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Enzymes in Sustainable Lignocellulose Processing and Degradation

DISSERTATION

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I

Preamble

This thesis covers enzymatic strategies in production, functionalization and degradation of cellulose based materials. Therefore, it is divided into five sections, starting with an introduction and followed by four scientific publications highlighting special aspects of cellulose modification by enzymes.

The introduction gives a short historical overview about discovery and research on cellulose, followed by a description of the complex molecular and macroscopic structure. Later, its natural function in the complex plant matrix of lignocellulose is discussed, as well as its processing and modification by humankind.

The first publication outlines the application of enzymes for cellulose processing in the pulp and paper industry. It focuses on an extensive basic characterization to understand the composition and behavior of commercial enzyme formulations and applies them on different pulp materials to investigate the effects theses enzymes can have on the refining process in a paper mill.

In the second publication, an environmentally friendly process to functionalize cellulose in its paper form with covalently coupled proteins is investigated. Paper sheets can be equipped with properties of proteins and enzymes to develop new products made from paper e.g. innovative packaging material or biosensors. We investigated the probability to couple hydrophobic proteins to make paper more resistant against water.

A global problem regarding the limited natural degradation of the cellulose derivate cellulose acetate is addressed in the third publication. Cellulases and esterases were characterized according to their potential to act on cellulose acetate. Esterases deacetylate cellulose acetate and convert it back into degradable cellulose. Cellulases cleave the backbone of the polymer and enable by this an enhancement of natural degradation mechanisms.

The fourth paper presents an approach using waste paper material as newspaper for the production of algae biomass. Algae can be cultivated on sugars which can be produced from cellulose by enzymatic hydrolysis. Algae are a valuable but underused product with multiple potential applications as animal feed, food supplement or bulk chemicals.

After the publications, a general conclusion summarizing the perspectives of enzyme-catalyzed cellulose modification is given. In the end, list of publications, oral presentations and grants are given in the appendix section.

II

Abstract

Enzymes, in their enormous variety represent a powerful tool to remodel many production processes used today in a traditional sense towards a more near-natural status. Their potential in cellulose processing is elucidated in this thesis, covering the fields of production, modification and recycling of certain cellulose-based materials. Impact of enzymes on production processes of pulp and paper is shown as well as the development of paper with new functions. Further, possibilities to degrade cellulose, its derivatives and transform their monomers into valuable materials is shown. Paper refining is the process of modifying cellulose fibers to form a strong but flexible paper sheet *via* a fibrillation process. Enzymes showed effects on the pulp as increasing the degree of refining and on produced paper sheets as decreasing the air permeability. It was obvious, that an extensive characterization and a deeper understanding of the enzymatic mechanism of action on pulps is crucial for their routine application on a paper machine.

Innovative materials from renewable resources as cellulose have a great potential to replace fossil-based products as certain plastics. Therefore, these new materials must provide the same functionalities as the traditional products. Making pulp and paper more hydrophobic can help to introduce its use as packaging material in fields where plastic foils are nowadays in use. This was achieved by a two-step process, first oxidizing the paper without breaking the backbone, followed by coupling a protein to the oxidized paper. Produced paper sheets, showed an enormous increase in hydrophobicity. Additionally, a model protein (BSA) was coupled and therefore the

ability of this approach to produce different and individualized protein-cellulose materials was proven.

Due to the production scale of cellulose and its derivatives in the million tons range worldwide every year, occurrence of huge amounts of waste is unavoidable. Often, traditional systems cannot scope with all the different varieties of waste or are socioeconomically not feasible. Enzymes can help to degrade cellulose derivatives which are naturally nondegradable. Cellulose acetate is a manmade cellulose product, without a natural degradation route. The potential of different esterases was proven to deacetylate cellulose acetate into cellulose, enabling its natural degradation. Further, the potential of cellulases was investigated and revealed action on cellulose acetate by breaking the backbone and reducing the polymer's chain length. The shorter a cellulose acetate chain, the easier the natural degradation is.

The sugars bound in cellulose and lignocellulose can act as carbon source for different microbial production processes. Due to the rigid structure of cellulose, efficient techniques to obtain glucose in high concentrations are critical. Enzymatic hydrolysis using cellulases is an applicable approach also on an industrial scale. A medium for algae cultivation from newspaper hydrolysate mixed with digestate from anaerobic digestion was developed. Algae biomass was produced under sufficient consumption of glucose, xylose and ammonium from the medium.

III

Kurzfassung

Enzyme in ihrer enormen Vielfalt, repräsentieren ein mächtiges Werkzeug viele traditionelle Produktionsprozesse umzuwandeln, hin zu naturnäheren Verfahren. Ihr Potential in den Bereichen Produktion, Modifikation und Recycling wird in dieser Arbeit beleuchtet. Behandelt wird der Einfluss von Enzymen auf die Produktion von Papier und Zellstoff als auch die Entwicklung von Papier mit neuen Funktionalitäten mithilfe von Enzymen. Des Weiteren werden Möglichkeiten Cellulose und Cellulose Derivate wie Cellulose Acetat abzubauen untersucht, und die Umwandlung ihrer Monomere in wertvolle Materialien wird gezeigt. Papiermahlung verursacht die Fibrillierung von Fasern und dient zur Modifizierung von Cellulose um stärkeres aber auch flexibleres Papier zu erhalten. Enzyme zeigten einen Einfluss auf Zellstoff, wie einen erhöhten Mahlgrad und auf Papierblätter, wie eine verringerte Luftdurchlässigkeit. Es war offensichtlich, dass eine umfassende Charakterisierung und ein tieferes Verständnis für die Mechanismen auf Fasen essentiell sind, um Enzyme routinemäßig in einer Papiermaschine einzusetzen.

Innovative Materialien aus erneuerbaren Ressourcen wie Cellulose haben großes Potential fossile Produkte wie Plastik zu ersetzen. Hierzu müssen diese Materialien dieselben Funktionen aufweisen wie die traditionellen Produkte. Zellstoff und Papier wasserabweisender zu machen kann helfen den Einsatz als Verpackungsmaterial in Bereichen in denen aktuell Plastikfolien verwendet werden zu forcieren. Das wurde mittels eines zweistufigen Verfahrens erreicht, dass Cellulose zuerst oxidiert, ohne den Cellulose Strang zu zerstören, und dann Proteine auf diese oxidierte Cellulose bindet.

Die so hergestellten Papierblätter wiesen eine stark erhöhte hydrophobizität auf. Zusätzlich wurde ein Modellprotein gebunden und so gezeigt, dass der präsentierte Ansatz sich dazu eignet verschiedene individualisierte Protein-Cellulose Materialien zu erzeugen.

Aufgrund des weltweiten Produktionsvolumens von Cellulose im Bereich von Millionen Tonnen im Jahr, ist das Auftreten von großen Mengen Abfall unvermeidbar. Häufig können traditionelle System mit den vielen verschiedenen Varietäten nicht umgehen, oder sind unter wirtschaftlichen und sozialen Aspekten unrentabel. Enzyme können helfen nicht abbaubare Cellulose Derivate zu zersetzen. Cellulose Acetat ist ein künstliches Cellulose Produkt ohne natürlichen Abbauweg. Esterasen bewiesen ihr Potential Cellulose Acetat zu deacetylieren und so zu Cellulose umzuwandeln, wodurch ein natürlicher Abbau möglich wird. Des Weiteren wurde das Potential von Cellulasen untersucht und diese zeigten ihr Vermögen die Cellulose Acetat Kette zu brechen und so die Länge der Polymere zu reduzieren. Je kürzer die Cellulose Acetat Kette ist, umso einfacher ist ihr natürlicher Abbau.

Die in Cellulose und Lignocellulose gebundenen Zucker können als Kohlenstoffquelle in verschiedenen mikrobiologischen Produktionsprozessen genutzt werden. Durch die rigide Struktur von Cellulose sind Prozesse zur Gewinnung von Glucose in hohen Konzentrationen entscheidend. Enzymatische Hydrolyse mit Cellulasen ist ein geeigneter Ansatz und im industriellen Maßstab anwendbar. Ein Medium zur Algenkultivierung aus Altpapier Hydrolysat, gemischt mit Gärrest aus anaerober Gärung wurde entwickelt. Algenbiomasse wurde produziert bei gleichzeitigem hinreichendem Verbrauch von Glucose Xylose und Ammonium aus dem Medium.

