %, 91 % and 99 %. The triplicate of 20 mg loading contained two replicates calculated to yields of 92 % and 99 % and one replicate calculated to 66 %.

Table 4.3. Enzymatic conversion of ball milled white cotton substrate for 48 hours with 5 % dry matter content and 2 mM ascorbic acid. Various loadings of Celluclast were applied and products were analysed by HPLC.

Celluclast loading (mg/g)	Conversion (%) into sugars		
	Total	Glucose	Cellobiose
2	60 ± 2	28 ± 1	31 ± 1
4	76 ± 4	44 ± 2	32 ± 2
8	89 ± 3	61 ± 2	28 ± 1
12	96 ± 2	71 ± 2	25 ± 0
16	92 ± 6	71 ± 5	21 ± 2
20	86 ± 14	69 ± 10	17 ± 5

Celluclast® shows different loading-response development glucose production and for total conversion, and the decision of what enzyme loading to use in time course experiments (see sections 4.4 and 4.5) was based on the latter. The conversion yields of Celluclast enzyme loading assays are shown in Table 4.3. An enzyme loading of 8 mg was chosen for further use in analyses with Celluclast®, this is discussed further in Chapter 5.2.1.

4.4. Time course analysis with Celluclast®

Time course experiments were set up to assess ideal duration for efficient conversion of the textiles. The conversion over time was investigated by time course analysis with Celluclast® at pH 5.0 with 5 % dry matter content with 8 mg enzyme loading. White cotton substrate with and without ball milling were analysed, as well Avicel as a control substrate.

As Figure 4.4 illustrates, conversion of all three substrates increase over time. The ball milled white cotton shows a total conversion of 67 ± 1,1 % after 24 hours, and at 96 hours degradation the conversion was 88 ± 2.3% (Table 4.4 a). For glucose production, the steepest part of the curve in Figure 4.4.1 is between 24 and 48 hours. At the same time, cellobiose has a decrease. Conversion of the substrate into glucose reaches its maximum at 75 ± 2,4 % after 96 hours (Table 4.4.b). The laboratory standard, Avicel, has a maximal total conversion at 53 ± 3,4 % after 96 hours.

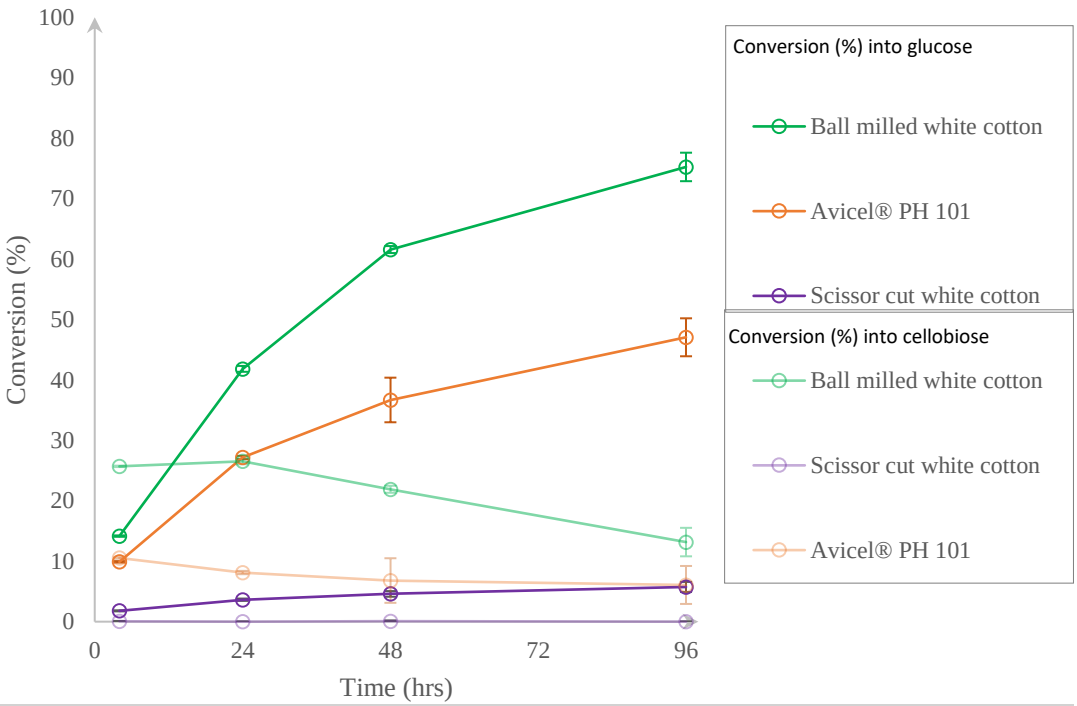


Figure 4.4: Conversion of cotton textiles and Avicel by Cellluclast. Conversion of WD, BWD and Avicel into glucose and cellobiose by 8 mg Cellucllast per g glucan , measured by HPLC. Reaction pH 5.0 and 5 % dry matter content.

The ball milled white cotton substrate shows the highest conversion for all time points in the analysis, in average 1.9-fold higher than Avicel. The scissor cut white cotton substrate, has a lower conversion at all time points, 19-fold lower than the ball milled substrate. Controls without enzyme and blind samples without substrate contained no detectable sugars. After 0 hours the conversion of the samples is assumed to be 0 % as for the controls

Table 4.4: Total conversion (a) conversion into glucose (b) of cotton substrates and Avicel by Celluclast with enzyme loading was 8 mg protein per g substrate, pH 5.0 and 5 % dry matter content. Quantification of glucose and cellobiose was obtained by HPLC. The ratio between conversion of the three substrates are presented.

a)

	Total conversion (%)							
Time (hrs)	4		24		48		96	
Ball milled textile (BWD)	38.6	± 0.4	67	± 1.1	82.4	± 0.7	88	± 2.3
Avicel® PH 101	20.4	± 0.3	35.3	± 0.4	43	± 4.3	53	± 3.4
Scissor cut textile (WD)	1.9	± 0.1	3.6	± 0.2	4.7	± 0.6	5.7	± 0.8

b)

	Conversion (%) into glucose							
Time (hrs)	4		24		48		96	
Ball milled textile (BWD)	14.2	± 0.1	41.8	± 0.5	61.6	± 0.6	75	± 2.4
Avicel® PH 101	9.9	± 0.1	27.2	± 0.3	37	± 3.7	47	± 3.1
Scissor cut textile (WD)	1.8	± 0.1	3.6	± 0.2	4.6	± 0.5	5.7	± 0.8

4.5. Time course analysis with Cellic®CTec2

Several time course experiments were carried out using Cellic® CTec2, known to be an efficient enzyme cocktail for converting cellulose into glucose, shown for Avicel and various types of pre-treated cellulosic biomass.

4.5.1. Comparing cotton from white denim before and after ball milling

Cellic® CTec2 was used in an analysis under similar conditions as Celluclast®, at pH 5.0 with 5 % dry matter content. However, the enzyme loading was 4 mg and 2 mM ascorbic acid was present. BWD, WD and Avicel were analysed. Figure 4.5.1 shows the conversion during the time course, and Table 4.5.1 present the conversion yields with standard deviations.

After 24 hours, the ball milled white denim showed a conversion of 92 ± 5.2 %, and the conversion after 48 hours displayed similar conversion with 92 ± 4.7 % (Figure 4.5.1). At 96 hours degradation, the

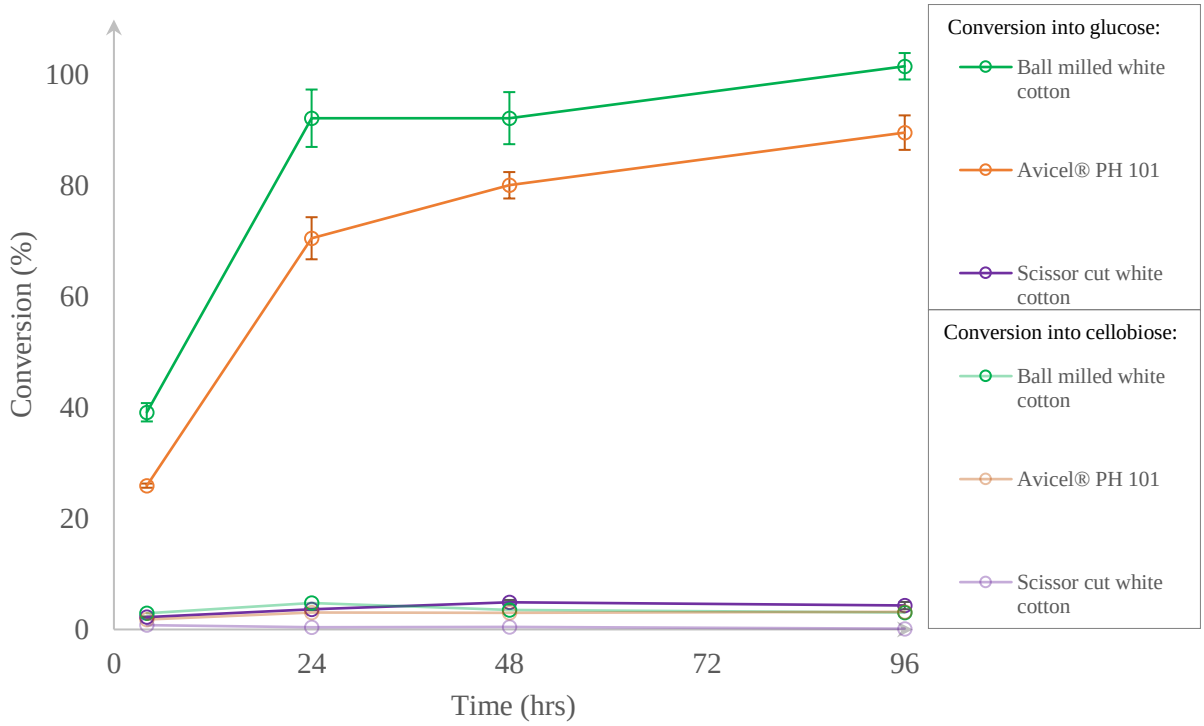


Figure 4.5.1: Conversion of cotton substrates and Avicel into glucose and cellobiose by Cellic® CTec2. WD, BWD and Avicel were converted by 4 mg enzyme per g glucan measured by HPLC. Ascorbic acid concentration was 2 mM, pH 5.0 and 5 % dry matter content.

reaction products show slightly higher conversion and seems to reach a theoretical maximum, showing 102 ± 2.4 %. The steepest part of the curve is clearly observed between 4 and 24 hours. Conversion after 4 hours seems to produce less than half of theoretical possible yield, with 39 ± 1.7 % conversion.

Avicel had a maximal conversion at 93 ± 2.8 % after 96 hours. With 27.7 ± 0.7 %, 74 ± 3.9 % and 83 ± 2.4 % after 4, 24 and 48 hours, respectively, a similar trend in conversion over time between Avicel and BWD was observed, although higher at all time points for BWD.

All analysed samples in this experiment contain less than 2.5 g/L cellobiose, which is 5 % of the dry matter content. Blind samples without enzyme contained no detectable sugars. Blind samples without substrate contained 0.1 g/L cellobiose and 1 g/L glucose, which was subtracted in calculations.

Table 4.5.1: Conversion of cotton substrates and Avicel by Cellic® CTec2 with enzyme loading 4 mg protein per g cellulose, pH 5.0 and 5 % dry matter content. Quantification of sugars was obtained by HPLC.

	Total conversion (%)							
Time (hrs)	4		24		48		96	
Ball milled white cotton	39	± 1.7	92	± 5.2	92	± 4.7	102	± 2.4
Avicel® PH 101	27.7	± 0.7	74	± 3.9	83	± 2.4	93	± 2.8
Scissor cut white cotton	3.0	± 0.5	4.0	± 0.5	5.4	± 0.8	4.4	± 0.9

4.5.2. Time course analysis with variable dry matter concentrations

An experiment with increased dry matter of BWD over 96 hours was carried out to investigate the capacity of Cellic® CTec2 to convert the substrate at increased substrate concentrations. The experiment was set up as for 4.5.1, except for concentration of substrate and enzyme. The enzyme loading used was consistently 4 mg per g glucan. The results from the substrate conversion is shown in Figure 4.5.2. Conversion after 4, 24, 48 and 96 hours when the dry matter content was 10 % was 49 ± 1.6 %, 92 ± 3.1 %, 85 ± 1.3 % and 95 ± 5.2 %, respectively. At 20 % dry matter, the conversion was 43 ± 1.3 %, 78 ± 2.1 %, 78 ± 1.8 % and 87 ± 1.3 %.

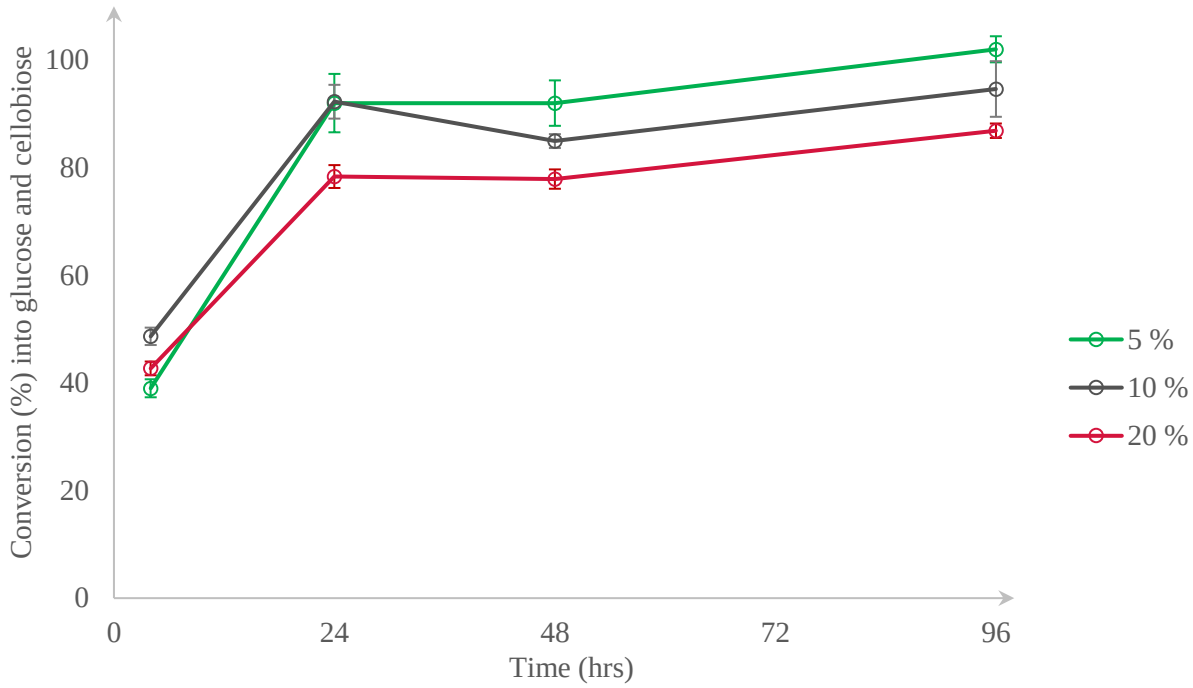


Figure 4.5.2. Conversion of BWD into glucose and cellobiose by 4 mg/g Cellic® CTec2, 2 mM ascorbic acid, pH 5.0 and varying dry matter content.