1

Aim of the Thesis

The aim of this thesis was to develop different enzymatic processes to create sustainable alternatives in cellulose processing. Approaches regarding the full lifespan of cellulose-based materials were investigated, including production, use and recycling/biodegradation of cellulose and its derivatives. Enzymes in pulp and paper production should help to design more energy efficient and environmentally friendly processes. This requires a closer look into the characteristics of enzyme formulations that already exist, as well as an in-depth view on the effects of enzymes on the properties of paper sheets. Functionalization of paper materials is prolonging the product's lifespan and can be used to replace materials that are harmful to the environment. Further, novel applications involving paper are conceivable and materials individually functionalized for a certain customer represent a high value application. Innovative utilisation of paper residues, a bulk product from the industry, by its conversion to high value biomass closes the carbon cycle and enables the implementation of a fully integrated biorefinery concept. Due to the enormous production capacity of the paper industry, even small changes of processes can have substantial influences on global issues as greenhouse gas emissions and energy saving efforts. Moreover, there is a social engagement for meaningful usage of all resources, no matter of its origin or utilization.

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2

Introduction

Cellulose, the most abundant biopolymer on our planet, mainly derives from the cell wall of wooden plants. There, it forms in combination with lignin and hemicellulose the bulk part of plant biomass. In nature, pure cellulose can occur as the seed hairs of the cotton plant or as a product of some bacteria but is rare compared to its combined form. An annual synthesis of 10^{11}-10^{12} t is estimated by photosynthesis in plants only [1]. Generally, polysaccharides fulfil three major tasks in nature, building strength, providing energy and manage moisture distribution [2]. The earliest use of cellulose by humankind, besides incineration, is reported for rope and cordage in 4500 BC from hemp, and papyrus production in ancient Egypt from sedge around 3000 BC. Modern history started in 1873 when the French chemist Anselme Payen identified cellulose, whereas the term cellulose is in use since 1839 [3]. Today, papermaking is an important branch in cellulose processing. The first patent for a paper machine was accorded in 1798 and first recycling paper was produced only two years later [4]. Actually, in Europe, 1255 paper machines produce 42,8 million tons pulp and paper at 897 mills [5]. Further, cellulose is a widely used raw material in different industrial sectors which process it to manifold products in large amounts. For example, the annual production of cellulose acetate (CA), an ester of industrial importance, is around 1.5 billion pounds [6]. In 2017 the world largest factory for dissolving wood pulp had a capacity of 300,000 tons per year [7]. Derivatization and solubilisation processes of cellulose became important form the early 1850s until today. The first observed derivatization was the formation of explosive cellulose nitrate (often misnamed as

nitrocellulose) in 1845 [8]. Later, in the early 20th century another derivate, cellophane, became important for its transparency [9]. Dissolving processes do not chemically modify the cellulose subunits, but rather change properties of the hydrogen bonding system of the polymer. In the viscose process, raw materials as cotton or wood cellulose, are dissolved in carbon disulfide and a yarn can be spun out to the solution. Despite all disadvantages, the viscose process is still of enormous importance for global regenerated cellulose industry [8].

2.1 Cellulose structure

Cellulose is a linear homopolymer of glucose subunits linked via β 1-4 glycosidic bonds. Each glucose molecule in the chain is thereby rotated by 180°, which makes in a strict sense cellobiose the repeating unit in cellulose and not glucose. This is a major difference to polymers like starch and callose were the single sugar is the repeating unit [10]. The functional groups of the cellulose molecule deserve further attention. A carbonyl group can be found at one end of the cellulose chain. This end is called reducing end, whereas the other end is referred to non-reducing end. The reducing end is more present as hemiacetal than as aldehyde and can be oxidized to its corresponding aldonic acid. It is the starting point of many degradation processes (peeling of) in cellulose [11]. The structure of the unbranched cellulose molecule with its reducing and non-reducing end is given in Figure 1.

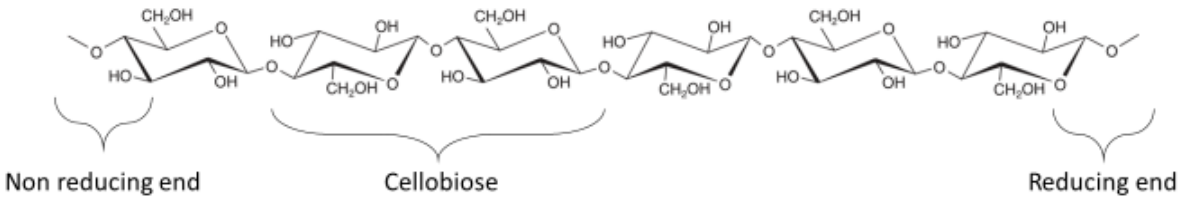


Figure 1. Structure of a cellulose chain with indications of cellobiose as repeating unit and the reducing and non-reducing end. Adapted from [12].

The hydroxyl groups of the polymer are oriented in an equatorial position along the chain [13]. The characteristic pyranose ring structure, consisting of five C atoms and one O atom, is locked into a chair conformation. The distance of the two oxygen atoms (O_1O_4 vector) of the glyosidic bond is at a maximum in this formation. A transformation into another confirmation (e.g. boat) due to a stretching force is therefore prohibited [14]. The typical chair formation and rigidity of cellulose is illustrated in Figure 2.

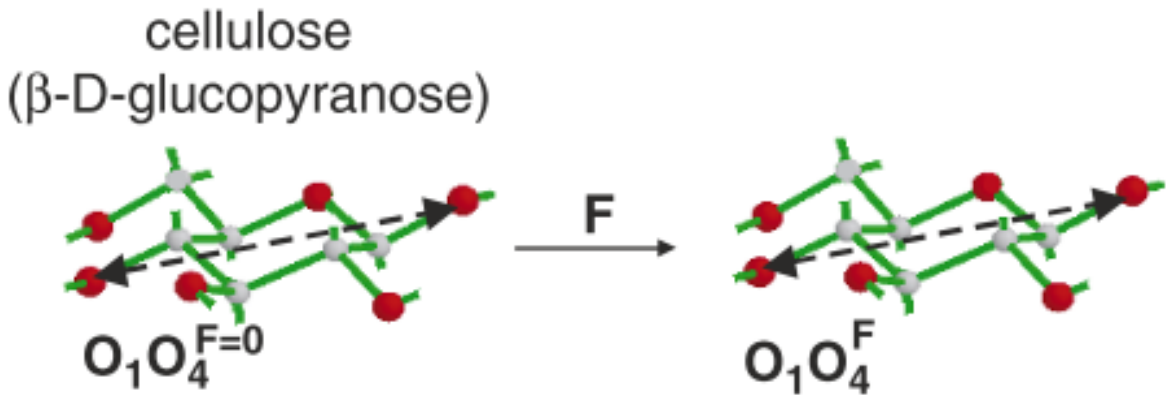


Figure 2. Typical chair formation of a glycopyranose ring in cellulose. Oxygen atoms are marked in red, carbon atoms are grey and bonds are green. Absence of a conformation changes when a force (F) is applied to the structure (F=0 →F) is indicated on the right. The distance of O1O4 does not change, causing high rigidity of the cellulose chain [14].

AFM studies using ab initio calculation of the O_1O_4 vector revealed a length of 5,417 Å. This is the molecular basis for the known rigidity of the cellulose polymer [14]. Hydroxyl groups of cellulose are of enormous meaning for the understanding of cellulose structure and purpose, regarding two distinct functions. Firstly, intra and inter-molecular hydrogen bonds determine the macromolecular structure and properties. Secondly the three hydroxyl groups per glucose unit work as functional groups. Intramolecular hydrogen bonds exist between the OH group of the third carbon atom (C3) and the oxygen atom of the next pyranose ring (O5´). Further, another hydrogen bond can be formed between the C6 hydroxyl group and the C2´ hydroxyl group of a neighboring subunit in the same chain. Hydrogen bond pattern depends on the structure of the cellulose chain investigated. Two of the three OH groups act as secondary alcohols (C2 and C3), whereas the C6 atom carries a primary alcohol [15]. The OH groups are the starting point of several modification and functionalization reactions with partly enormous commercial and historical relevance. Nitration, first performed 1833 with nitric acid [3], methylation using sodium hydroxide and dimethyl sulfate [16] as well as acetylation, with acetic anhydride and sulfuric acid [17] are only some of the possible modifications of cellulose.

2.1.1 Complex macromolecular structure

From the beginning of its biosynthesis, cellulose is organized as a union of several chains called microfibrils [10]. The chain length ranges from a few hundred pyranose rings in plants up to 10,000 found in some algae [18]. Orientation of chains and complex bonding patterns cause multiple possible formations of cellulose called polymorphs. Cellulose I is the natural form and by far, the most frequent one [3]. Two distinct crystalline allomorphs, namely cellulose Iα and Iβ exist. In cellulose Iα one chain exhibits a triclinic crystal system of parallel chains. In Cellulose Iβ the crystal structure is monoclinic and includes 2 chains [19]. A monoclinic system is one of seven crystal systems with a parallel shaped cell which has edge vectors a, b and c of unequal length. Only the angle between a and b is not 90˚[20]. For Cellulose Iβ one chain is located at vector c and the second chain crosses the center point of the plane of vector a and vector b [21]. A triclinic system, also with the vectors a, b and c, has three sides unequal in lengths and 3 unequal non-orthogonal angles [20]. A description of the three dimensional structure of the crystalline systems and location of the cellulose chains is illustrated in Figure 3. This means for the structure of cellulose Iα, that a chain is located at vector c, the neighboring chain is also located at vector c of a second crystal system but shifted. Both allomorphs have parallel orientation of the chains, meaning the beta glycosidic bonds have the same direction. Further, two neighboring chains are displaced by ¼ of the length of c, whereas c displays a subunit length (cellobiose) [18].

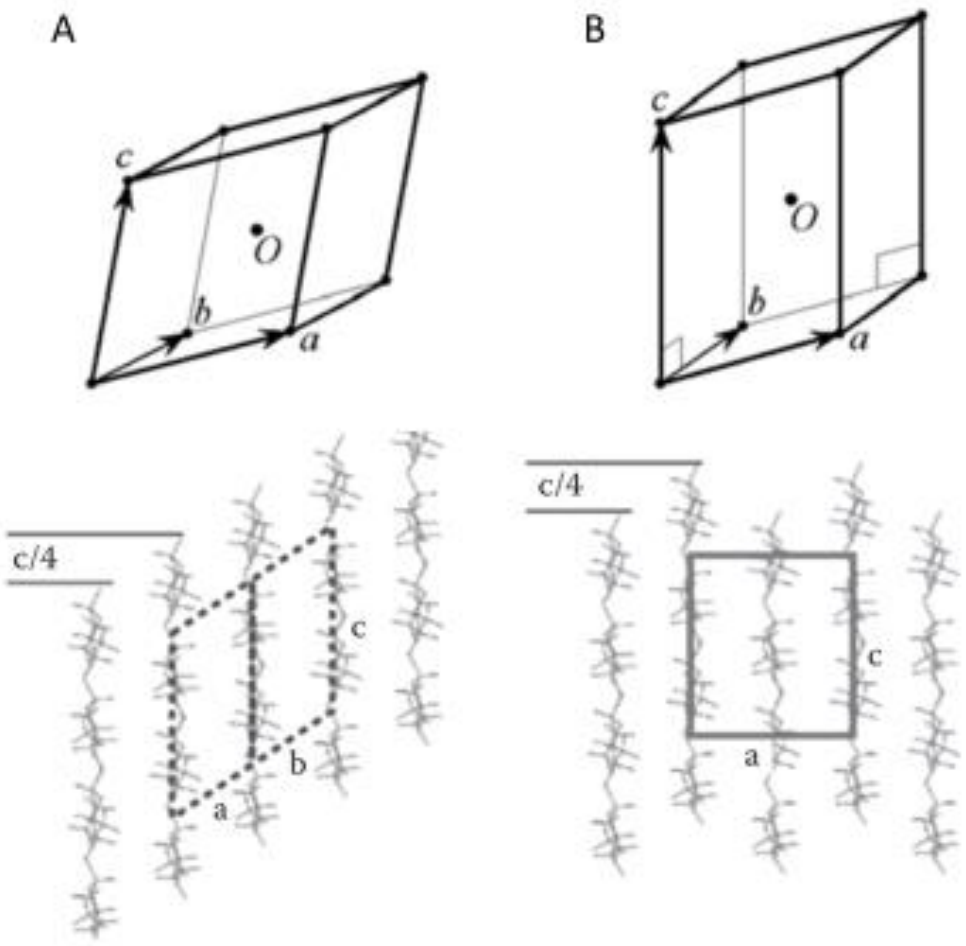


Figure 3. Three-dimensional crystal structure of a triclinic (A) and monoclinic (B) crystal system with the corresponding cellulose type cellulose Iα (A) and Iβ (B). The vectors are indicated by a, b and c. The center point is O. The crystal structure for the repeating unit (cellobiose) is marked as grey box for cellulose Iβ and as dotted box for cellulose 1α. Shift of neighboring chains is labeled as c/4. Figure adapted from [20] *and* [18].

An interesting aspect of cellulose behavior linked to macromolecular properties is its solubility. The Lindman hypothesis links solubility behavior to the amphiphilic character of cellulose [2]. Hydrogen bonds are present in cellulose between chains in one plane. Water as solvent could interfere with these existing hydrogen bonds and make cellulose soluble. If this would be the only present interaction cellulose would be water soluble. Reality shows a different behavior of cellulose, therefor other interactions must be present. The missing solubility relates to the hydrophobic properties of the glucopyranoside ring between C-H groups, where hydrophobic interactions occur. This results in a sheet like structure stabilized by hydrophobic interactions [22]. Objection about differences in strength of hydrogen bonds and van der Waals attraction, hydrogen bonds are stronger, can be rejected by the fact that more potential hydrophobic van der Waals positions for interaction exist than hydrogen bond positions

[2]. This means a potential solvent has to prevent both types of interactions to dissolve cellulose. Different substances like NaOH causing swelling or ionic liquids dissolving cellulose are known [22]. Summarizing, its amphiphilic properties and complex crystal structure cause heterogeneity and versatility of cellulose.

Besides the crystalline allomorphs, more unstructured version and regions of cellulose exists, called amorphous cellulose. Amorphous cellulose is twisted and exposes more hydrophilic parts to its surroundings. Further, less hydrogen bonds imparting a higher flexibility of amorphous cellulose were contemplated [23]. This is linked with a decrease in the elastic modulus, thermal expansion coefficient and bond energies. A transition of crystalline cellulose *via* a paracrystalline state into amorphous cellulose was observed. Transition of crystalline cellulose to amorphous cellulose can be achieved by heat, as visible in Figure 4. Thereby, the change in structure are related to a loss in hydrogen bonds between OH group of carbon 3 and the O5 as well as between the OH group of C2 and O6 [24].

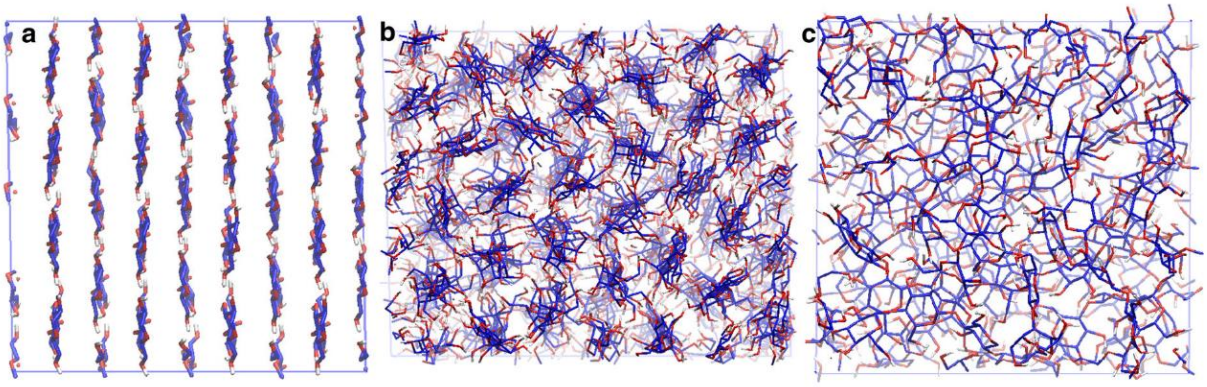


Figure 4. Simulated crystalline structure of cellulose in a temperature range from 250 (a) to 650 K (b), over a total recording time of 9 ns. Crystalline Iβ cellulose (a), paracrystalline cellulose (b) and amorphous cellulose (c).The simulation box is given as square around the cellulose [24].