4.5.3. Comparison of conversion with and without ascorbic acid

LPMOs have been shown to increase the efficiency of crystalline biomass to soluble sugars (Chylenski et al. 2017b; Johansen 2016; Villares et al. 2017). Since LPMOs depend on an external electron donor, the presence and absence of ascorbic acid (AscA) was investigated by analysing the conversion of WD, BWD and Avicel into glucose during 96 hours with and without 0.2 mM AscA Figure 4.5.3.

In the absence of AscA, BWD was converted with 41 ± 2.1 % after 4 hours, whereas the conversion without AscA was 37 ± 1 %, whereas 41 ± 2.1 % was converted in the presence of AscA after the same time. The slightly higher conversion in the presence of AscA was observed only after 4 hours, while later time points showed slightly higher conversion in the absence of AscA. Overall, the conversion did not significantly differ for BWD.

After 4 hours the conversion of BWD was 3.1 ± 0.3 % and 3.5 ± 0.1 % in the presence and absence of AscA, respectively. After 24, 48 and 96 hours, the conversion did not show any difference.

For Avicel, the conversion yields in the presence of AscA were 21.2 ± 0.7 %, 60.7 ± 0.6 %, 61 ± 8.6 % and 87.3 ± 0.5 %, after 4, 24, 48 and 96 hours, respectively. In absence of AscA, the conversion was similar after 4 hours, showing 20.1 ± 0.6 %, while at all other time points it was significantly lower. After 24, 48 and 96 hours, the conversion was 46 ± 3.0 %, 58 ± 2.5 % and 79 ± 2.1 %.

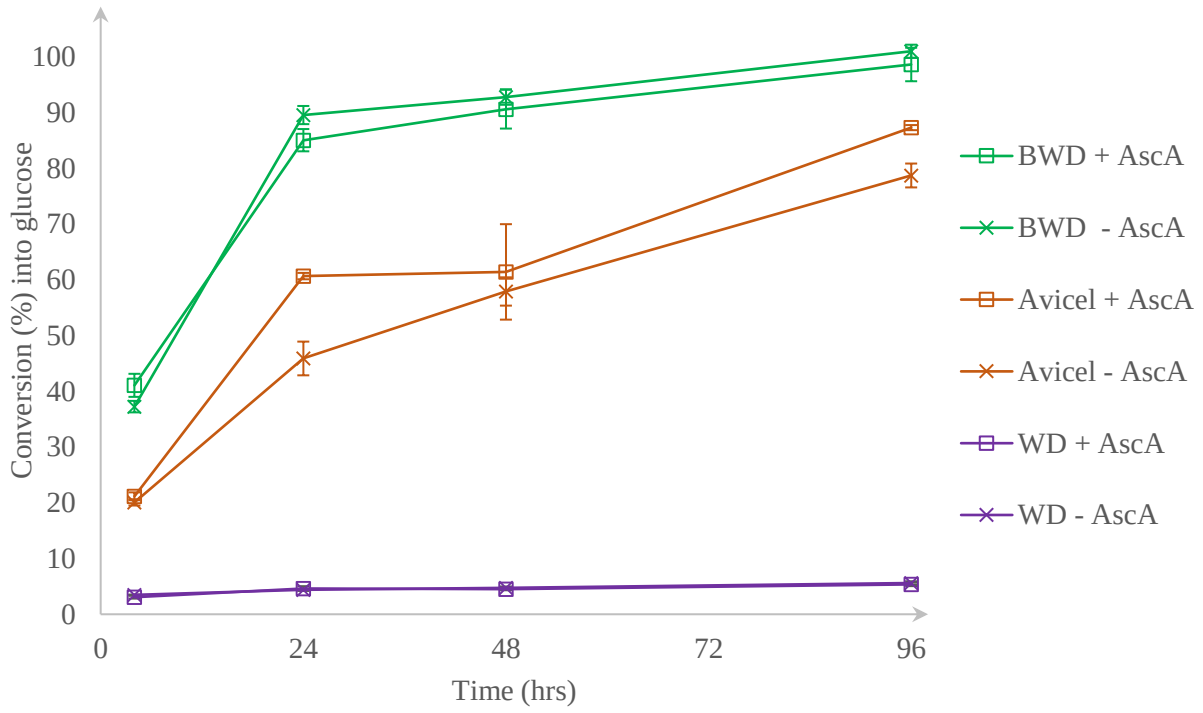


Figure 4.5.3 Conversion of cotton substrates and Avicel by Cellic® CTec2, with and without ascorbic acid (2 mM). Glucose is measured by HPLC. Enzyme loading was 4 mg protein per g glucan, pH 5.0 and 5 % dry matter content.

4.5.4. Comparison of degradation of cotton from undyed and indigo dyed denim

The content of the indigo dye stuff (described in section 1.5.2) and the part of denim production process called mercerization (1.3.3) are variables that might affect enzymatic conversion of denim fabrics. An experiment for comparing ball milled white denim and ball milled blue denim was carried out to investigate the conversion efficiency under the same conditions.

Enzymatic conversion of BBD was also investigated at 5 % DM with 4 mg Cellic® CTec2 per g glucan. Conversion of BBD into glucose (Figure 4.5.4) was 47 ± 2.3 % after 4 hours, 97.8 ± 0.5 % after 24 hours and 102 ± 2.8 after 48 hours. In the same experiment, BWD was converted with 39.7 ± 0.6 %, 80.0 ± 0.2 % and 90 ± 3.2 %.

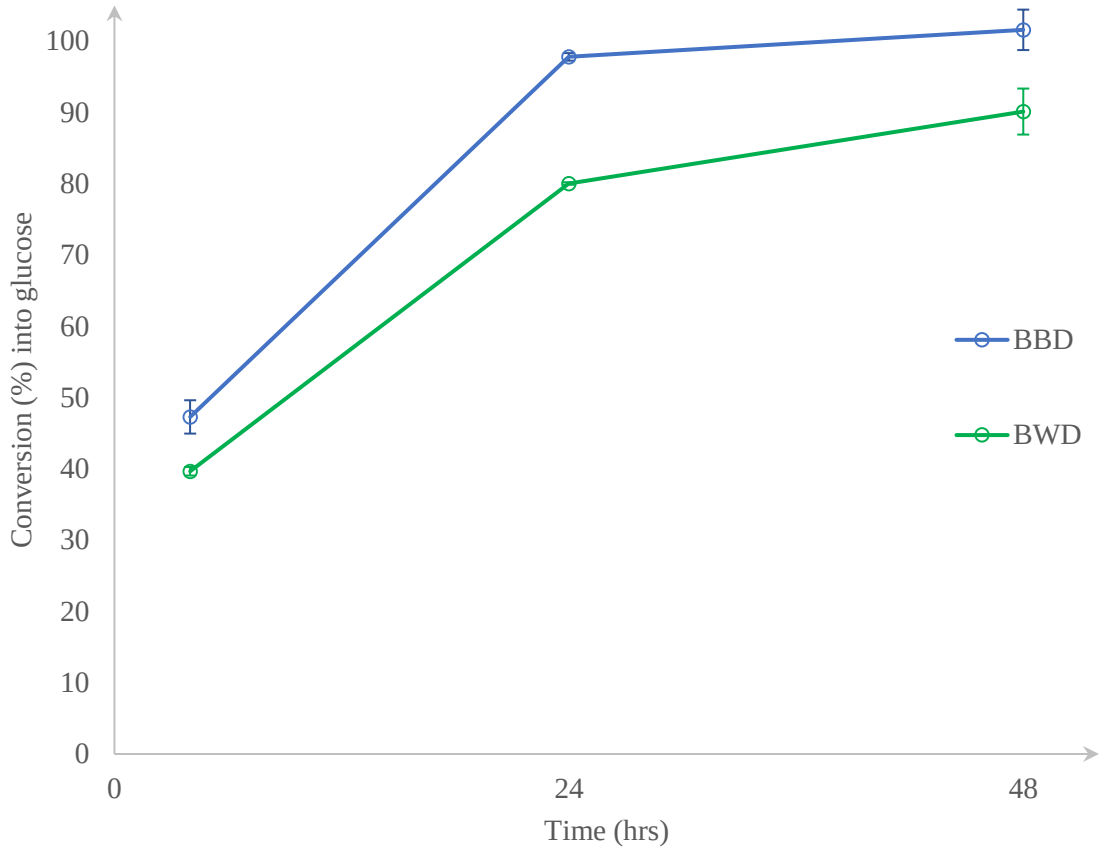


Figure 4.5.4. Conversion of BWD and BBD into glucose by Cellic® CTec2, measured by HPLC. Enzyme loading was 4 mg protein per g substrate, ascorbic acid concentration 2 mM, pH 5.0 and 5 % dry matter content.

4.6. *Ta*LPMO9A reactions

As already noted, addition of LPMOs to cellulose hydrolysis reactions has been shown to increase the conversion rate of the reaction. It was therefore of interest to investigate whether the cotton textile substrates were good substrates for LPMOs. In order to achieve such an analysis, the purified, recombinant LPMO, *Ta*LPMO9A from the fungus *Thermoascus auraantiacus* was used in reactions with cotton textile substrates. All denim substrates (WD, BWD, BD and BBD), Avicel and phosphoric acid-swollen cellulose (PASC) were incubated with *Ta*LPMO9A and ascorbic acid as electron donor.

4.6.1. Detection of oxidized oligosaccharides

Reaction products were analysed by MALDI-TOF. For the white denim substrate that was not ball milled, no peaks associated with oxidized cello-oligosaccharides were identified. Assigned mass values in the spectra are presented with the associated compounds in Table 4.6.1.

The ball milled substrate showed several m/z signals that might represent oxidized cello-oligosaccharides were observed (Figure 4.6.2). Due to the large background noise caused by the MALDI-TOF matrix in the low m/z region (up to m/z~ 400), products with an expected mass higher than 900 (i.e. DP higher than 6) were analysed. The mass spectrum resulting from LPMO activity on BWD (Figure 4.6.1), BBD (Figure 4.6.2), BD (Figure 4.6.4), Avicel (Figure 4.6.5) and PASC (Appendix S-7) contained peaks of mass per charge that correspond to the adducts of certain oxidized oligosaccharides, which structures are shown in Table 4.6.1.a. Spectra of substrate WD contained no such peaks (Figure 4.6.3). Lithium adducts are not included in the assessment, as Lithium is assumed not to be present in the reactions. The mass of a C1 oxidized lactone is equal to the mass of the C4 oxidized ketoaldose of the same DP, and geminal diols are equal to the aldonic acids of the same DP. Lactones and geminal diols are not assigned, as the equilibrium lies towards the aldonic acid and keto aldose sides of the reactions (pH~6.5).

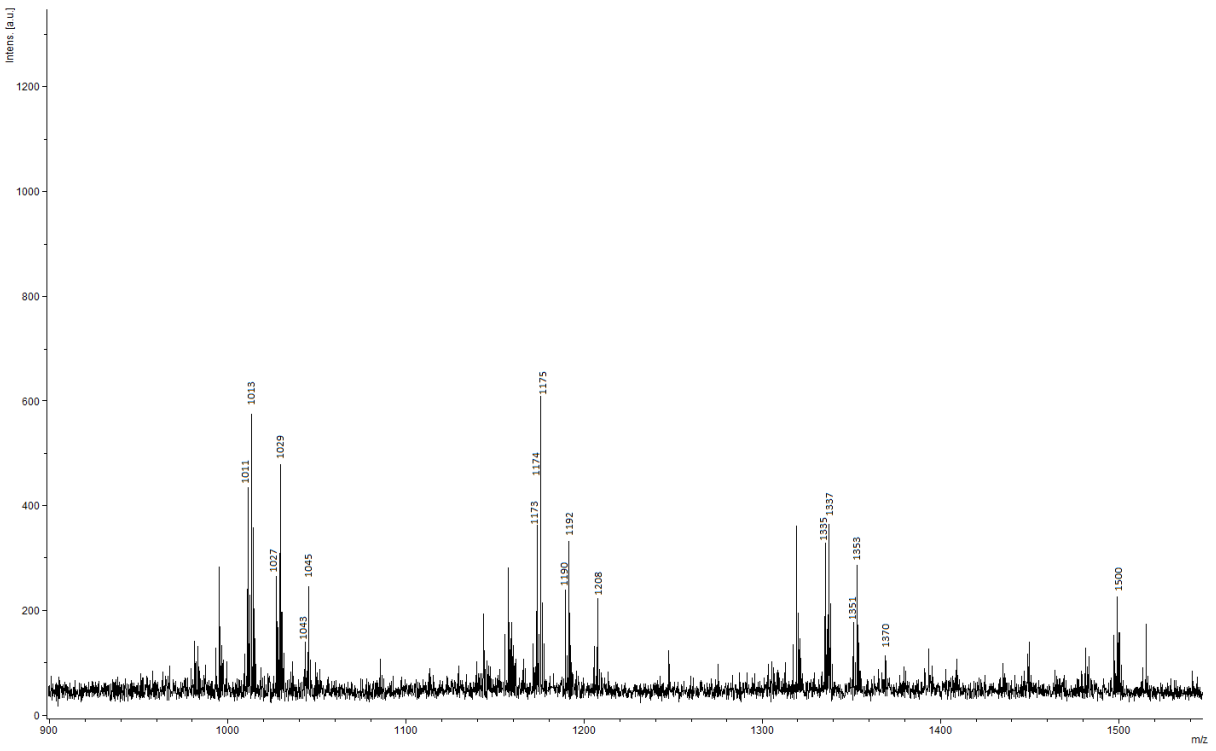


Figure 4.6.1.1:. MALDI-TOF analysis of oxidized products in BWD. The figure shows mass spectrum of ball milled white cotton treated with *Ta*LPMO9A in pH=6,5, 45°C at 1 000 rpm for 20 hours.