2.2 Biosynthesis and cell structuring

A transmembrane structure with a diameter of approximately 25-30 nm is responsible for cellulose biosynthesis in higher plants. This complex, called hexameric rosette, contains six so called ’lobes ’, containing again six cellulose synthase enzymes, each synthesizing one cellulose chain from the cytoplasm into the cell wall (36 cellulose chains/rosette). Gens necessary for cellulose synthesis (CesA family) are conserved across different plant species, whereby a complete rosette is required for proper cellulose formation. One rosette with six lobes therefore forms 36 cellulose chains

resulting in a microfibril [12]. Four main compartments are known forth cellulose synthase. A Zn binding domain (N-terminal) in the cytosol, responsible for dimerization/oligomerization of the enzymes to form the complex, followed by two transmembrane domains, represent compartment one and two. The third is a large cytoplasmic central loop, responsible for substrate binding and polymerization. The end forms six transmembrane domains with the C-terminus in the cytosol. Three different isoforms of the enzyme are necessary to form a functional complex. [25]. It is worth to mention, that polymerization and crystallization are processes separated during cellulose synthesis, whereby the crystallization seems to be limiting towards the synthesis. Crystallization is a three step process: (a) monomolecular glucan chain sheets form by van der Waals forces; (b) sheets associate into mini-crystals by H-bonding and (c) mini-crystals convergence into the native crystalline microfibrils (=sub elementary fibrils) [2]. A cross section of the cellulose synthase rosette is shown in Figure 5.

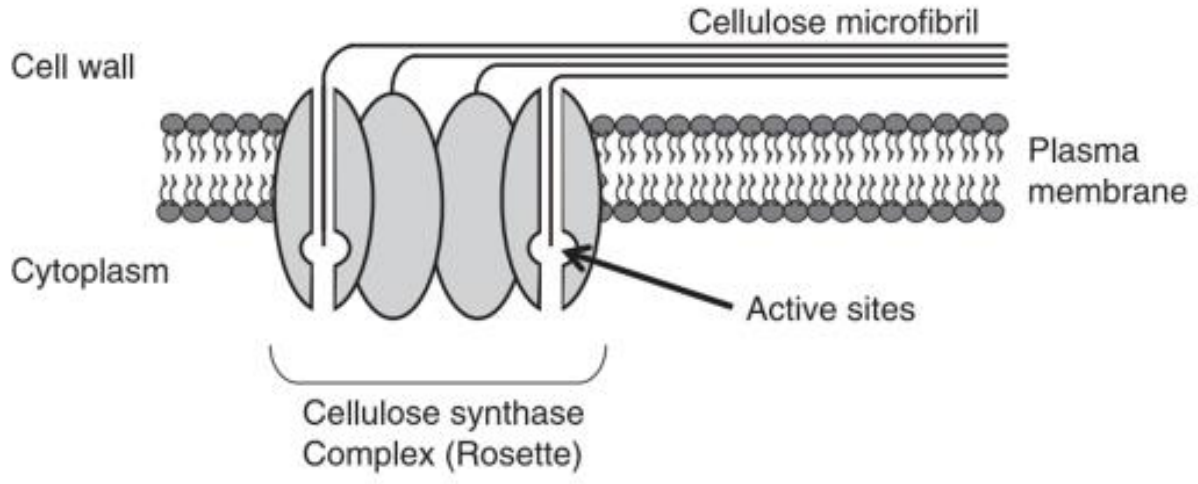


Figure 5. Graphic representation of the cellulose synthase complex in the cross section of a plasma membrane of a plant cell. Each rosette contains six lobes. Each lobe contains six cellulose synthases. Each single cellulose synthase polymerizes one cellulose chain from the cytoplasm into the cell wall. The chains form a microfibril directly after synthesis [12].

A microfibril can be up to 7 µm in length. Thereby the deposition is not random, rather orientation is perpendicular to the axis of the cellular expansion. Complex mechanisms present in cells as well as structures outside of the membrane direct cellulose orientation. Two major factors directing orientation were identified. On one side, presence and orientation of microtubular within the cells is interfering with the movement of the cellulose synthase. On the other side, when the microtubular

structure is missing, an intrinsic ability of self-organization was recorded. This would mean, that orientation of already existing fibers is influencing orientation of newly polymerized fibers, or another mechanism independent from cellulose and microtubular is existing [26]. Microtubular structure is not only important for directing operational enzymes, but also plays a role in directing newly produced enzymes form the Golgi apparatus *via* vesicles [25]. Orientation of cellulose in cell walls is important for the understanding of cell architecture and different degrees of robustness in plants. The general composition of the cell wall, as cellulose cross linked via hemicellulose embedded in a gel like matrix of pectin is of importance. Further, the orientation of cellulose is crucial. Cells with a mean orientation of cellulose fibers were identified as well as cells where cellulose was oriented randomly [27]. For plants and compartments of plants containing additionally lignin, the architecture is even more complex. New cellulose material in the primary cell wall is oriented rather perpendicular to the cell axis. During growing, fibers tilt towards a more parallel alignment. Thereby, this change in orientation is a function of cell elongation, meaning a more parallel structure for highly elongated cells. Tilting is stronger during cell elongation the more the fiber was tilt before growing. The secondary cell wall consists of different layers. The number of layers and the dominant angle varies between different plant species and the respective layer. Figure 6 six shows the number of layers and cellulose orientation for a wood cell (Figure 6A) and a bamboo cell (Figure 6B). For the wood cell (Figure 6A), three layers are visible. For S1 and S3 the angle towards the cell axis is high, whereas the dominant layer (S2) shows more parallel cellulose fibers. Number and distance control the orientation of cellulose fibers in the layers as well as geometrical constrains during growth. Other factors influencing the layer structure regarding thickness and cellulose orientation are plant age and stress factors. Younger, and more flexible plants, form microfibrils with a higher angel (perpendicular to cell axis) than mature plants. An orientation more parallel to the cell axis is more stress resistant for the same level of strain compared to high microfibril angels. The bamboo cell (Figure 6B) shows more layers with heterogeneous cellulose orientation between the layers, explaining bamboos flexibility and strength. As for a wood cell, the P layer is more disorder [28].

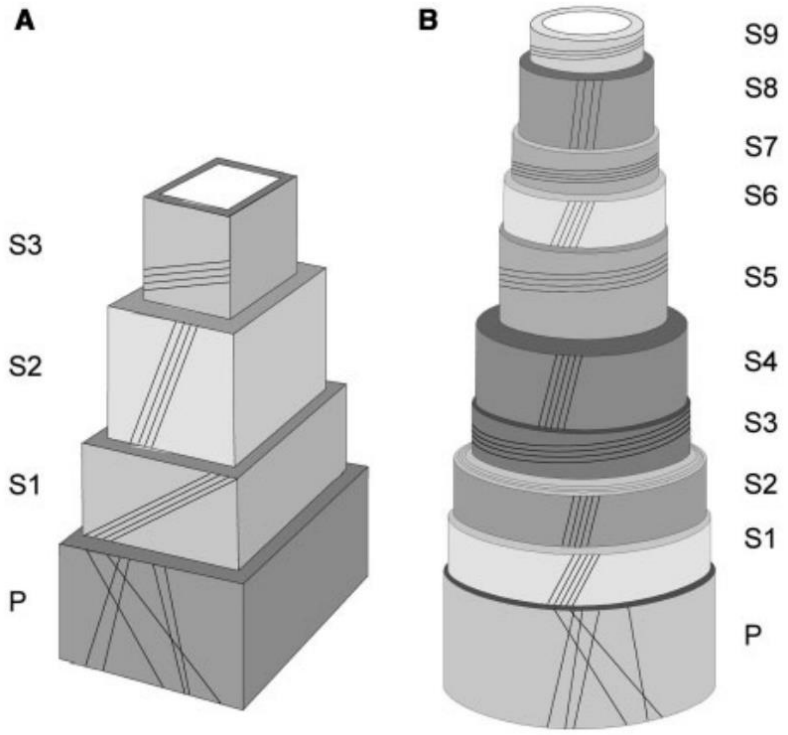


Figure 6. Layer structure of a wood cell (A) and a bamboo cell (B) with cellulose fibril coordination (black lines). A wood cell shows three layers (S1 – S3). The bamboo cell shows 9 layers. The primary cell wall as outmost structure (P) shows a more disorder pattern of fibers, whereas within the inner layers the cellulose structures are parallel within the layer and differ strongly between layers [28].

Not only crystalline areas determine cellulose structure, amorphous regions are of importance too. Amorphous regions (unstructured) and structured regions (crystalline) occur in the same microfibril on a regular pattern. Additionally, amorphous regions are the attacking points of acid hydrolysis [29] and starting point of many enzymatic degradation processes [30]. The pattern and distribution of amorphous cellulose is complex and enormously influences cell morphology, despite it represents only 1% of cellulose. In higher plants occurrence of amorphous regions was reported every 100-150 nm [31]. It was also shown that the outer regions of microfibrils were more disordered, due to direct contact with hemicellulose. Frequency and distribution of amorphous cellulose is always related to physiochemical and mechanical factors as occurrence of pectin or osmotic pressure of the cell [24]. For the S2 layer of softwood an amount of amorphous cellulose of 40–60% was reported. Due to its more hydrophilic properties, amorphous cellulose plays a role in water uptake and swelling, and therefore also porosity. Water uptake causes volumetric strain, which weakens mechanical strength resulting in a rubber like behavior at high moisture rates. Further, the increased hydrophobicity is linked to higher capability of transporting water [32].

2.3 Pulping, bleaching and sheet formation

As mentioned before, cellulose occurs most often in combination with other substances forming lignocellulose [1]. Next to cellulose, hemicellulose and lignin are major constituents, which need consideration during industrial processing. Hemicellulose is a branched polysaccharide polymer. Its chain length varies between 100 – 200 subunits [33]. Different types with different linkages exist: β-(1→4) linked mannans, xylans, xyloglucans and glucomannans (in all terrestrial plants) as well as β-(1→3,1→4)-glucans (amongst other in poales) [34], whereby the main sugars are glucose, mannose, xylose, galactose and arabinose. Hemicellulose accounts for 20 – 30% of total mass and occurrence of different types varies by wood type (softwood or hardwood), tissue and species [35][36]. It fulfills several crucial functions in plants like causing flexibility of the cell wall. Furthermore, it functions as an adhesive between cellulose and lignin. Since it works as media in the cell wall a function in structuring and formation of cellulose microfibrils was recorded. Additionally, hemicellulose is hygroscopic and swells when absorbing water, causing a reduction in interaction between hemicellulose. This confers hemicellulose a role in the water household and softening of wood material [37]. Hemicellulose is usually amorphous and therefore easy to separate from the lignocellulose material. Hemicellulose is a byproduct in kraft pulp mills. There, hydrogen production from hemicellulose could help to realize a biorefinery concept on an industrial scale. Further, utilization of rare sugars and their derivatives as platform chemicals or additives in the pharmaceutical industry or food industry were reported [35].

Lignin, the second most abundant biopolymer, accounts for 30% of terrestrial carbon in the biosphere. Lignin is crucial for land plants, since it provides strength, stiffness and structural integrity. It is a racemic aromatic heteropolymer derived from three different hydroxycinnamyl alcohol monomers [38]. Its formation is also a major reaction towards various biotic and abiotic perturbations, such as wounding, pathogen infection and metabolic stress. Lignin composition is quite heterogeneous and differs between species, cell types, cell wall layers and individual bearing [39]. Figure 7 illustrates a plant cell wall, a macrofibril, a cellulose microfibril with surrounded lignin and hemicellulose and the molecular structure of the cellulose molecule. A detail of the microfibril emphasizes differences in crystallinity and hydrogen bonds between chains.

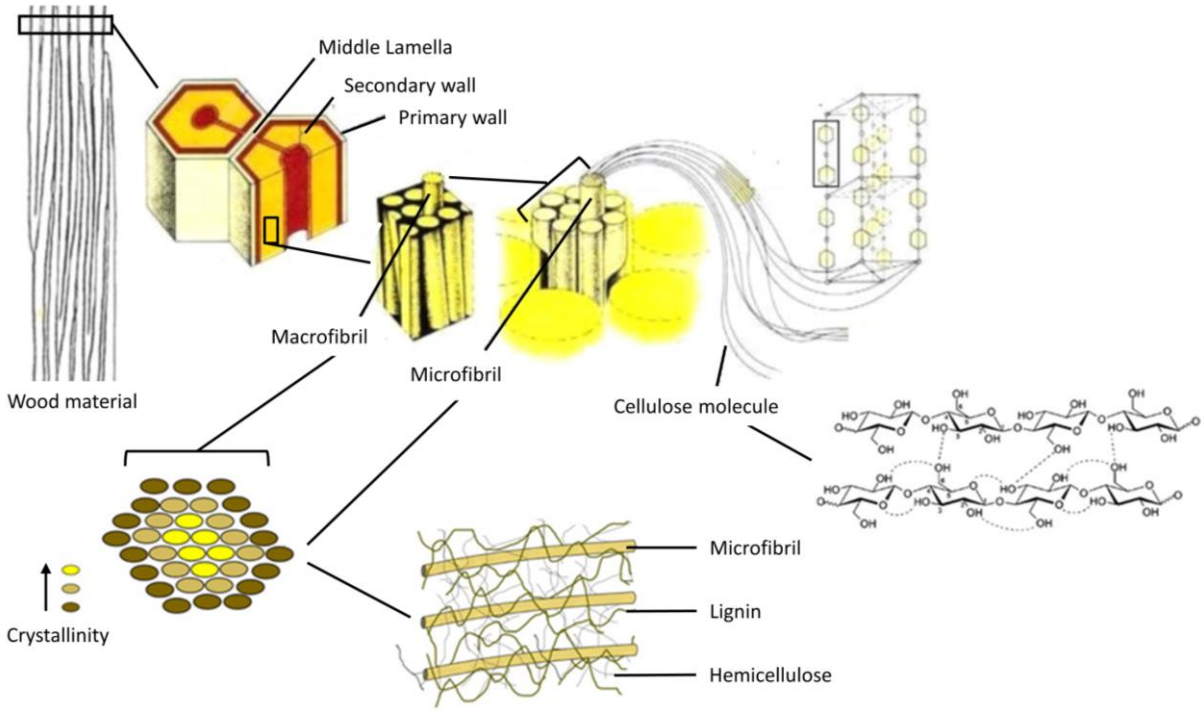


Figure 7. Structure of a plant cell wall. Cellulose chains form microfibrils, which form bundles called macrofibril. Centered microfibrils in a macrofibril show a higher crystallinity. Macrofibrils are surrounded by lignin and Hemicellulose. Macrofibrils form the largest part of the plant cell wall, the secondary wall. The secondary wall is surrounded by the middle lamella and the primary wall. Image based on work from Tsuchida et. al. and Gharehkhani et.al. [40] [41].

Pulping is a process to liberate more or less discrete fibers from lignocellulose materials as wood or others by chemical, mechanical or intermediate forms of treatment. The main process is delignification, meaning lignin is converted into soluble components and passed over into a liquid removable from the remaining cellulose. The lignin content is monitored by the kappa number (κ). The higher κ is, the higher is the lignin content of a specific pulp. Common chemical pulping processes are the Kraft process, also called sulfate process, and the sulfite process [42]. Chemical pulps are more flexible and conformation behavior and material strength of paper sheets is qualitatively high. The Kraft process is globally dominant. Sodium hydroxide, and sodium sulfide are used for treating the wood chips, this process is referred as cooking. When only sodium hydroxide is used, the process is called soda cooking. The active chemicals in delignification are OH^- and HS^-, whereby sodium sulfide delignifies and sodium hydroxide keeps the lignin in solution. Kraft pulps show a high variety, they are used as sacking paper or cardboard material, showing higher lignin contents. But also, bleachable Kraft pulps exist, exhibiting a lower lignin content. It has to be mentioned, that pulping is not capable of total lignin removal, depending on the field of application,

a bleaching process is required and often mandatory. Delignification via the Kraft process can be separated into three phases. First, in the initial phase, lignin and carbohydrates get dissolved in an equal manner to about 20%. A district solubilisation of lignin takes place in the second phase (bulk phase) until 90% of the lignin is dissolved. The final phase, to remove the remaining lignin is usually skipped, since fiber damage is a strong side effect in this phase. Further, a topochemical effect influences delignification, meaning different temperature and time are necessary to dissolve lignin from different compartments of the cell wall. Pulping with the Kraft process causes depolymerization of the carbohydrates starting from the reducing end. This reaction is refers to as peeling reaction [43].