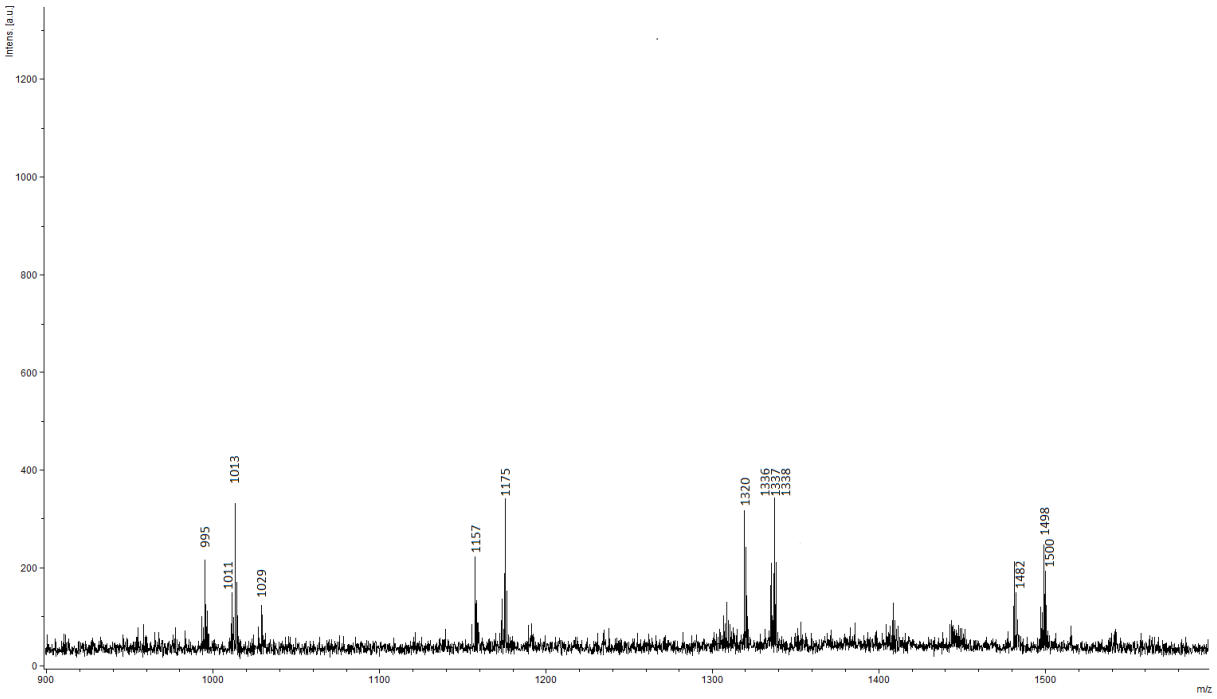


Figure 4.6.1.2: MALDI-TOF analysis of oxidized products in BBD. The figure shows mass spectrum of BBD treated with *Ta*LPMO9A in pH=6,5, 45°C at 1 000 rpm for 20 hours.

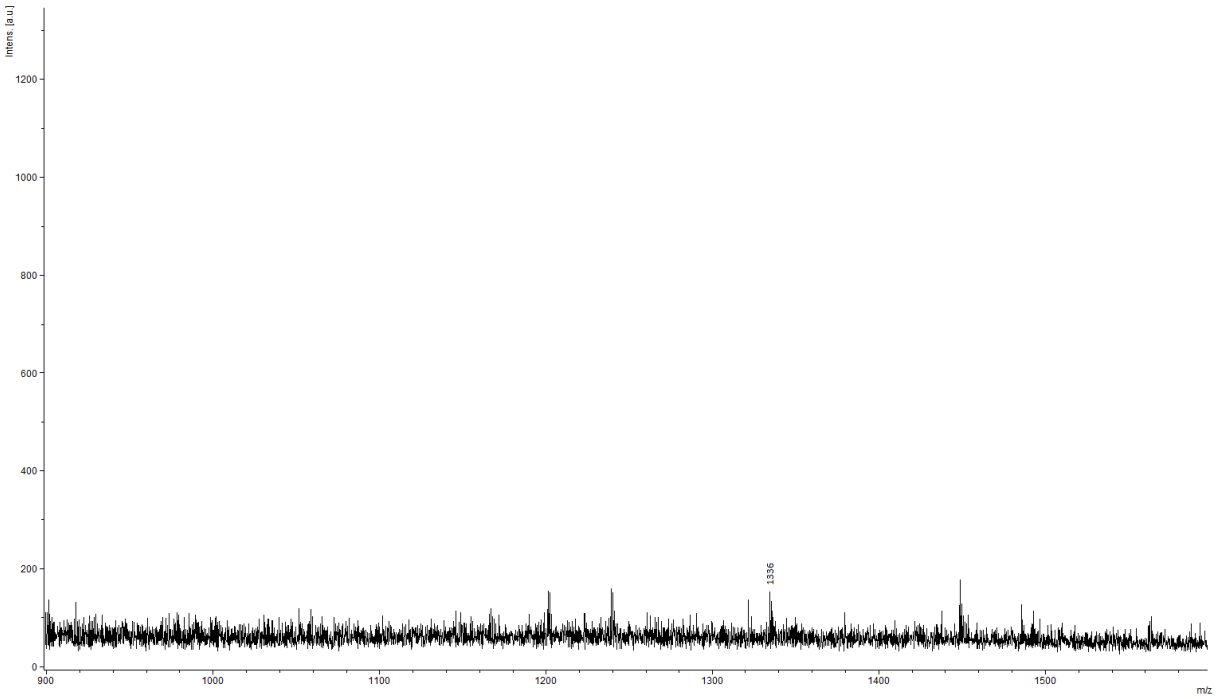


Figure 4.6.1.3: MALDI-TOF analysis of oxidized products in WD. The figure shows mass spectrum of white denim fabric treated with *Ta*LPMO9A in pH=6,5, 45°C at 1 000 rpm for 20 hours.

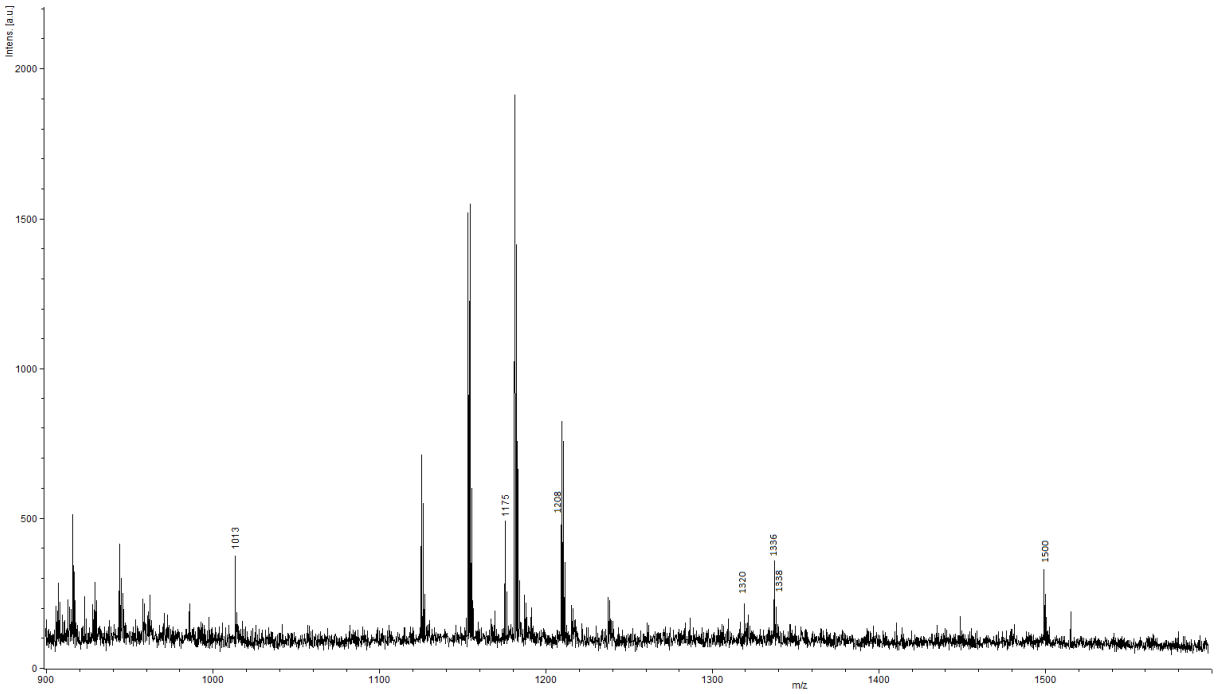


Figure 4.6.1.4: MALDI-TOF analysis of oxidized products in BD. The figure shows mass spectrum of blue denim fabric treated with *Ta*LPMO9A in pH=6,5, 45°C at 1 000 rpm for 20 hours.

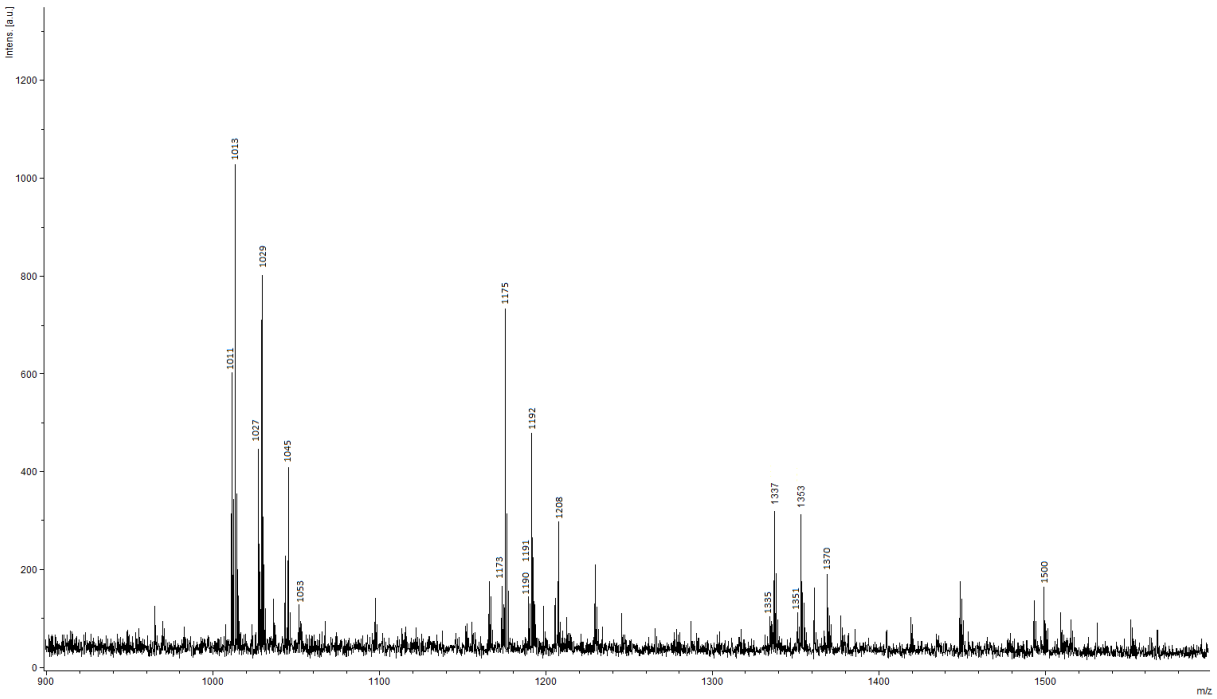
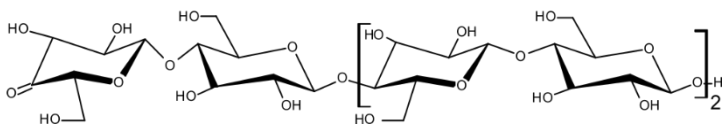
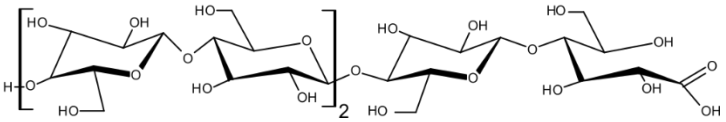
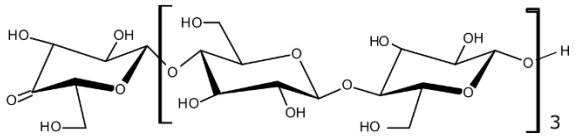
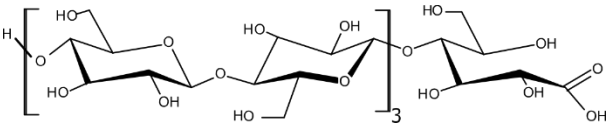
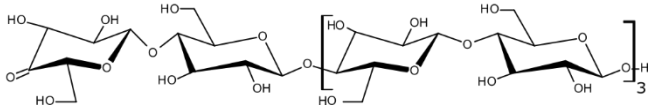
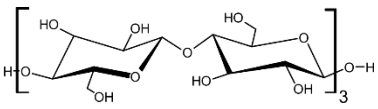
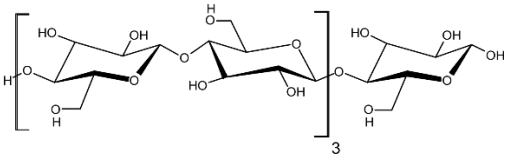
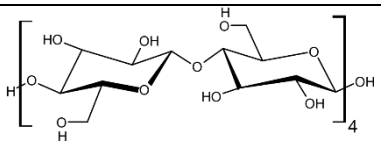


Figure 4.6.1.5. MALDI-TOF analysis of oxidized products in Avicel. The figure shows mass spectrum of Avicel treated with *Ta*LPMO9A in pH=6,5, 45°C at 1 000 rpm for 20 hours.

Table 4.6.1.a Structure of oxidized cello-oligosaccharides indicated by mass spectra to be present in reactions where cotton substrates were incubated with *Ta*LPMO9A in pH=6,5, 45°C at 1 000 rpm.

DP	m/z	Structure	Referred to as
6	1011.8		$[Glc4KGlc_5+Na]^+$
6	1027.9		$[Glc4KGlc_5+K]^+$
6	1029.8		$[Glc_5Glc1A+Na]^+$
6	1045.9		$[Glc_5Glc1A+K]^+$
6	1052.8		$[Glc_5Glc1A-H+2Na]$
7	1173.9		$[Glc4KGlc_6+Na]^+$
7	1190.0		$[Glc4KGlc_6+K]^+$
7	1191.9		$[Glc_6Glc1A+Na]^+$
7	1208.1		$[Glc_6Glc1A+K]^+$
7	1214.9		$[Glc_6Glc1A-H+2Na]$
8	1336		$[Glc4KGlc_7+Na]^+$
8	1352.2		$[Glc4KGlc_7+K]^+$

b. Structure of native cello-oligosaccharides indicated by mass spectra to be present in reactions where cotton substrates were incubated with *Ta*LPMO9A in pH=6,5, 45°C at 1 000 rpm. Because $m(K^+) = m(O^{2-}) + m(Na^+) = 39\ g/mol$, the mass of any aldonic acid-sodium adduct equals the mass of the corresponding potassium-native adduct (e.g. m/z=1192).

DP	m/z	Structure	Referred to as
6	1013.8		$[Glc_6 + Na]^+$
6	1029.9		$[Glc_6 + K]^+$
7	1176		$[Glc_7 + Na]^+$
7	1192		$[Glc_7+K]^+$
8	1338		$[Glc_8 + Na]^+$
8	1354.2		$[Glc_8+K]^+$

4.6.2. Comparing quantities of oxidized dimer in substrates

HPAEC-PAD was used to separate native cello-oligosaccharides and oxidized sugars in the reactions containing textile cotton substrates and *Ta*LPMO9A. Figure 4.6.2 shows the separation of soluble oligosaccharides coming from reactions with in BWD, WD, BBD, BD, PASC, Avicel, compared to a standard of oxidized cellobiose (i.e. the geminal diol with DP 2 at pH 13 in this analysis) and native oligomers of DP 2-5. The ketoaldose from the standard was eluted after 25 minutes. The peaks corresponding to native oligomers and peaks that most likely correspond to oxidized products of higher DP were significantly more intense for PASC than the few peaks separated for textile substrates.

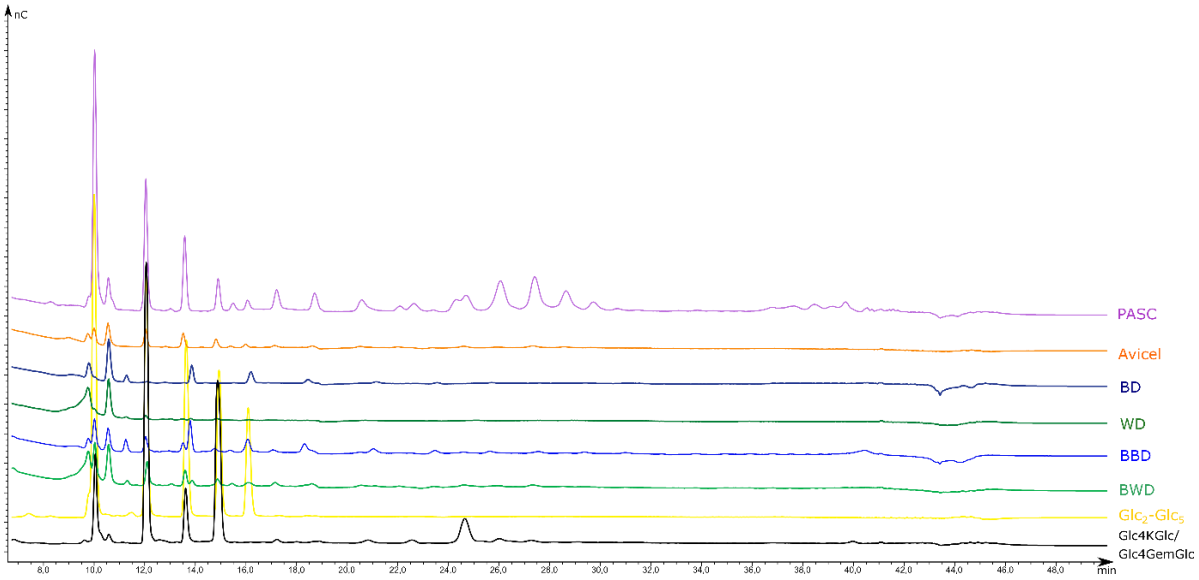


Figure 4.6.2 a. Analysing for oxidized dimer by IEC. HPAEC-PAD chromatograms of BWD, BBD, WD, BD, Avicel and PASC treated with *Ta*LPMO9A in the presence of ascorbic acid, at pH=6,5, 45°C and 1 000 rpm for 20 hours.

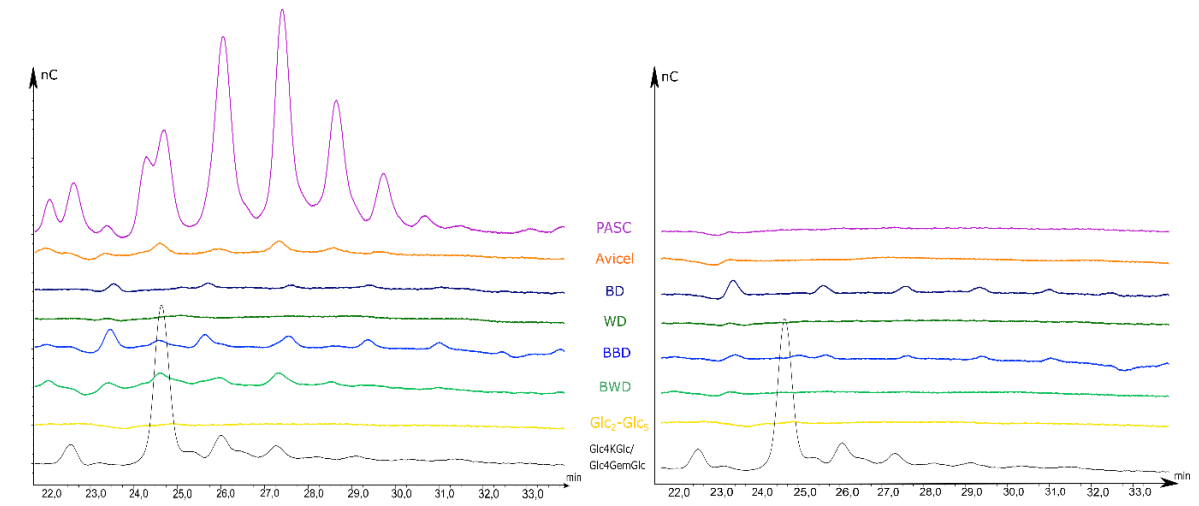


Figure 4.6.2 b. The peaks corresponding to oxidized oligosaccharides in samples with *Ta*LPMO9A and controls.

5. Discussion

The objective of this study was to investigate the hydrolysis efficiency of cotton textiles pretreated by ball milling. The substrates were initially characterized by a compositional analysis determining cellulose, ash and water content. The hydrolysis yields of two commercial enzyme cocktails were investigated, including several experiments to optimize the method for obtaining high conversion with low input (costs and chemicals) by decreased enzyme loading and increased dry matter. In order to avoid interfering factors present in the cotton textile, the experiments were initially done with a white cotton fabric from a denim garment of pure cotton. Later, we wanted to investigate whether cotton from denim coloured by indigo showed similar conversion yield, due to indications of affinity between indigo and cellulases in the literature and differences in the production of denim fabrics. Finally, reactions with LPMO as the sole enzyme activity were conducted and analysed qualitatively to observe whether significant LPMO activity occurs in the degradation of the textiles. The main aim of the present MSc project was to develop an efficient method for enzymatic conversion of cotton cellulose to glucose.

5.1. Substrate composition

In order to assess accuracy of this study, the laboratory standard Avicel was included in the compositional analysis for glucan quantification as well as the enzymatic hydrolysis. Avicel is assumes to consist of pure cellulose (Gupta & Lee 2009; Jeihanipour & Taherzadeh 2009) or nearly pure cellulose (Yang et al. 2006) in the literature. More specifically, the glucan content Avicel PH-101 has been determined to 92.2 %, with 2.1% xylan, 0.9 % % lignin and 0.1 % mannan, as percentage of total solids (Chylenski et al. 2017b). In this study, the glucan content of Avicel PH-101 was determined to 92.5 ± 0,5 % of total solids (Figure 4.1). Lignin and hemicellulose content was not determined because the cotton textiles are assumed to not contain lignin nor hemicellulose. The glucan content given as percentage of total sample weight was determined to be 96,8 % in Avicel PH-101 (Peciulyte et al. 2017). Similarly, 95.6 ± 0.5 % was the fraction of total sample weight in this analysis (Table 4.1). The accuracy of glucan content determination might be influenced by errors in practical weighing, pipetting of samples or the quantification by HPLC (e.g. the dilution of calibration standards). Additionally, the batch differences are unnkown. The determination of total solid contents of the substrates also has an impact on the glucan content when the calculations of glucan content (Equation 3.2.2) are based given as percentage of total solids. The accuracy of the glucan quantification in this analysis is assumed to be sufficient based on the high siilarity of the determined values with values found in the literature.

The compositional analysis included white denim fabric (WD), ball milled WD (BWD), blue denim fabric (BD) and ballmilled BD (BBD). The glucan content (fraction of total solids) of WD and BWD were determined to 91 ± 2.4 % and 96 %, respectively. The determination was carried out with WD and

calculated separately according to the different total solid contents. The content of total solids in the substrates were 97,4 ± 0,2 % and 93 ± 2 % for BWD and WD, respectively. Likewise, the glucan content was 88 ± 0,1 % and 88,7 for BD and BBD, and the total solid contents 96.0 ± 0.1 % and 95.4 ± 0.2 %, respectively. The glucan content and the total solid content of each substrate is used to set up enzymatic conversion experiments and to calculate the conversion yields. First, the total solids content was used to ensure the right dry matter content (DM) in reactions (for details, see Appendix S1- S7). The DM is further used in the calculations of conversion yield (Equation 3.6.3.2), where the glucan content is also a variable in the formula. From the glucan quantification, the standard deviations give an uncertainty on the second numeral digit for both textile substrates. The standard deviations give a rather small effect on the further calculations for all except BWD. The high standard deviations in total solid content after ball milling were most likely due to irregular absorbance of water. The accuracy of enzymatic conversion yields might be more reduced for BWD than the other substrates due to higher uncertainty in total solid content. The uncertainty of glucan and water contents in the textile and the variation in enzymatic conversion yield within a triplicate was assumed to account for the same range of dispersion. However, the accuracy of conversion yields might be affected from the dispersion, because the total solid and glucan content determined in the compositional analysis are used to set up and to calculate yields for the enzymatic hydrolysis experiments. The standard deviation for cellulose content in Avicel is 0,5 %, which indicated that the higher standard deviations in the cotton substrates are not due to analysis method, but rather due to irregularities in the material. The accuracy of the analysis can be assessed by comparison with the literature. However, it is likely that denim fabrics vary in composition due to differences in production and in the source of the raw cotton. The cellulose content of blue denim fabric has previously been estimated to be 93 ± 1 % (of total solids) by a determination protocol with small differences to the protocol applied in this work (Jeihanipour & Taherzadeh 2009; Ruiz & Ehrman 1996; Sluiter 2012). The cellulose content obtained by Jeihanipour and Taherzadeh is higher than what was determined for blue denim fabric in this study (89 ± 1.5 %). Whether the difference is caused by differences in the textile composition or analytical errors is unknown, it might also be a combination.

The ash content of the substrates is not known to have any impact on the enzymatic hydrolysis. In the present study, ash content was determined to study the differences of the cotton substrates, and in order to determine the mass balance. Other, unidentified compounds, account for 6 -7 % of total sample weight in both substrates.

5.2. Conversion of cotton textiles by commercial cocktails

5.2.1. Enzyme loading

Ball milled white cotton was converted into glucose and cellobiose by Cellic® CTec2 and Celluclast® at 5 % DM for 48 hours, using 2 mg, 4 mg, 8 mg, 12 mg, 16 mg and 20 mg protein per g glucan in substrate. A low loading of enzyme is beneficial in industrial appliance, since the enzyme component usually represent the highest expense in industrial conversion of cellulosic biomass (Barakat et al. 2013). Conversion over 80 % are often considered as industrially acceptable minimum yields, e.g by the National Renewable Energy Laboratory, NREL (Chylenski 2017).

Celluclast® converted BWD into glucose and cellobiose, resulting in approximately equal amounts of the two products at 2 mg enzyme loading (Table 4.3). At loadings higher than 4 mg, the trend observed was a decrease of the dimer and an increase of the monomer, likely to be caused by enzymatic cleavage of cellobiose into glucose by β-glucosidase activity. This indicated a low content of β-glucosidases (BGs) in Celluclast®, which has previously been shown in several studies (Cannella et al. 2012; Peciulyte et al. 2015; Rodrigues et al. 2015). Moreover, Rodrigues and colleagues also demonstrated that the BGs of Celluclast® are less stable under the given reaction conditions than the BGs in Cellic® CTec2. Higher loadings of the cocktail necessarily provide more BGs, which seemed to cause the decreasing and almost linear trend of cellobiose concentration. Total conversion increased from 60% to 89% for the lowest loadings. Likewise, conversion into glucose increased from 28% to 61%. The glucose concentration and the total conversion increased until 12 mg loading that gave 96 ± 2 % total conversion. Higher loadings did not show significant increase in conversion, neither what regards total conversion nor glucose production, as shown in Figure 4.3.2. When degrading BWD with 20 mg enzyme loading, relatively large standard deviations were observed (the total conversion yield was calculated to be 86 ± 14%, see Table 4.3). One of the replicates (66%) had a calculated yield significantly lower than the two other replicates of the same triplicate, and lower than the calculated average values representing lower enzyme loadings. Assessing the lower replicate yield as an outlier would give an average yield of 96 ±3 % (instead of 86 ± 14%) for 20 mg enzyme loading, which corresponds well to the assumption that a maximal conversion yield indicated at 12 mg loading does not decrease by increasing the loading. However, the two remaining replicates alone are believed to not represent the dispersion and all three replicates are kept.

In order to select an enzyme loading value for time course hydrolysis experiments with Celluclast®, 8 mg loading was selected, for two reasons. Firstly, because the industrially preferred 80 % conversion yield was reached using this loading (Figure 4.3.2), considering both glucose and cellobiose. Secondly, it was interesting to investigate whether 61 ± 2 % glucose yield would increase up to 80 % with time under a consistent enzyme loading. Further investigation of lower Celluclast® loadings with additional BGs might be of interest for further research.

In the experiment determining the optimal enzyme loading of Cellic® CTec2 for BWD, 4 mg Cellic® CTec2 per g glucan resulted in satisfactory yields (i.e. over 80 % conversion, more specifically 88 ± 2 % (Figure 4.3.1). Moreover, the incline of the conversion curve had a decrease between 4 and 8 mg loading, clearly illustrated by the fact that increasing the loading by double give approximately 5 % higher conversion, while increasing by the same factor from 2 to 4 mg gave 32 % higher conversion. For these two reasons, 4 mg Cellic® CTec2 per g glucan was selected for further hydrolysis experiments.

A trend similar to the dose-response curve in Figure 4.3.1 has been seen in degradation of pretreated Norway spruce and sugarcane bagasse, where analysis at 5 % DM for 48 hours has been investigated by 2 mg, 4 mg, 6 mg, 8 mg, 10 mg, 16 mg and 20 mg Cellic® CTec3 per g glucan (Chylenski et al. 2017a). When increasing from 2 mg to 4 mg loading, the conversion of substrate into glucose was doubled for spruce and increased with almost 50 % for sugarcane bagasse. The incline of the loading curve decreased at the higher loadings in the degradation of pretreated lignocellulosic biomass with Cellic® CTec3, which is claimed by the producer to have a 1.5 times performance in conversion of hemicellulose and cellulose to fermentable sugars (Novozymes). The pretreatment utilized for Norway spruce and sugarcane bagasse

5.2.2. Effect of pretreatment

The reactions with 5% DM and 8 mg/g loading of Celluclast® resulted in significantly higher conversion of BWD than WD (Figure 4.4). Total conversion of BWD substrate was 19-fold higher than for WD, calculated as an average of the ratio for each time point (Table 4.4 a). At the time point where total BWD conversion normally exceeds 80 % (after 48 hours), the yield of BWD conversion was 17- fold higher than the conversion of WD. Glucose production was also more efficient for the ball milled substrate, although the ratios are smaller (Table 4.4 b). At most, the ratio of WD:BWD was 1:13, which was observed after 48 and 96 hours hydrolysis. Cellic® CTec2 gave on average 21-fold higher conversion for the cotton when it was ball milled (Figure 4.5 and Table 4.5). After 24 hours 80 % conversion is exceeded. At this time point, the ratio is 1:25. Ball milling of this denim fabric (WD) unambiguously increases the saccharification by both enzyme cocktails under the given conditions.