The second, less dominant process is called sulfite pulping [43]. According to the Confederation of European Paper Industries (CEPI), only 6% of the chemical pulp is produced via the sulfite process [5]. Sulfurous acid (H_2SO_3) and bisulfite ions (HS^-) are the acting chemicals. This process can be performed in an acidic environment (pH 1-2) with acid sulfite and at more neutral pH (7-9) with neutral sulfites. Counter ions as calcium, magnesium, sodium or ammonium are required. Chemical recovery was a major problem in the beginning, later solved by using magnesium hydroxide or sodium hydroxide and the establishment of recovery technologies for these substances. An increase in lignin solubility is reached via its sulfonation. Further, acid hydrolysis takes place for lignin as well as for carbohydrates. Therefore, high amounts of monomeric sugars can be found in the pulping liquor. Most of them, like xylose, derive from the easily accessible hemicellulose. Hexoses present in the liquor can be used theoretically for ethanol fermentation by microorganisms. More products deriving from acid hydrolysis, depending on pH, are furfural and hydroxymethylfurfural. Degree in sulfonation increases with an increase in pH, reaching a plateau at neutral or slightly alkaline conditions. Approximately 30 sulfonate groups per 100 phenylpropane units of lignin can be found when the plateau is reached [43].

Additionally, to the chemical reactions described above, a mechanical and/or thermal contribution is of enormous importance for pulp and paper production. This combination of methods results in different types of pulp referred to according to the production process. Thermomechanical pulp (TMP), Refiner mechanical pulp (RMP), and Chemo thermomechanical pulp (CTMP) are the most important types of pulp [42].

A refiner is used for the mechanical modification of fibers. Thereby the changes of fiber characteristics are extremely complex. Fibers are cut and shortened and so the fines concentration increases. Further external fibrillation and internal fibrillation occurs. External fibrillation is the partial removal of the fiber wall, whereby the remaining particles stay attached to the fiber. Internal fibrillation refers to the delamination and swelling of fibers. Further refining can cause curls, nodes, compressions and abrasion on fibers. These changes are important for the later sheet formation, since fibrillated fibers are more flexible and their bonding surface increases. Refining influences the following parameters of a paper material: tensile stiffness, tensile strength, fracture toughness, burst strength, and internal bonding strength increases. Whereas, air permeability, bulk, absorbency, opacity, and light scattering decreases [44]. Differences between refined and unrefined fibers are visible in Figure 8. The number of connections between fibers strongly increases for refined pulp.

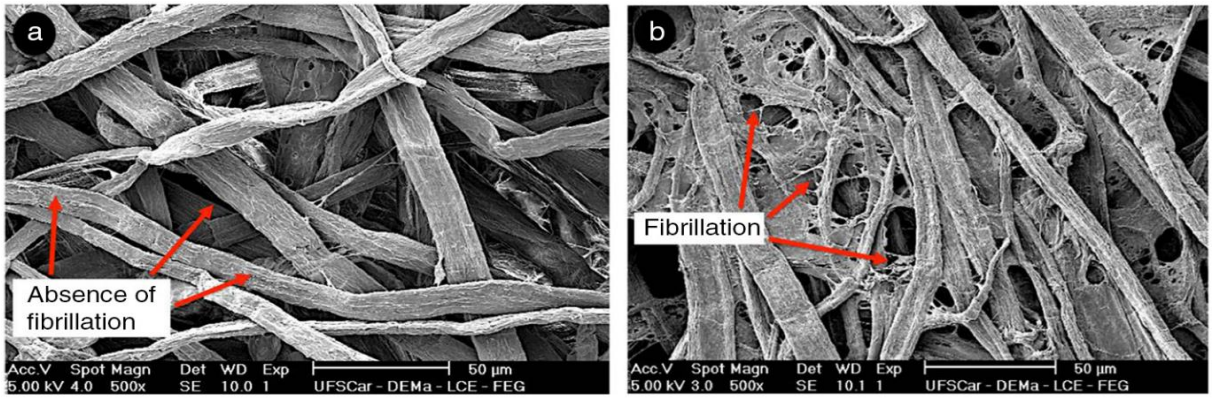


Figure 8. Scanning electron microscopy (SEM) of unrefined (a) and refined (B) bleached eucalyptus fibers [45].

Refining is a mechanical treatment of fibers between two parallel grooved plates, called stator and rotor. The stator is fixed, whereas the rotor moves bedside the stator. Refining of a single fiber can be divided into three different phases. In the pick-up phase fibers accumulate between the edges of the bars. In the second phase a compression of fibers takes place when the bars of the rotor are succinct with the bars of the stator. When the rotor bars move on, shear forces are dominant. Fibers hit between bar surfaces and bar edges, edges and edges as well as fibers hit each other. Different theories exist to describe the refining process. The most common and reliable theory is the specific edge load (SEL) theory. It describes the amount of effective energy deployed per unit edge length of crossing bars. SEL as all the widely used

theories relies on the energy input. In reality, force and not energy is responsible for altering the fiber structure. Therefore, newly developed theories include fiber distribution, refiner gaps and the amount of fibers to calculate direction and strength of forces acting on fibers. Figure 9 shows a visualization of a single fiber within a refiner. The three stages (pick-up, compression and final phase) are shown with their corresponding rotor position towards the stator. Further, the direction of different forces can be seen. Forces act parallel, vertical and angular to the fiber length axis. Different refiners for multiple applications with a wide range of capacity exist. For lab applications the PFI mill is most common. In this device, a steel roll with bars and a rotating disk with a smooth bed is responsible for the refining process. Both parts rotate with different velocity into the same direction. The PFI mill is characterized by a high energy input coupled with a low refining intensity. Bar and groove dimension for industry refiners can differ. Disk, conical and cylindrical geometries are in use. Energy saving, homogeneity of the refining and capacity are the most important factors for industry refiners [41].

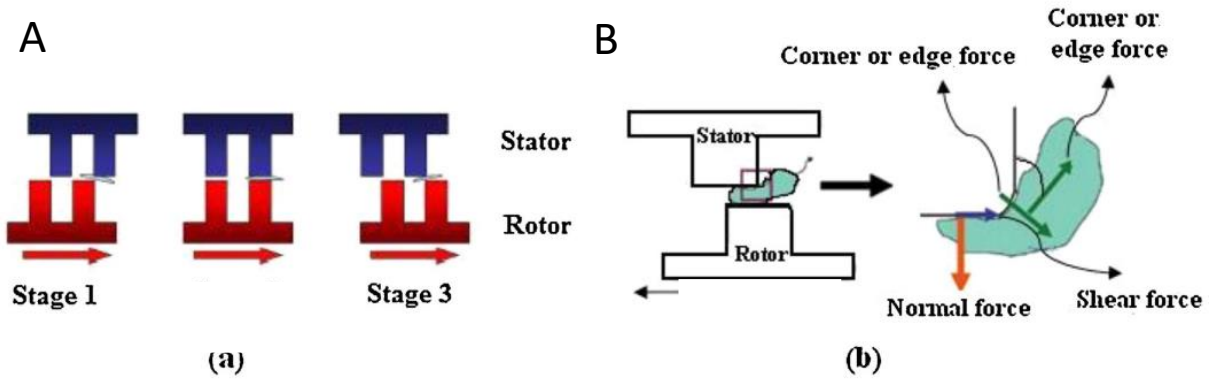


Figure 9. Refining mechanism for a single fiber between the bars of a rotor (red) and a stator (blue) in a refiner (A). Type and direction of forces present within a single fiber during refining (B) [41].

For certain paper grades the pulp has to be bleached. Bleaching increases sheet brightness, contrast when printed, removes impurities and helps to prevent aging of paper. Before actual bleaching, an oxygen delignification takes place [43]. At first, chlorine-based bleaching was the dominant process. Bleaching in general aims the oxidation and removal of impurities without effecting the cellulose structure. Chlorine in pulp bleaching produced environmentally harmful byproducts such as dioxins. Therefore, elemental chlorine was replaced by chlorine dioxide (elemental chlorine-

free, ECF) and later total chlorine-free (TFC) processes were introduced using oxygen, ozone, or hydrogen peroxide [33].

The actual sheet formation takes place in a paper machine. The three major steps in paper production from a pulp slurry are forming, pressing and drying [46]. In the beginning, pulp is applied at a low consistency. Most water is removed in the first two stages, which operate by vacuum (wire section) and pressure applied by cylinders (press section) [47]. Drying removes less than 1% of the water that remains but consumes an enormous part of the energy required in papermaking. Therefore, energy recovery is a major issue in paper production [46]. Next to forming a sheet, different types of alterations can take place in the paper machine. Starch and pigments can be added to the paper, altering color, strength or stiffness of the final product [48]. Coatings containing additives like latex binders and polymeric thickeners can alter the surface of paper. Recent research showed the ability of enzymatically polymerized lignin to act as more environmentally friendly coating [49]. Figure 10 illustrates the dimensions and complexity of a modern paper machine including all steps from applying the pulp to coating and rolling the finished paper. Modern machines can operate up to a speed of 130 km h^{-1} [47].

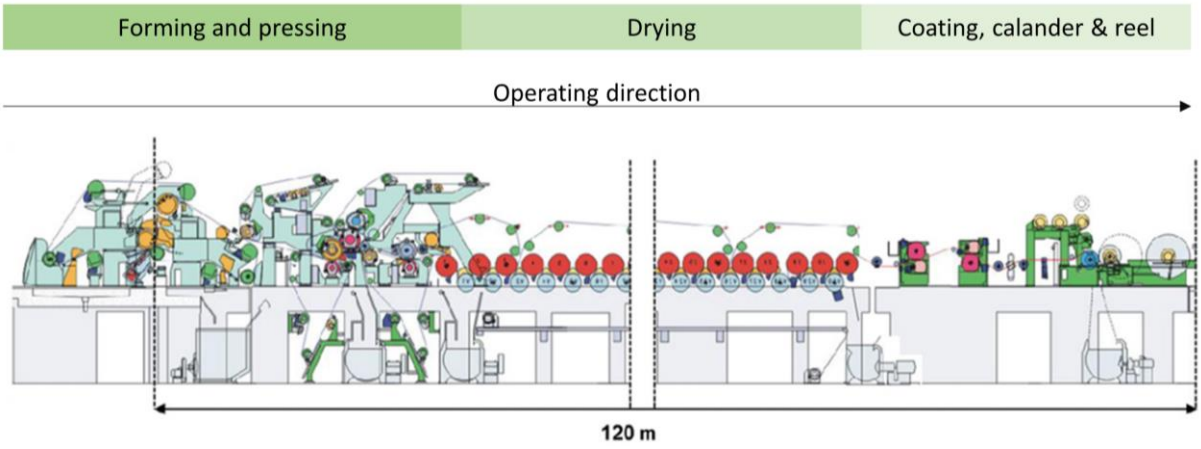


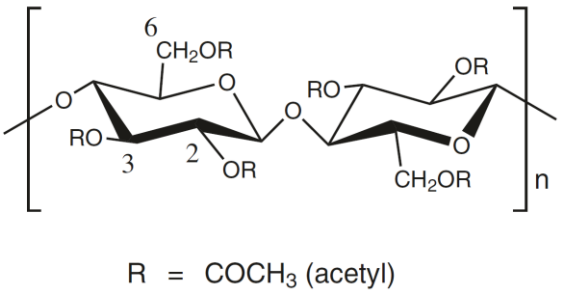
Figure 10. Paper machine, producing an endless paper sheet with up to 130 km h^{-1}. A pulp slurry is applied on the left and transported across the machine. Water is removed, in the beginning by vacuum and pressing forces (forming and pressing) and later by thermal drying (drying). With several techniques, other materials can be applied to the paper to change the sheet properties and surface characteristics. Finally the endless paper band is rolled up on a spindle on the right [47].

2.4 Cellulose derivatives and functionalization

Properties like hydrophilicity, biocompatibility, stereoregularity, and the ability to form superstructures impart cellulose ideal markings for functionalization and altering its structure and properties. The tree hydroxyl groups per glucose are generally accessible for chemical reactions. Reactions present are often typical conversions for primary and secondary alcohols [50].

2.4.1 Cellulose esters

Next to classic paper manufacturing, described above, pulp is the starting material for several industrial processes aiming on chemical alterations of the pulp´s cellulose and its properties. An important cellulose derivate with an industrial history since the 1920/30´s is cellulose acetate (CA). A pre-treatment of the used pulp is essential to successfully acetylate the material. During the pre-treatment all the hydroxyl groups get accessible thanks to a mechanical disintegration and swelling of the pulp. For swelling water and water with acetic acid can be used, but also sodium hydroxide and NH_3 showed to swell the pulp to a large extent. Degradation reaction of cellulose by a swelling agent as NaOH have to be considered. After the mechanical disintegration and swelling of the pulp sulfuric acid as a catalyst is required to produce cellulose triacetate (fully acetylated cellulose). The catalyst level can range from below 2% up to 15% and has next to temperature and performed numbers of cycles (number of catalyst additions) effects on the produced cellulose triacetate. This process is called “Acetic Acid Process”. Another process industrially applied used acetic anhydride and sulfuric acid and was called “Heterogeneous (Non-Solvent) Process”. The last commercial factory closed in the late 90´s in the US and this process showed never responsible for more than 10% production, globally. To produce cellulose acetate with a certain degree of substitution (DS) cellulose triacetate is hydrolyzed by an acid. Therefore, usually 5–15% water is required. The water content, low temperatures and longer duration helps to prevent breakup of the backbone structure. Finally, a precipitation followed by washing and drying the cellulose acetate is performed. Figure 11 illustrates the chemical structure of cellulose acetate with the positions an acetylation can take place (C2, C3 & C6).



R = $COCH_3$ (acetyl)

Figure 11. Structure of cellulose acetate (CA). Acetyl groups can be found on position C2, C3 and C6 of the backbone [51].

According to the precipitation setup different types of CA can be obtained. Pressing out the water produces CA flakes, whereas diluting the solution results in a fluffy material [52]. Cellulose from cotton and wood are the main sources for cellulose for CA production. However, research showed the ability of different materials to serve as a substrate for CA production. Biswas et. al. showed the ability to produce CA from wheat straw, corn fiber and rice hulls after a pre-treatment hydrolyzing hemicellulose with the heterogeneous (non-solvent) process [53]. CA is used in consumer products as cigarette butts [54], but plays also an important role in industrial applications such as membranes for drinking water filtration [55]. Additionally, other derivatives also exist like cellulose nitrate, the only inorganic ester produced on an industrial scale. Historically it was used as an effective explosive and as a film material. Nowadays it is still in use as an explosive and as a component in protective and coating lacquers. Other inorganic esters are cellulose sulfates (sulfuric acid for esterification) and cellulose phosphate (phosphoric acid for esterification). An overview about different inorganic esters is given in Table 1. Different organic esters are in use on an industrial scale for diverse applications. The functionalization for all compounds discussed takes place at C2, C3 or C6 (compare Figure 11). Next to CA, described above, cellulose acetate propionate and cellulose acetate butyrate are of interest. These compounds are referred to as cellulose mixed esters. Theoretically, highly esterified cellulose derivate can be produced form multiple fatty acids. Due to steric hindrance and reaction kinetics aliphatic acids with up to four carbon atoms are suitable. An overview about the most important organic esters is given in Table 1. Cellulose acetate phthalates are an interesting product, since they can be used as coating agent in drug delivery systems. Tablets are covered by a thin film, which disintegrates slowly in the stomach and fast in the small intestine where the substance is released. Cellulose acetate propionate and butyrate esters are versatile in their properties. The higher the amount

of acetyl groups is, the more brittle the material is. The application ranges from polarizing glasses to insulating films and compounds in coatings. An interesting application is the use as protection film for screens, where they reduce the angle the screen content can be read, increasing privacy and security e.g. for automated teller machines (ATM) [56].

Table 1. Inorganic and organic cellulose esters and a description of their production form cellulose [56].

Type	Name	Description
Inorganic	Cellulose halogenides	Transesterification of cellulose esters with hydrohalic acids
	Cellulose borates	transesterification of methyl and n-propyl borate with cellulose
	Cellulose titanate	Reaction of Cellulsoe with titanium tetrachloride in DMF, chlorinated anhydridesor chlorinated ester anhydrides
	Cellulose nitrite	Reacting cellulose with nitrosyl compounds (proton acceptor) and a suitable solvent for the resulting ester.
	Cellulose xanthate	Reacting alkali cellulose with carbon disulfide, which dissolves in dilute sodium hydroxide (represents the base of the viscose process).
Organic	Cellulose acetate phthalates	prepared from hydrolyzed cellulose acetate and phthalic anhydride providing a carboxyl-functionalized cellulosic
	Cellulose acetate propionate	Anhydride of acetic acid and propionic acid (sulfuric acid, catalyst). Proportion of anhydride/propionic acids determines substitution
	Cellulose acetate butyrate	Acetic acid and butyric acid with sulfuric acid as catalyst. Proportion of acetic acid and butyric acids determines the substitution proportion.