In 1974, waste cotton among other residues were digested by *Trichoderma viride* (Mandels et al. 1974) with the aim to convert cellulosic waste into glucose and to use cellulosic waste as medium to produce cellulases. For the cotton samples, the conversion yield ranged from 2-92 %. The most efficient pretreatment for enhanced enzyme susceptibility was ball milling. Fibrous cotton waste was converted without and with ball milling as pretreatment, where the cotton had a conversion yield 5-fold higher after milling (55 % compared to 10 %). Despite lack of information of the cotton type and structure as

well as information about the ball mill type, the trend is clearly in accordance with the results presented in this work. Avicel was converted under the same conditions, with a conversion yield of 33 %.

Jeihanipour & Taherzadeh converted cotton from blue jeans at 3% DM with 20 FPU Cellulases and 30 IU BGs per g cellulose for 48 hours (Jeihanipour & Taherzadeh 2009). They found that pre-treatment was necessary to achieve a conversion into glucose higher than 23,6 %. Avicel was converted 39 % into glucose at the same time point and under the same conditions. They describe the cotton samples as "shredded and ground to small size", and the cellulose content was determined to be 93 ± 1 % before pretreatment. In order to increase the conversion, they pretreated the cotton with phosphoric acid and sodium hydroxide at 0°C, 23°C and 100°C for 3 hours, which increased the conversion up to 99 % (based on the cellulose content after pretreatment). The solvent NMMO was later applied as pretreatment for blended (50/50) cotton and polyester fibres, where the cellulose was converted up to 95,8 % after 48 hours by the same enzymes and loading at 5% DM (Jeihanipour, A. et al. 2010). The textile substrate was cut to small pieces of approximately 9 cm^2 prior to pretreatment. Enzyme activity studies were not a part of investigating enzymatic conversion of WD and BWD in the present study, and the activity of the applied enzyme cocktails is unknown. However, based on other batches of the same cocktails, information about enzyme activity might be used as rough estimates. 4 mg/g glucan of Cellic® CTec2 corresponds to a cellulase (EG and CBH) activity of 3 FPU per g glucan and a BG content of 68 U per g glucan (Cannella et al. 2012). A loading of 8 mg/g glucan Celluclast® is likewise estimated to 4 FPU per g glucan and a 1 U of BG per g glucan. The loading of enzymes appears to have been significantly higher in the experiments of Jeihanipour & Taherzadeh than for experiments with the two cocktails used in the present study.

Cellic® CTec2 has also been used to degrade cotton gin dust and cotton gin waste (Fockink et al. 2015). The dust and waste removed from cotton at this initial step of textile production contains recalcitrant lignin and hemicellulose, which might hamper efficient hydrolysis. Alkaline pretreatment was used to reduce the lignin content in the biomass and increase the glucan content. The hydrolysis was carried out with the pretreated substrates containing 89 % (CGD) and 81 % (CGW) glucan and an enzyme loading of 85 mg per g DM. After 96 hours, the conversion of the substrates was 54 % at the most. This was the CGD with the most severe pretreatment (4 % NaOH at 120 °C) at 15 % DM, which decreased lignin and hemicellulose the most. The enzyme loading was 76 mg per g cellulose for the CGD, which is a tenfold higher than the loading applied on cotton textiles in this thesis. CGD contains short fibres recovered from filter screens during the ginning process, and might be or varying particle size. The difference in enzyme loading needed to convert CGD and BWD may be a consequence of differences in available surface area (i.e. particle size). It is possible that a reduction in particle size would result in lower required Cellic® CTec2 loading per glucan concentration. It is also possible that further decreasing or even removing the content of lignin and hemicellulose could have the same effect, possibly

conducted by more severe alkaline pretreatment or by utilizing hemicellulases and enzymes degrading lignin.

In the present study, ball milling of the white cotton substrate resulted in a powder measured to have a size distribution with sauter mean diameter of 12,5 µm (see Table 4.2). Sofla and co-workers extracted cellulose from lignocellulosic biomass by chemical and mechanical treatment, resulting in nanocrystals and nanofibrils, respectively (Sofla et al. 2016). The mechanical treatment was carried out under wet conditions in a shaker mill, which is a vibratory ball mill. The frequency of impact between balls and sample is higher for the shaker mill, while the speed of the balls is higher for the planetary mill. (Aliofkhazraei 2015; Li 2016). In the comparative analysis of Sofla et al., they extracted cellulose from lignocellulosic biomass (sugarcane bagasse) by chemical and mechanical treatment, resulting in cellulose nanocrystals (CNC) and nanofibrils (CNF), respectively. The size of CNC was 160-400 nm in length and 20-30 nm in diameter, while CNF had length about 2000 nm and diameter of 50 nm. Sofla et al. also measured the crystallinity by X-ray powder diffraction and compared with lignocellulose and extracted cellulose and found that the crystallinity was not reduced after ball milling by comparing CNF and purified cellulose. If this is the case for cotton milled in a planetary ball mill, the increased enzymatic digestibility of the substrates is not due to change in crystallinity. Sofla and colleagues along with other researchers, saw that crystallinity of CNC increases due to chemical remove of amorphous cellulose. This might explain why the ball milled cotton was more efficiently degraded than microcrystalline cellulose Avicel in the time course experiments in the present study, although the mill differs and the treatment of Avicel is not available. Other studies have shown a reduction in crystallinity of cellulose after ball milling (Barakat et al. 2013).

It is apparent that ball milling of cellulosic substrates results in a reduction in particle size and a concomitant increase in productive binding sites for cellulolytic enzymes (Nakagawa et al. 2011; Suckling et al. 2017).

Considering the large effect of ball milling on the conversion yields obtained in this study, and assuming cellulase loading in this study and in the literature is comparable, the results presented in the present MSc thesis indicate that planetary ball milling is a pretreatment capable of increasing enzymatic digestibility of cotton textiles as much as the discussed solvents (phosphoric acid, sodium hydroxide and NMMO).

5.2.3. Effect of increased dry matter in reactions

Increasing the dry matter content in enzymatic hydrolysis is beneficial in industrial process because it reduces the costly downstream process of water removal and more substrate can be converted per volume and per time. On the other hand, products and other compounds have been seen to inhibit

enzymatic hydrolysis at high substrate loadings and mixing of the reactions might in some reactions be hindered from increased viscosity.

An experiment with BWD of various dry matter contents was set up to compare the conversion yield. Without increasing the enzyme loading, the conversion of cellulose from BWD was investigated and compared at 5 %, 10% and 20% DM. Figure 4.5.2 shows the conversion of BWD into glucose and cellobiose after 4, 24, 48 and 96 hours. The difference between the conversion yield of reactions at different dry matter content is small compared to the difference of substrate concentration. After 96 hours, a 4-fold increment of the substrate concentration gives a conversion yield without significant decrease.

Several experiments have been successful in finding a pretreatment compatible with increasing the dry matter of enzymatic hydrolysis reactions. Rana and colleagues (Rana et al. 2012) investigated the digestibility of loblolly pine pre-treated by wet oxidation and steam explosion by Cellic® CTec2. One of the main objectives in that study was obtaining high yields of glucose with increased dry matter content in reactions. The glucan content of the pretreated substrate was 50 % of the total solids. Rana and colleagues achieved the highest conversion yield seen for loblolly pine, with 96 % conversion at 25 % dry matter in reactions, when applying Cellic® CTec2 at a loading of 60 mg per g cellulose, which is 15-fold higher than the loading that gives equal conversion of the textile substrates in this study. However, 25 % DM in reactions of ball milled cotton textiles is not investigated. Assuming assessments regarding pretreatment are correct, these results suggest that the loading of Cellic® CTec2 needed to convert high substrate concentrations of cotton textiles is significantly lower than for pine, when optimal pretreatment is applied.

When Fockink and colleagues degraded cotton gin dust with dilute alkali pretreatment, they compared dry matter contents of 5 % (pretreated with 2 % NaOH) and 15 % (pretreated with 4 % NaOH) in reactions, with a loading of 85 mg Cellic® CTec2 per g dry matter (Fockink et al. 2015). For the two dry matter contents, the conversion yield was 49 % and 54 %, respectively. It is difficult to say whether product concentration mattered significantly as inhibiting factors or whether lignin and hemicellulose were the main cause of limiting the conversion. However, their results indicate higher conversion at 15 % DM than at 5 % DM, indicating that inhibition from high substrate concentrations was not an issue. Experiments with high dry matter content in conversion of cotton textiles have seemingly not been published.

It is not known in which degree conversion per time changes in further increasing DM (over 20 %) when converting ball milled cotton textiles. The viscosity in reaction solution might increase and reduce the mass transfer rate of the cellulases. However, it is likely that if DM *and* enzyme loading are increased, the conversion might remain higher than 80 % until a certain point. It would be interesting to investigate

this further. Additionally, a financial analysis should be carried out to assess the costs of increased loading and the potential cost reduction of high substrate concentration.

5.2.4. Comparison of blue and white denim fabric

Blue denim makes up a major part of textiles produced, hence a large part of landfilled and/or incinerated textiles are composed of this material. Affinity between indigo and cellulases has been indicated (Campos et al. 2000), but the literature lacks information on whether indigo has impact on hydrolysis of textiles by cellulases. Another difference between blue and white denim, is that coloured denim undergoes mercerization, which changes the crystallinity of the cellulose (from type I to type II, see section 1.2.2 for more information). The crystallinity type and index are not known for the substrates in this study. However, it is likely that a white denim fabric contains more cellulose type I while blue fabrics consist of more cellulose type II because of mercerization which is a key step for dye absorption during the production. For these reasons, it was of interest to investigate whether processing and hydrolysis differed when comparing blue denim and white denim. The experimental strategy to investigate this was comparing conversion yield of 5 % DM of BWD and BBD with 4 mg loading of Cellic® CTec2 per g glucan. The results from this experiment are illustrated by Figure 4.5.4. Conversion yields of BBD after 4, 24 and 48 were significantly higher than the conversion yields of BWD under the same conditions at the same time points. This is an indication that the presence of indigo had no inhibiting effect on the hydrolysis reactions.

The results shown in Figure 4.5.4 further indicate that indigo coloured denim fabrics are more digestible than white denim fabrics. It should be noted that the particle size distribution was different for BBD and WD (Figure 4.2). The surface area moment mean was lower for BBD than for BWD, which might be the explanation for increased accessibility for enzymes. The volume/mass moment mean, however, was slightly higher for BBD than for BWD. This is most likely due to different densities of the substrates, which is unknown. If the higher digestibility is partly due to the supramolecular structure of the cellulose is not known, but mercerization of cotton yarn has previously been shown to enhance accessibility for cellulases (Gisela & Zeronian 1994). Cellulase mechanisms and the correlation between cellulase hydrolysis and supramolecular structure of cellulosic materials are not fully understood (Peciulyte et al. 2015). Further experiments for investigating the significance of the modified crystal structure from mercerization in enzymatic conversion of ball milled cotton textiles might measure the crystallinity, whereas one technique is in fact based on protein binding methodology where cellulose-directed carbohydrate-binding modules are used to indicate degree of cellulose crystallinity (Kljun et al. 2011). Another strategy could be to investigate digestibility of cotton textiles before and after mercerization.

5.3. Effect of lytic polysaccharide monooxygenases on cotton substrates

Activity assays followed by mass spectrometry demonstrated that *Ta*LPMO9A produced several compounds corresponding to m/z values of oxidized oligosaccharides from BWD (Figure 4.6.1.1) and

Avicel (Figure 4.6.1.5). The dominant peak signals observed indicated ketoaldoses of cellohexaose (Glc_5Glc4K), celloheptaose (Glc_6Glc4K) and cellooctaose (Glc_7Glc4K) as well as the native forms of these (Glc_6-Glc_8). There were also clear indices on presence of aldonic acid of the same oligosaccharides (Glc_5Glc1A, Glc_6Glc1A and Glc_7Glc1A). Because the sodium adduct of any aldonic acid of an oligosaccharide has equal mass values to the potassium adduct of the native oligosaccharide (Table 4.6.1), only the potassium adducts are assessed as clear indications of oxidized products. Another indication of LPMO activity for BWD and Avicel, is that the signals detected for native oligosaccharides in potassium- and sodium adducts were not detected in substrate controls without enzyme. The spectra of BWD and Avicel were similar, i.e. the same m/z values were detected with relatively similar intensities. Although this is not a quantitative analysis, the similarity of the spectra of BWD and Avicel indicates a similar LPMO effect on the two substrates. Avicel has previously demonstrated to be a compatible substrate for LPMOs (Chylenski et al. 2017b).

Detected peak signals in BBD (Figure 4.6.1.2) associated with oxidized oligosaccharides were fewer with less intensity. Ketoaldoses of cellohexaose, celloheptaose and cellooctaose and the native forms of these were detected, while aldonic acids were not detected. The C1-oxidizing activity of *Ta*LPMO9A has previously been observed to be lower than the C4-oxidizing activity (Petrović unpublished). The reason why the activity on C1 seems to be absent for BBD is unknown. For BD (Figure 4.6.1.4), however, an aldonic acid of celloheptaose, as well as the ketoaldose of cellooctaose were observed. For WD (Figure 4.6.1.3), one signal peak associated with native and oxidized oligosaccharides was detected (ketoaldose of cellooctaose) with weak intensity in only one replicate. There is no indication that this was a product arising from LPMO activity. Oxidized oligosaccharides were expected to be produced from phosphoric acid swollen Avicel (PASC), as previously shown by Petrović and co-workers. Signals corresponding to oxidized and native peaks were clear in spectra from PASC, including aldonic acids and ketoaldoses (see Appendix S9). The acid swelling treatment is changing the cellulose microstructure, by halving the DP, severely decreasing the crystallinity and increasing the surface area per volume (SSA) (Andersen et al. 2008).

From the separation of dimeric ketoaldose from all textile samples, Avicel and PASC, the only signals that were sufficiently higher than the noise (S/N seemingly higher than standard deviation), were for PASC, Avicel, BWD and BBD Figure 4.6.2). The reactions were not hydrolysed by cellulases in order to enhance production of dimers for neither substrates. Nevertheless, all replicates showed significantly higher peaks for PASC (Appendix S9) than all other substrates, included Avicel, for which peaks of approximately the same intensity as BWD and BBD were separated.

It is apparent that *Ta*LPMO9A had an effect on BWD, BBD and BD, while activity on WD is not clearly indicated in this study. It is known that ball milling decreases DP (Sofla et al. 2016) and increases SSA (Peng et al. 2013). Crystallinity has been seen to change and to remain unchanged, depending on type

of material and analysis method. Higher LPMO activity on PASC than Avicel might have the same explanation as the observed higher activity on milled denim fabric than un-milled denim fabric. Overall, the results (from Section 4.6) suggest that substrate availability, in BWD, BBD, Avicel and PASC enhanced by small particle size and associated high specific surface area, enhances LPMO activity.

5.3.1. Effect of ascorbic acid in Cellic® CTec2 reactions

An experiment with 4 mg loading of Cellic® CTec2 and 5 % DM was set up with WD, BWD and Avicel in the presence and absence of ascorbic acid. The aim of the experiment was to investigate whether the hydrolysis of the textile was enhanced by addition of ascorbic acid, which has importance for understanding the significance of LPMOs present in the cocktail for the specific substrates.

When comparing the results obtained from reactions with and without ascorbic acid, the ball milled cotton substrate displays slightly higher conversion without ascorbic acid at 24 hours, while the conversion does not differ significantly at the other time points (Figure 4.5.3). The scissor cut cotton does not show different conversion in reactions with and without ascorbic acid. Avicel shows a higher conversion when ascorbic acid is applied, which is in agreement with previous studies (Chylenski 2017). There are two possible explanations for why the presence of ascorbic acid makes a difference for Avicel saccharification and not for the cotton substrates. Firstly, it is possible that the denim fabric for unknown reasons is not a compatible substrate for the LPMOs in the cocktail, and therefore do not need any reducing agent, while Avicel does. Secondly, the substrate might contain a reductant, being any product capable of providing the reaction with molecular oxygen or hydrogen peroxide.

5.4. Comparison of enzyme cocktails

Ballmilled cotton from white denim fabric was converted 80 % of theoretical yield of glucose with Cellic® CTec2 when applying minimum 4 mg enzyme per substrate over 24 hours, under the given conditions. Celluclast® converted the same substrate with 80 % when the loading was 8 mg and hydrolysis went on for 96 hours. Even though an increase of enzyme loading seems to approach 80 % conversion, the results indicate that time is the crucial requirement for this combination of enzyme cocktail and substrate.

Enzymatic activity of the two cocktails was measured by Canella and colleagues, and it appeared that that the BG content in Celluclast® was 1,0 U and 68 U, respectively (Cannella et al. 2012). According to what they found, BG are barely present in low doses of Celluclast®. The activity (FPU/g) of EGs and CBHs in Celluclast® was approximately 65 % of the activity in Cellic® CTec2. The results of Canella and co-workers might not be valid for the cocktail batch used in this thesis, although used as a rough

estimate, they correspond well to the main difference when comparing textile conversion yields by the two cocktails. A low BG content in a cellulase cocktail increase the content of cellobiose relative to glucose in the reactions, which is thought to inhibit hydrolysis significantly (Kristensen et al. 2009; Tolan, J. S. 2002)

Another difference in the composition of the two cocktails, is the ability of oxidative cleavage in Cellic® CTec2. Cannella et al. reported a decrease in BG activity from C1-oxidized sugars due to slow rate of the cleavage of cellobionic acid, giving slower production of glucose. The substrate analysed was pretreated wheat straw, and they saw an overall synergistic effect of the LPMO content in Cellic® CTec2, despite the inhibition. For the ball milled cotton, Cellic® CTec2 gives equal conversion with and without ascorbic acid, indicating that the LPMO activity is small or absent. Reactions from the experiment were not analysed for gluconic acid and cellobionic acid, but the inhibiting effect is not likely to occur/be a significant obstacle for efficient conversion.

5.5. Feasibility and further research

This study has focused on denim fabrics as a representative cotton textile for conversion to glucose by ball milling and enzymatic hydrolysis. Several aspects regarding feasibility and financial viability are not discussed here. One important limitation to highlight is the potential difference between laboratory scale and industrial scale. The ball mill used throughout this study has a nominal volume of 500 mL, which is strictly limited to utilization in laboratory scale. Investigation of cotton textile milling in larger ball mills would be necessary research for assessing the feasibility of the method. What regards conversion of 10 % or 20 % dry matter, the use of a thermomixer was a sufficient stirring method in the lab, while upscaling might lead to difficulties or high costs for mixing the reactions well when the viscosity increases. The representability of denim for all cotton textiles might be inaccurate. In order to apply the method industrially, it would be necessary to investigate ball milling and digestibility by the enzyme cocktails for several types of cellulosic textiles, as well as textiles of blended fibres, which often contains non-degradable polymers (Hasanzadeh et al. 2018). Indications that ball milling of cotton textiles increase accessibility for LPMOs potentially means that less intensive or shorter time of milling combined with LPMO rich enzyme cocktails might be an alternative to the suggested method, which remains to be investigated.

A major obstacle for viability of enzymatic conversion of lignocellulosic biomass is the costs of the enzymes (Merino & Cherry 2007). Cellulase cocktails of higher cost-efficiency might be developed as a consequence of intensive research on the field. Since the enzymes in a cocktail work collectively in the cellulose conversion, improved enzyme composition for an optimal synergy is a key to increase efficiency and lowering costs. Producing substrate-specific cocktails might also be beneficial, for instance an LPMO-free cocktail might be a cheaper and equally efficient alternative for cotton textile

conversion. Optimal synergy in a cocktail might reduce the need for pre-treatment of cotton textiles before enzymatic conversion. Recycling the cellulases has been discussed as a strategy to reduce the costs (Gomes et al. 2018). Recycling of Celluclast® was studied by Gomes and co-workers and assessed to be efficient during 4 rounds of hydrolysis of 22 % DM of paper sludge in reactions, when 50 % of the utilized enzyme cocktail was fresh and 50 % was recycled. Cellic® CTec2 was significantly more difficult to recycle, which was previously showed by Rodrigues and co-workers (Rodrigues et al. 2015).

Figure 5.4 illustrated possible pathways of pathways to produce biofuels, power and heat from biomass. As illustrated in the Figure, denim fabric degradation has also been investigated as a substrate in biogas production, which is a method of biomass conversion also based on digestion of biomass by hydrolysis, followed by further conversion (acidogenesis, acetogenesis and methanogenesis) into methane. The products are valuable as biofuels and generation of power, while the solid residues make a good alternative to conventional agricultural fertilizers.

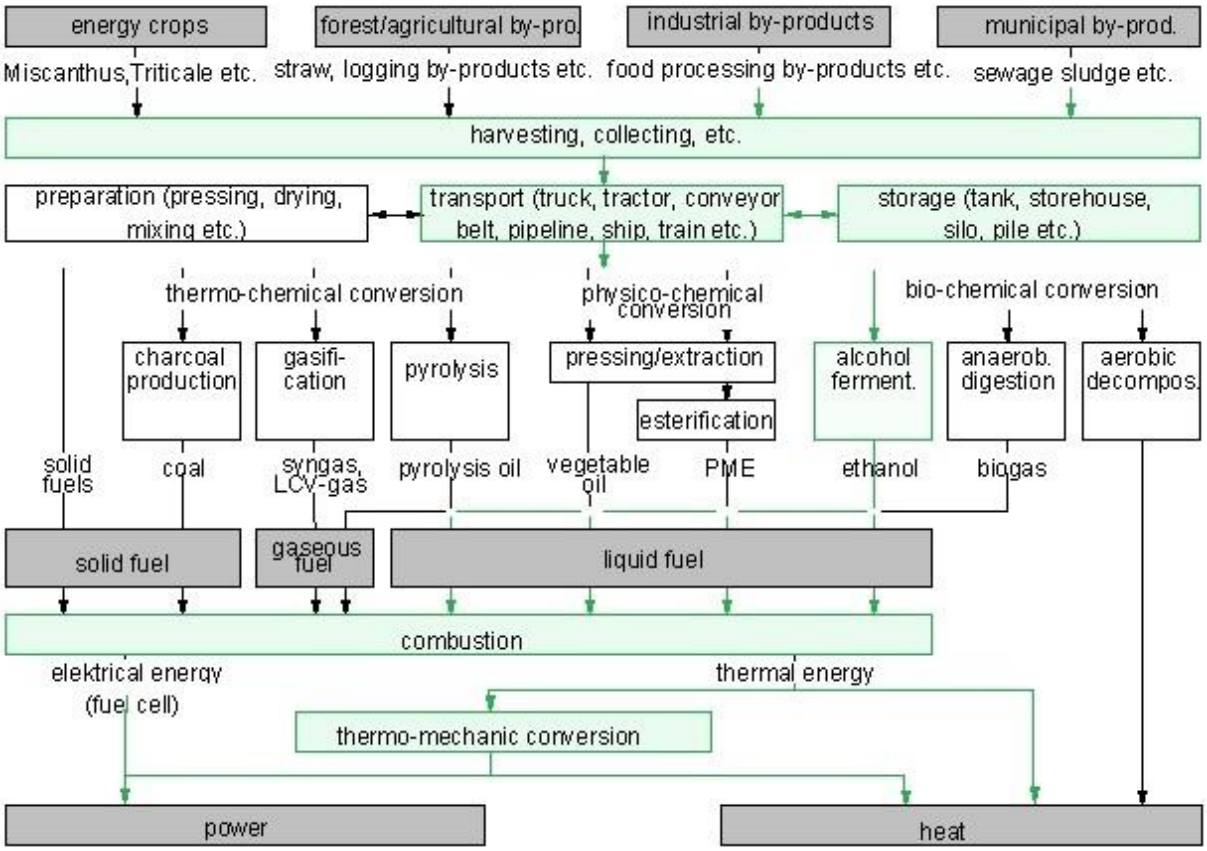


Figure 5.4. Schematic view of value-added production from first and second generation fuels. Utilization of cotton textiles from the industry and from municipal by-products might be produced into power and heat by several pathways. The method studied in this thesis belongs to the category marked green. Figure source: Food and Agricultural Organization of the United (FAO 2004).

The initial step in biogas production from cotton textiles is reducing the cellulose crystallinity by pretreatment. Alkaline treatment of cellulose is known to enhance digestibility of crystalline cellulose by a change in the crystal structure. Sodium carbonate may be used for avoiding the corrosive effects of strong alkali (Hasanzadeh et al. 2018). NMMO has been assessed as a good alternative to the other solvents because of high potential for recycling of the solvent and none indicated corrosive or hazardous effects. Sodium bicarbonate pretreated denim fabric and NMMO pretreated denim fabric have been converted to biogas with high yields (Hasanzadeh et al. 2018; Jeihanipour, Azam et al. 2010). It would be interesting to investigate whether biogas production is enhanced by ball milling. Certain pretreatment chemicals leave residues that inhibit glucose fermentation. Comminution is not assumed to introduce any inhibition issue for fermentation.

The role of textile waste as a source for second generation biofuels may be compatible with several pathways, although the method described in this study focuses on glucose production, which main usage is fermentation into ethanol, which might be converted into liquid fuels.

6. Conclusion

The aim of this study was converting cellulosic fibres from cotton to glucose without chemical pretreatment. Investigation of saccharification of cotton substrate pretreated by planetary ball milling showed that reduction in particle size significantly increased the digestibility of the substrate with two commercially available enzyme cocktails.

The surplus of cotton textiles is currently landfilled and incinerated in most countries, although the energy recycling of incineration is shown to be small. Reported methods for conversion of cotton involve chemical pretreatment for reducing the crystallinity in order to allow enzymes to access and catalyse cellulose depolymerization. Methods applying solely mechanical pretreatment prior to efficient conversion (over 80 % of theoretical yield) of cotton cellulose have apparently not been reported. For lignocellulosic biomass degradation in general, ball milling has been reviewed to be a too energy demanding pretreatment technique and cost-efficient only if combined with other pretreatment methods, even though ball milling of crystalline cellulose has been observed to produce nanofibrils with simple operation and relatively low costs. In this study, ball milled denim fabric of substrate concentrations up to 10 % (w/v dry matter content) resulted in 90 % conversion into glucose after by 24 hours incubation with 4 mg Cellic® CTec2 per g glucan. At 5 % DM the respective conversion yields for ball milled white and blue (indigo dyed) denim were 90 % and 100 % of theoretical maximal conversion.

Activity assays demonstrated that *Ta*LPMO9A produced several oxidized oligosaccharides from both denim fabrics studied when pretreated by ball milling. In substrates without pretreatment, however, small amounts of oxidized oligosaccharides were detected in blue denim and none in white (undyed)

denim. This indicates that the applied pretreatment in this study increased the availability for LPMOs and for cellulases. Ascorbic acid did not enhance hydrolysis by Cellic® CTec2 for cotton substrates. By assays with and without ascorbic acid and by comparison with Celluclast®, the LPMO content of Cellic® CTec2 was assessed to not be of significant importance for the efficient degradation.

7. References

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Contents

Methods

Equations for S1, S2, S3, S4, S5, S6 and S7:

[1] Calculated as follows: $\frac{substrat[mg]}{5{,}0 \cdot \frac{1}{DM(\%)}} \cdot \frac{glucan\ content\ fraction}{0{,}9115} = Factor$

[2] Calculated as follows:

$500\ \mu L \cdot Factor = Total\ volume\ [\mu L]$

[3] Calculated as follows:

$V_E\ [\mu L] \cdot Factor = volume\ of\ enzyme\ dilution\ added\ [\mu L]$

where V_E is the volume that would be added to a sample with Factor=1

[4] Calculated as follows:

$V_t - V(AscA)\ [\mu L] - V(enzyme)\ [\mu L] - \mathrm{V}(substrate)\ [mg] = \mathrm{V}(buffer)[\mu L]$

Table S-1: Loading experiment with Cellic® CTec2

Table S-1: details from the setup of the enzyme loading experiment with Cellic CTec2.

Experimental								Control calculations				
Sample		Substrate [mg]	[3]**CTec2 0,9115 g/L** [µL]	[4]**50 mM SoAcpH5 buffer** [µL]	AscA (100 mM) [µL]	[2]V_T [µL]	[1]factor	substrate [g]	enzyme/ glucan [g/g]]	enzyme/ volume [g/L]	DM	AscA [mM]
Std 1	Glc1 48 hrs	2,5	**0**	**488**	10	500				0,000	0,5 %	
Std 2		5,2	**0**	**485**	10	500				0,000	1,0 %	
Std 3		25	**0**	**465**	10	500				0,000	5,0 %	
Std 4		50,5	**0**	**440**	10	500				0,000	10,1 %	
BWD 1	4 mg 48 hrs	26,8	**50**	**410**	10	496	0,99	0,023	0,0040	0,182	5,0 %	2,0
BWD 2		27	**50**	**413**	10	500	1,00	0,023	0,0040	0,182	5,0 %	2,0
BWD 3		27,5	**51**	**420**	10	509	1,02	0,023	0,0040	0,182	5,0 %	2,0
BWD 4	8 mg 48 hrs	28	**104**	**376**	10	518	1,04	0,024	0,0080	0,365	5,0 %	1,9
BWD 5		26,9	**100**	**361**	10	498	1,00	0,023	0,0080	0,365	5,0 %	2,0
BWD 6		26,5	**98**	**356**	10	490	0,98	0,022	0,0080	0,365	5,0 %	2,0
BWD 7	12 mg 48 hrs	26,1	**145**	**302**	10	483	0,97	0,022	0,0120	0,547	5,0 %	2,1
BWD 8		27,6	**153**	**320**	10	511	1,02	0,023	0,0120	0,547	5,0 %	2,0
BWD 9		27,5	**153**	**319**	10	509	1,02	0,023	0,0120	0,547	5,0 %	2,0
BWD 10	16 mg 48 hrs	27,5	**204**	**268**	10	509	1,02	0,023	0,0160	0,729	5,0 %	2,0
BWD 11		26,6	**197**	**259**	10	492	0,98	0,022	0,0160	0,729	5,0 %	2,0
BWD 12		26,4	**195**	**257**	10	489	0,98	0,022	0,0160	0,729	5,0 %	2,0
BWD 13	20 mg 48 hrs	27,7	**256**	**219**	10	513	1,03	0,023	0,0200	0,912	5,0 %	2,0
BWD 14		26,3	**243**	**207**	10	487	0,97	0,022	0,0200	0,912	5,0 %	2,1
BWD 15		26,9	**249**	**212**	10	498	1,00	0,023	0,0200	0,912	5,0 %	2,0
BWD 16	2 mg 48 hrs	26,4	**24**	**428**	10	489	0,98	0,022	0,00200	0,091	5,0 %	2,0
BWD 17		27	**25**	**438**	10	500	1,00	0,023	0,00200	0,091	5,0 %	2,0
BWD 18		27,2	**25**	**441**	10	503	1,01	0,023	0,00200	0,091	5,0 %	2,0
BWD 0	0	32,3	**0**	**555**	10	598	1,20	0,027	0,00000	0,000	5,0 %	1,7
E	0	0	**100**	**390**	10	500	0,00	0,0	0,0	0,365	0,0 %	2,0

Table S-2: Time course experiments with Cellic® CTec2

Table S-2 a: details from the setup of time course experiment with Cellic CTec2 at 4 mg loading and 5 % DM.

Experimental								Control calculations			
Sample		Substrate [mg]	[3]**CTec2 0,9115 g/L** [µL]	[4]**50 mM SoAcpH5 buffer** [µL]	AscA (100 mM) [µL]	[2]V_T [µL]	[1]factor	substrate [g]	enzyme/ glucan [g/g]]	enzyme/ volume [g/L]	DM
Std 1	Glc 24 hrs	1,5	**0**	**489**	10	500		0,000	0,000	0,000	0,3 %
Std 2		5	**0**	**485**	10	500		0,000	0,000	0,000	1,0 %
Std 3		27,1	**0**	**463**	10	500		0,000	0,000	0,000	5,4 %
Std 4		49,6	**0**	**440**	10	500		0,000	0,000	0,000	9,9 %
Std 5	Glc 96 hrs	4,9	**0**	**485**	10	500		0,000	0,000	0,000	1,0 %
Std 6	Glc_2 24 hrs	1,7	**0**	**488**	10	500		0,000	0,000	0,000	0,3 %
Std 7		5,2	**0**	**485**	10	500		0,000	0,000	0,000	1 %
Std 8		25,1	**0**	**465**	10	500		0,000	0,000	0,000	5 %
Std 9		51,4	**0**	**439**	10	500		0,000	0,000	0,000	10 %
BWD 1	BWD 4 hrs	26,3	**97**	**353**	10,0	486,7	0,97	0,022	0,00400	0,182	5,00 %
BWD 2		27,8	**103**	**374**	10,0	514,4	1,03	0,023	0,00400	0,182	5,00 %
BWD 3		30,3	**112**	**408**	10,0	560,7	1,12	0,026	0,00400	0,182	5,00 %
BWD 4	BWD 24 hrs	28,9	**107**	**389**	10,0	534,8	1,07	0,024	0,00400	0,182	5,00 %
BWD 5		25,5	**94**	**342**	10,0	471,9	0,94	0,022	0,00400	0,182	5,00 %
BWD 6		28,2	**104**	**379**	10,0	521,8	1,04	0,024	0,00400	0,182	5,00 %
BWD 7	BWD 48 hrs	27,1	**100**	**364**	10,0	501,5	1,00	0,023	0,00400	0,182	5,00 %
BWD 8		27,5	**102**	**370**	10,0	508,9	1,02	0,023	0,00400	0,182	5,00 %
BWD 9		26,9	**100**	**361**	10,0	497,8	1,00	0,023	0,00400	0,182	5,00 %
BWD 10	BWD 96 hrs	26,4	**98**	**354**	10,0	488,5	0,98	0,022	0,00400	0,182	5,13 %
BWD 11		27,4	**101**	**368**	10,0	507,0	1,01	0,023	0,00400	0,182	5,00 %
BWD 12		27,4	**101**	**368**	10,0	507,0	1,01	0,023	0,00400	0,182	5,00 %
BWD 0	BWD control	27	**0**	**463**	10,0	499,6	1,0	0,023	0,00000	0,000	5,00 %
1	Avicel 4 hrs	26,3	**103**	**377**	10,0	516,7	1,03	0,024	0,00400	0,182	4,93 %
2		27,9	**110**	**401**	10,0	548,2	1,10	0,025	0,00400	0,182	4,93 %
3		26,3	**103**	**377**	10,0	516,7	1,03	0,024	0,00400	0,182	4,93 %
4	Avicel 24 hrs	25,8	**101**	**370**	10,0	506,9	1,01	0,023	0,00400	0,182	4,93 %
5		26	**102**	**373**	10,0	510,8	1,02	0,023	0,00400	0,182	4,93 %
6		25,7	**101**	**368**	10,0	504,9	1,01	0,023	0,00400	0,182	4,93 %
7	Avicel 48 hrs	25,9	**102**	**371**	10,0	508,9	1,02	0,023	0,00400	0,182	4,93 %
8		26,2	**103**	**376**	10,0	514,8	1,03	0,023	0,00400	0,182	4,93 %
9		25,7	**101**	**368**	10,0	504,9	1,01	0,023	0,00400	0,182	4,93 %
10	Avicel 96 hrs	26	**102**	**373**	10,0	510,8	1,02	0,023	0,00400	0,182	4,93 %
11		25,4	**100**	**364**	10,0	499,0	1,00	0,023	0,00400	0,182	4,93 %
12		25,9	**102**	**371**	10,0	508,9	1,02	0,023	0,00400	0,182	4,93 %
13	Avicel control	26	**102**	**373**	10,0	510,8	1,0	0,023	0,00400	0,182	4,93 %
WD 1	WD 4 hrs	26,8	**104**	**381**	10,0	521,9	1,04	0,024	0,00400	0,182	5,00 %
WD 2		25,3	**99**	**359**	10,0	492,7	0,99	0,022	0,00400	0,182	5,00 %
WD 3		27,3	**106**	**388**	10,0	531,7	1,06	0,024	0,00400	0,182	5,00 %
WD 4	WD 24 hrs	28	**109**	**398**	10,0	545,3	1,09	0,025	0,00400	0,182	5,00 %
WD 5		25,1	**98**	**356**	10,0	488,8	0,98	0,022	0,00400	0,182	5,00 %
WD 6		27,2	**106**	**387**	10,0	529,7	1,06	0,024	0,00400	0,182	5,00 %
WD 7	WD 48 hrs	24,9	**97**	**353**	10,0	484,9	0,97	0,022	0,00400	0,182	5,00 %
WD 8		25,7	**100**	**365**	10,0	500,5	1,00	0,023	0,00400	0,182	5,00 %
WD 9		27,5	**107**	**391**	10,0	535,6	1,07	0,024	0,00400	0,182	5,00 %
WD 10	WD 96 hrs	24,5	**95**	**347**	10,0	477,1	0,95	0,022	0,00400	0,182	5,00 %
WD 11		26,1	**102**	**371**	10,0	508,3	1,02	0,023	0,00400	0,182	5,00 %
WD 12		27,1	**106**	**385**	10,0	527,8	1,06	0,024	0,00400	0,182	5,00 %
WD 13	WD control	24,1	**94**	**341**	10,0	469,4	0,9	0,021	0,00400	0,182	5,00 %
E	Enzyme	0	100	390	10,0	500,0					
E2	Enzyme	0	100	390	10,0	500,0					

Table S-2 b: details from the setup of time course experiment with Cellic CTec2 at 4 mg loading and 5 % DM for BBD and BWD.

Sample		Substrate [mg]	[3]**CTec2 0,9115 g/L** [µL]	[4]**50 mM SoAcpH5 buffer** [µL]	AscA (100 mM) [µL]	[2]V_T [µL]	[1]factor	substrate [g]	enzyme/ glucan [g/g]]	enzyme/ volume [g/L]	DM
BWD 1	BWD 4 hrs	26,9	**100**	**361**	10	498	1,00	0,023	0,00400	0,182	5,00 %
BWD 2		27,2	**101**	**365**	10	503	1,01	0,023	0,00400	0,182	5,00 %
BWD 3		27	**100**	**363**	10	500	1,00	0,023	0,00400	0,182	5,00 %
BWD 4	BWD 24 hrs	28,1	**104**	**378**	10	520	1,04	0,024	0,00400	0,182	5,00 %
BWD 5		27,6	**102**	**371**	10	511	1,02	0,023	0,00400	0,182	5,00 %
BWD 6		27,7	**103**	**372**	10	513	1,03	0,023	0,00400	0,182	5,00 %
BWD 7	BWD 48 hrs	27,6	**102**	**371**	10	511	1,02	0,023	0,00400	0,182	5,00 %
BWD 8		27,3	**101**	**367**	10	505	1,01	0,023	0,00400	0,182	5,00 %
BWD 9		27,3	**101**	**367**	10	505	1,01	0,023	0,00400	0,182	5,00 %
BWD 0	BWD control	30,3	**0**	**520**	10	561	1,12	0,026	0,00000	0,000	5,00 %
BBD 1	BBD 4 hrs	26,4	**98**	**355**	10	490	0,98	0,022	0,00400	0,182	5,18 %
BBD 2		26,1	**97**	**351**	10	484	0,97	0,022	0,00400	0,182	5,18 %
BBD 3		25,9	**96**	**349**	10	481	0,96	0,022	0,00400	0,182	5,18 %
BBD 4	BBD 24 hrs	25,9	**96**	**349**	10	481	0,96	0,022	0,00400	0,182	5,18 %
BBD 5		26	**96**	**350**	10	482	0,96	0,022	0,00400	0,182	5,18 %
BBD 6		25,6	**95**	**344**	10	475	0,95	0,022	0,00400	0,182	5,18 %
BBD 7	BBD 48 hrs	25,7	**95**	**346**	10	477	0,95	0,022	0,00400	0,182	5,18 %
BBD 8		25,6	**95**	**344**	10	475	0,95	0,022	0,00400	0,182	5,18 %
BBD 9		26,7	**99**	**360**	10	495	0,99	0,023	0,00400	0,182	5,18 %
BBD 0	BBD control	27,2	**0**	**467**	10	505	1,01	0,023	0,00000	0,000	5,18 %

Table S-3: Time course experiment with Cellic® CTec2 at various dry matter contents

Table S-3: details from the setup of time course experiment with Cellic CTec2 at various DM.

Sample		Substrate [mg]	[3]**CTec2 1.823 g/L** [µL]	[4]**50 mM SoAcpH5 buffer** [µL]	AscA (100 mM) [µL]	[2]V_T [µL]	[1]factor	substrate [g]	enzyme/glucan [g/g]]	enzyme/volume [g/L]	DM
Std 1	Glc 24 hrs	6	**0**	**484**	10	500				0,000	1,20 %
Std 2		25,5	**0**	**465**	10	500				0,000	5,10 %
Std 3		48,9	**0**	**441**	10	500				0,000	9,78 %
Std 4		104	**0**	**386**	10	500				0,000	20,80 %
E1	Control		**100**	**390**	10	500					
E2	Control		**200**	**290**	10	500					
1	BWD10 4 hrs	53	**98**	**329**	10	490	0,98	0,045	0,00400	0,3646	10,00 %
2		54,5	**101**	**339**	10	504	1,01	0,046	0,00400	0,3646	10,00 %
3		53,8	**100**	**334**	10	498	1,00	0,045	0,00400	0,3646	10,00 %
4	BWD10 24 hrs	52,6	**97**	**327**	10	487	0,97	0,044	0,00400	0,3646	10,00 %
5		52,8	**98**	**328**	10	489	0,98	0,045	0,00400	0,3646	10,00 %
6		53	**98**	**329**	10	490	0,98	0,045	0,00400	0,3646	10,00 %
7	BWD10 48 hrs	54,4	**101**	**338**	10	503	1,01	0,046	0,00400	0,3646	10,00 %
8		53,5	**99**	**332**	10	495	0,99	0,045	0,00400	0,3646	10,00 %
9		53,5	**99**	**332**	10	495	0,99	0,045	0,00400	0,3646	10,00 %
10	BWD10 96 hrs	53,5	**99**	**332**	10	495	0,99	0,045	0,00400	0,3646	10,00 %
11		54,7	**101**	**340**	10	506	1,01	0,046	0,00400	0,3646	10,00 %
12		54,5	**101**	**339**	10	504	1,01	0,046	0,00400	0,3646	10,00 %
control	BWD10 48 hrs	54,3	**0**	**438**	10	502	1,0	0,046	0,00000	0,0000	10,00 %
			CTec2 0,9115 g/L								
14	BWD20 4 hrs	117,1	**217**	**198**	10	542	1,08	0,099	0,00400	0,7292	20,00 %
15		103,4	**191**	**174**	10	478	0,96	0,087	0,00400	0,7292	20,00 %
16		108,7	**201**	**183**	10	503	1,01	0,092	0,00400	0,7292	20,00 %
17	BWD20 4 hrs	106,1	**196**	**178**	10	491	0,98	0,089	0,00400	0,7292	20,00 %
18		109,2	**202**	**184**	10	505	1,01	0,092	0,00400	0,7292	20,00 %
19		112,8	**209**	**190**	10	522	1,04	0,095	0,00400	0,7292	20,00 %
20	BWD20 4 hrs	110,7	**205**	**187**	10	512	1,02	0,093	0,00400	0,7292	20,00 %
21		112	**207**	**189**	10	518	1,04	0,094	0,00400	0,7292	20,00 %
22		109,5	**203**	**184**	10	507	1,01	0,092	0,00400	0,7292	20,00 %
23	BWD20 4 hrs	106,5	**197**	**179**	10	493	0,99	0,090	0,00400	0,7292	20,00 %
24		107,3	**199**	**181**	10	496	0,99	0,090	0,00400	0,7292	20,00 %
25		110,7	**205**	**187**	10	512	1,02	0,093	0,00400	0,7292	20,00 %
control	BWD20 48 hrs	110,7	**0**	**391**	10	512	1,0	0,093	0,00000	0,0000	20,00 %
27	BWD5 24hrs	26,9	**50**	**411**	10	498	1,00	0,023	0,00400	0,1823	5,00 %
28	BWD10-8mg-24 hrs	55,2	**204**	**241**	10	511	1,02	0,047	0,00800	0,7292	10,00 %

Table S-4: Loading experiment with Celluclast®

Table S-4: details from the setup of loading experiment with Celluclast

Sample		Substrate [mg]	[3]**Celluclast 1.823 g/L** [µL]	[4]**50 mM SoAcpH5 buffer** [µL]	[2]V_T [µL]	[1]factor	substrate [g]	enzyme/ glucan [g/g]]	enzyme/ volume [g/L]	DM
Std 1	Glc 24 hrs	4,6	**0**	**495**	500				0,000	0,9 %
Std 2		28,7	**0**	**471**	500				0,000	5,7 %
Std 3		50,1	**0**	**450**	500				0,000	10,0 %
Std 4		99,9	**0**	**400**	500				0,000	20,0 %
Std 5	Glc_2 24 hrs	3	**0**	**497**	500				0,000	0,3 %
Std 6		5,4	**0**	**495**	500				0,000	1 %
Std 7		25,9	**0**	**474**	500				0,000	5 %
Std 8		51,5	**0**	**449**	500				0,000	10 %
BWD 1	2 mg 48 hrs	27,1	**25**	**449**	501	1,00	0,023	0,00200	0,091	5,00 %
BWD 2		27,9	**26**	**463**	516	1,03	0,024	0,00200	0,091	5,00 %
BWD 3		26,5	**25**	**439**	490	0,98	0,022	0,00200	0,091	5,00 %
BWD 4	4 mg 48 hrs	26,4	**49**	**413**	489	0,98	0,022	0,00400	0,182	5,00 %
BWD 5		26,4	**49**	**413**	489	0,98	0,022	0,00400	0,182	5,00 %
BWD 6		27,3	**51**	**427**	505	1,01	0,023	0,00400	0,182	5,00 %
BWD 7	8 mg 48 hrs	28,3	**105**	**391**	524	1,05	0,024	0,00800	0,365	5,00 %
BWD 8		26,3	**97**	**363**	487	0,97	0,022	0,00800	0,365	5,00 %
BWD 9		26,4	**98**	**364**	489	0,98	0,022	0,00800	0,365	5,00 %
BWD 10	12 mg 48 hrs	26,2	**145**	**313**	485	0,97	0,022	0,01200	0,547	5,00 %
BWD 11		27	**150**	**323**	500	1,00	0,023	0,01200	0,547	5,00 %
BWD 12		28,3	**157**	**338**	524	1,05	0,024	0,01200	0,547	5,00 %
BWD 13	16 mg 48 hrs	26,7	**198**	**270**	494	0,99	0,023	0,01600	0,729	5,00 %
BWD 14		27,7	**205**	**280**	513	1,03	0,023	0,01600	0,729	5,00 %
BWD 15		26,3	**195**	**266**	487	0,97	0,022	0,01600	0,729	5,00 %
BWD 16	20 mg 48 hrs	26,2	**242**	**216**	485	0,97	0,022	0,02000	0,912	5,13 %
BWD 17		26,7	**247**	**220**	494	0,99	0,023	0,02000	0,912	5,13 %
BWD 18		26,5	**245**	**219**	490	0,98	0,022	0,02000	0,912	5,13 %
BWD 0	0	27,7	**0**	**485**	513	1,03	0,023			5,00 %
E	0	0	**100**	**400**	500	0				

Table S-5: Time course experiment with Celluclast®

Table S-5: details from the setup of time course experiment with Celluclast.

Sample		Substrate [mg]	[3]**Celluclast 0.91 g/L** [µL]	[4]**50 mM SoAcpH5 buffer** [µL]	[2]V_T [µL]	[1]factor	substrate [g]	enzyme/ glucan [g/g]]	enzyme/ volume [g/L]	DM
Std 1	Glc 24 hrs	2,9	**0**	**497**	500				0,000	0,6 %
Std 2		8,2	**0**	**492**	500				0,000	1,6 %
Std 3		26,3	**0**	**474**	500				0,000	5,3 %
Std 4		49,9	**0**	**450**	500				0,000	10,0 %
Std 5		99,8	**0**	**400**	500				0,000	20,0 %
Std 6	Glc2 24 hrs	3	**0**	**497**	500				0,000	0,6 %
Std 7		5,4	**0**	**495**	500				0,000	1,1 %
Std 8		25,9	**0**	**474**	500				0,000	5,2 %
Std 9		51,5	**0**	**449**	500				0,000	10,3 %
Std 10		99,8	**0**	**400**	500				0,000	20,0 %
BWD 1	BWD 4 hrs	26,5	**196**	**268**	490	0,98	0,022	0,0080	0,365	5,00 %
BWD 2		27	**200**	**273**	500	1,00	0,023	0,0080	0,365	5,00 %
BWD 3		26,8	**198**	**271**	496	0,99	0,023	0,0080	0,365	5,00 %
BWD 4	BWD 24 hrs	27,5	**204**	**278**	509	1,02	0,023	0,0080	0,365	5,00 %
BWD 5		26,7	**198**	**270**	494	0,99	0,023	0,0080	0,365	5,00 %
BWD 6		27,3	**202**	**276**	505	1,01	0,023	0,0080	0,365	5,00 %
BWD 7	BWD 48 hrs	29,8	**221**	**301**	551	1,10	0,025	0,0080	0,365	5,00 %
BWD 8		26,4	**195**	**267**	489	0,98	0,022	0,0080	0,365	5,00 %
BWD 9		26,3	**195**	**266**	487	0,97	0,022	0,0080	0,365	5,00 %
BWD 10	BWD 96 hrs	26,8	**198**	**271**	496	0,99	0,023	0,0080	0,365	5,00 %
BWD 11		27,7	**205**	**280**	513	1,03	0,023	0,0080	0,365	5,00 %
BWD 12		26,4	**195**	**267**	489	0,98	0,022	0,0080	0,365	5,00 %
BWD 0	BWD control	30,5	**0**	**534**	564	1,1	0,026	0,0000	0,000	5,00 %
1	Avicel 4 hrs	25,4	**200**	**274**	499	1,00	0,023	0,0080	0,365	4,93 %
2		25,9	**204**	**279**	509	1,02	0,023	0,0080	0,365	4,93 %
3		24,2	**190**	**261**	475	0,95	0,022	0,0080	0,365	4,93 %
4	Avicel 24 hrs	24,9	**196**	**269**	489	0,98	0,022	0,0080	0,365	4,93 %
5		25,1	**197**	**271**	493	0,99	0,022	0,0080	0,365	4,93 %
6		24,9	**196**	**269**	489	0,98	0,022	0,0080	0,365	4,93 %
7	Avicel 48 hrs	27,4	**215**	**296**	538	1,08	0,025	0,0080	0,365	4,93 %
8		28,8	**226**	**311**	566	1,13	0,026	0,0080	0,365	4,93 %
9		25,8	**203**	**278**	507	1,01	0,023	0,0080	0,365	4,93 %
10	Avicel 96 hrs	25,4	**200**	**274**	499	1,00	0,023	0,0080	0,365	4,93 %
11		25,1	**197**	**271**	493	0,99	0,022	0,0080	0,365	4,93 %
12		25,6	**201**	**276**	503	1,01	0,023	0,0080	0,365	4,93 %
13	Avicel control	27,1	**0**	**505**	532	1,1	0,024	0,0000	0,000	4,93 %
WD 1	WD 4 hrs	26,7	**208**	**285**	520	1,04	0,024	0,0080	0,365	5,00 %
WD 2		26	**203**	**278**	506	1,01	0,023	0,0080	0,365	5,00 %
WD 3		27,6	**215**	**295**	538	1,08	0,024	0,0080	0,365	5,00 %
WD 4	WD 24 hrs	26,2	**204**	**280**	510	1,02	0,023	0,0080	0,365	5,00 %
WD 5		27,9	**217**	**298**	543	1,09	0,025	0,0080	0,365	5,00 %
WD 6		26,5	**206**	**283**	516	1,03	0,024	0,0080	0,365	5,00 %
WD 7	WD 48 hrs	25,9	**202**	**277**	504	1,01	0,023	0,0080	0,365	5,00 %
WD 8		27,6	**215**	**295**	538	1,08	0,024	0,0080	0,365	5,00 %
WD 9		29,2	**227**	**312**	569	1,14	0,026	0,0080	0,365	5,00 %
WD 10	WD 96 hrs	31,5	**245**	**337**	613	1,23	0,028	0,0080	0,365	5,00 %
WD 11		30,8	**240**	**329**	600	1,20	0,027	0,0080	0,365	5,00 %
WD 12		28,7	**224**	**307**	559	1,12	0,025	0,0080	0,365	5,00 %
WD 13	WD control	25,2	**0**	**466**	491	1,0	0,022	0,0000	0,000	5,00 %

Table S-6: Reactions with *Ta*LPMO9A

Table S-6: details from the setup of LPMO analysis with Ta*LPMO9A*

Nr.	Sample	Substrate [mg]	[3]**TaLPMO9A 10 µM** [µL]	[4]**Buffer (70 mM TrisHCl-6,5)** [µL]	AscA (100 mM) [µL]	[2]V_T [µL]	[1]Factor	substrat [g]	enzym/glucan [g per g]	enzym/volum [g/L]	DM [%]	AscA [mM]	Tris-HCl [mM]
1	BWD	1,8	**17**	**146**	2,5	166,5	0,67	0,0015	0,0001	0,0000010	1,000	1,5	61,193
2		1,6	**15**	**129**	2,5	148,0	0,59	0,0013	0,0001	0,0000010	1,000	1,7	61,1
3	BWD control	1,7	**0**	**153**	2,5	157,3	0,6	0,0014	0,0000	0,0000000	1,000	1,6	68,1
4	Avicel	1,9	**19**	**164**	2,5	186,6	0,75	0,0017	0,0001	0,0000010	1,052	1,3	61,3
5		2,1	**21**	**181**	2,5	206,3	0,83	0,0019	0,0001	0,0000010	1,052	1,2	61,4
6	Avicel control	1,7	**0**	**163**	2,5	167,0	0,7	0,0015	0,0000	0,0000000	1,052	1,5	68,2
7	BBD	2,1	**19**	**171**	2,5	194,8	0,78	0,0018	0,0001	0,0000010	1,122	1,3	61,3
8		2,2	**20**	**179**	2,5	204,1	0,82	0,0019	0,0001	0,0000010	1,122	1,2	61,4
9	BBD control	2,1	**0**	**190**	2,5	194,8	0,8	0,0019	0,0000	0,0000000	1,122	1,3	68,3
10	WD	1,5	**15**	**127**	2,5	146,1	0,58	0,0013	0,0001	0,0000010	1,055	1,7	61,1
11		2,1	**20**	**179**	2,5	204,5	0,82	0,0019	0,0001	0,0000010	1,055	1,2	61,4
12	WD control	3,6	**0**	**344**	2,5	350,6	1,40	0,0032	0,0000	0,0000000	1,055	0,7	68,8
13	BD	1,5	**14**	**121**	2,5	139,2	0,56	0,0013	0,0001	0,0000010	1,122	1,8	61,0
14		2,1	**19**	**171**	2,5	194,8	0,78	0,0018	0,0001	0,0000010	1,122	1,3	61,3
15	BD control	2,7	**0**	**245**	2,5	250,5	1,00	0,0023	0,0000	0,0000000	1,122	1,0	68,5
16	Pasc[5]	2,5	**25**	**205**	2,5	253,7	1,01	0,0023	0,0001	0,0000010	0,985	1,0	56,6
17		2,5	**25**	**205**	2,5	253,7	1,01	0,0023	0,0001	0,0000010	0,985	1,0	56,6
18	Pasc control[5]	2,5	**0**	**230**	2,5	253,7	1,01	0,0023	0,0000	0,0000000	0,985	1,0	63,6

Equations for S-6:

[1] Calculated as follows: $\frac{substrat(mg)}{2{,}5 \cdot \frac{1}{DM(\%)}} = Factor$

[2] Calculated as follows: $250\ \mu L \cdot Factor = Total\ volume\ (\mu L)$

[3] Calculated as follows: $25\ \mu L \cdot Factor = enzyme\ volume\ (\mu L)$

[4] Calculated as follows: $V_t - AscA(\mu L) - enzyme\ (\mu L) - substrate\ (mg) = buffer(\mu L)$

[5]PASC was pipetted from a 12 % solution. 20.8 mL was used, because $20.8\ mL \cdot 0.12 = 2.5\ mg$

Results

Figure S-7: MS spectrum of PASC

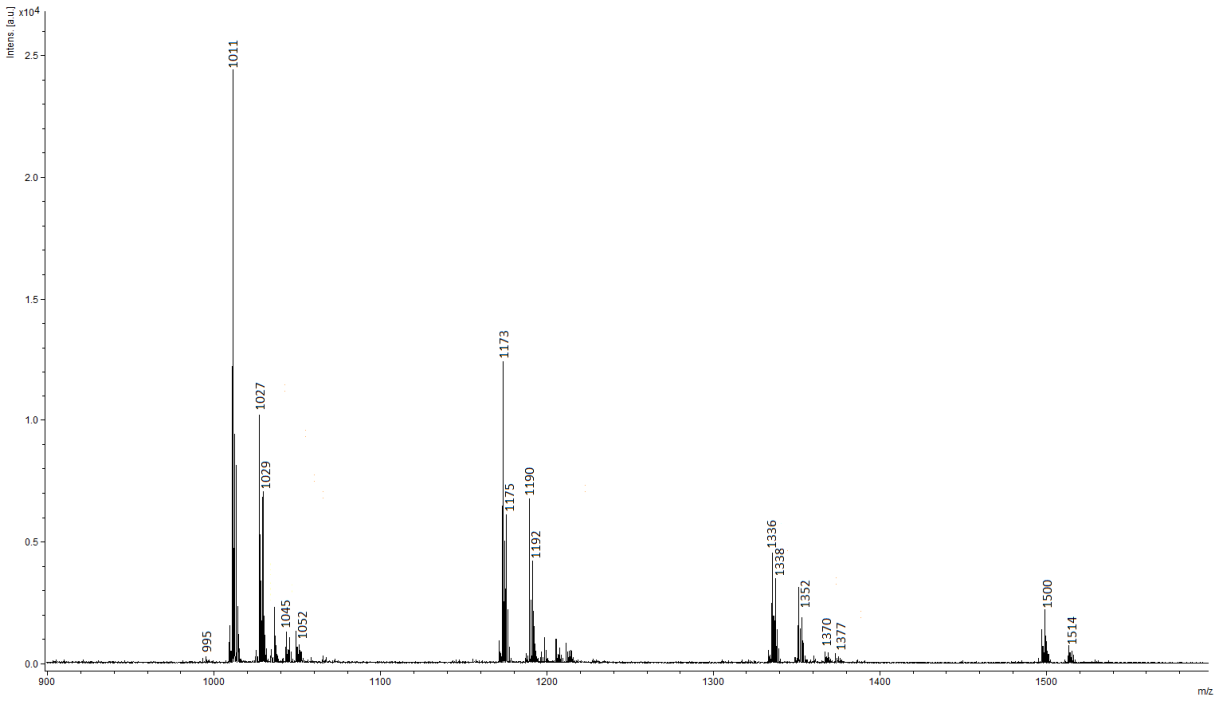

Figure S-7: MALDI-TOF analysis of oxidized products in PASC. The figure shows mass spectrum of BBD treated with *Ta*LPMO9A in pH=6,5, 45°C at 1 000 rpm for 20 hours.

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