2.4.2 Cellulose ethers

All cellulose derivatives discussed so far were insoluble in water. A widespread representative of a water soluble derivative is carboxymethyl cellulose (CMC). It is an ether with derivatization at O-2, O-3 and O-6 of the cellulose backbone. The water solubility is caused by the carboxymethyl groups (-CH_2-COOH) coupled. The degree of substitution (DS) describes the amount carboxymethyl groups coupled per glucose subunit. When all OH groups carry a carboxymethyl group the DS is 3. Usually the DS for CMC used in industry ranges from 0.4 to 0.8 meaning not each subunit carries at least one carboxymethyl, group [57]. On an industrial scale CMC is used in the food sector as thickener, emulsifier, and stabilizer or dispersing agent. Classical raw materials are paper sludge, wood residues, cotton linters and pure cellulose fibers. But also alternatives were investigated elaborating the potential of CMC production form residue streams as corn husk [58]. Besides these traditional raw materials bacterial cellulose is a promising substrate for high value CMC with a tailored DS. With this approach, expensive purification steps are not necessary. The carboxymethylation can follow a standard procedure as used for material of different origin. The first step is an alkalization performed in isopropanol with sodium hydroxide, followed by etherification with sodium monochloroacetate. The production process starting with cellulose is shown in Figure 12. The DS (max.3) could be controlled by adjusting the molar ratios of the used chemicals and the etherification time [59].

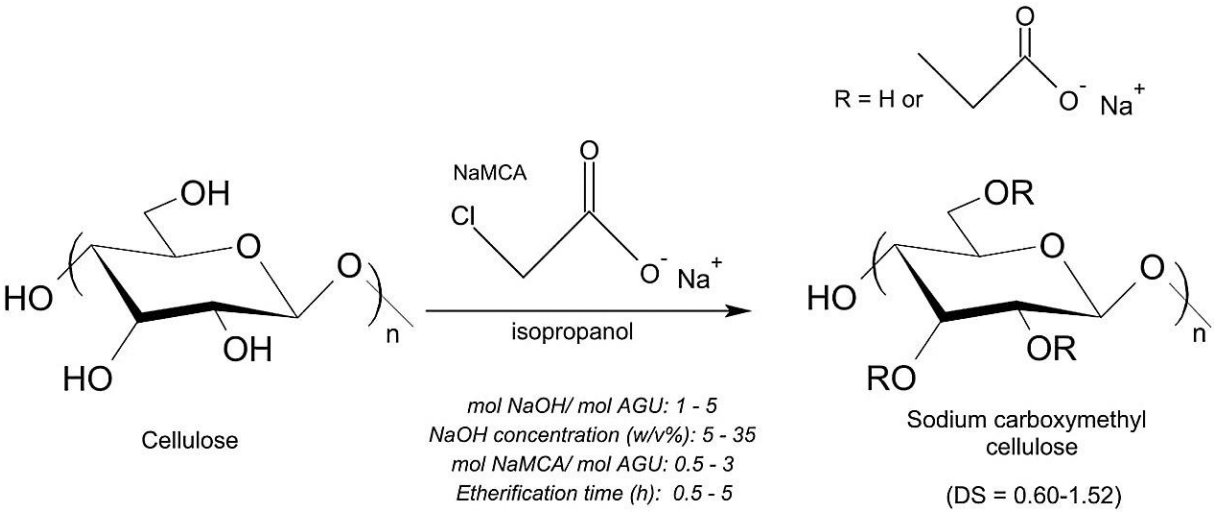


Figure 12. Production of carboxymethyl cellulose from cellulose. An alkalization with NaOH in isopropanol is followed by an etherification with monochloracetate [59].

Further, CMC is an important substrate in characterization and activity determination of cellulases, used since decades. Photometric detection of the reduced sugar form CMC is a very common method [60]. Furthermore, viscometric measurements can provide essential information. Hereby, the DS and DP are essential since high DS cause insufficient accuracy. Additionally, DS and DP should be high in uniformity. All these factors make CMC activity determinations in absolute terms difficult. Therefore, relative comparisons between enzymes measured with the same substrates should be preferred [61].

2.4.3 Unconventional functionalization approaches

Next to cellulose ethers and esters multiple more functionalization are known. They can be summarized as unconventional methods for cellulose alteration but rely as the approaches described above on the special properties of cellulose, first of all on the presence of reactive hydroxyl groups. Different solvents play an important role in these modifications. Dimethyl sulfoxide (DMSO)/paraformaldehyde (PF), chloral/dimethylformamide (DMF)/pyridine, trifluoroacetic acid (TFA), and DMF/N_2O_4 were investigated regarding their solubilisation properties. As acetylation agents, aromatic and unsaturated acid chlorides as well as anhydrides of different acids and free acids were investigated. The diversity in swelling agents/solvents and functional molecules attachable to cellulose offers a sheer endless variety in cellulose derived compounds. Chromophores, fluorophores, redox active groups and substituents with special magnetic, optical or biological functions are of interest. [50]. Furthermore, some methods use grafting and coating to functionalize cellulose sheets directly. Cusola et.al. prepared a so-called functionalized solution containing sonicated and enzymatically modified lauryl gallate and sulfonated kraft lignin. Applying this on filter paper sheets increased the surface hydrophobicity. The lignin was thereby used as dispersing agent and grafted lauryl gallate increased the paper hydrophobicity [62]. A completely different functionalization is the production of conductive electrodes made from paper printed with gold nanoparticles. After printing the gold particles to the surface and sintering it with infra-red radiation a self-assembled monolayer was formed by treating the paper sheet with 1-octadecanethiol. The thickness of this monolayer was 2.0 ± 0.4 nm. The conductivity of the paper electrodes was comparable to glass electrodes. Flexible and thin paper electrodes can be used in biosensors or electronic devices [63]. Many more functionalization approaches exist, using covalent chemical

coupling to cellulose or attachment (coating) to paper and paper like materials. Current research aims at various applications of functionalized materials. Setälä et. al. coupled nanoparticles made from lignin and tall oil fatty acid esters covalently to cellulose and proved their antimicrobial properties [64]. Thota et. al. coupled phytic acid by forming a carbamate linkage using 1,1´- Carbonyldiimidazole and Ethylenediamine to cotton. The so produced material exhibited flame retardant properties [65]. Gonçalves et. al. fused a carbohydrate-binding module with an odorant binding protein. The aim was to release the odor β-citronellol when wetting the cotton material [66].

2.5 Enzymes in lignocellulose processes

As mentioned above, traditional cellulose processing and converting is energy intensive and requires multiple chemicals. Further, manmade derivatives of cellulose might cause environmental damages when handled improperly. Enzymes can be a promising aid in converting production process towards sustainability and energy efficiency. For cellulose, hemicellulose and lignin, as main components of plant biomass [1], enzymes exist, altering these materials. Each component of lignocellulose has a corresponding group of enzymes namely cellulases for cellulose, hemicellulases for hemicellulose and laccases for lignin [67]. Fungi are a main group of organisms degrading lignocellulose biomass expressing all the enzymes necessary. In nature, lignin and hemicellulose forms a barrier around cellulose, compare chapter 2.3. In nature, soft- rot, brown-rot and white- rot fungi are mainly responsible for plant biomass degradation. A direct use of the fungi species is insufficient in an industrial context since it is to slow and the fungi would consume a noteworthy fraction of the biomass [68].

2.5.1 Cellulases

The term cellulase summarizes different single enzymes acting cooperative on the degradation of cellulose to glucose. The main enzymes are endoglucanases acting within the amorphous region producing cracks as starting point for other enzymes. Exoglucanases start on these cracks and release short chain fragments as cellobiose or cellodextrins. Cellobiohydrolase and cellodextrinase are part of this group of enzymes. Exoglucanases can work in in both directions, reducing to non-reducing end and the other way around. Finally, β-glucosidases cleave cellobiose to glucose. A group of enzymes active in cellulose degradation discovered later are polysaccharide monooxygenases (PMOs). In contrast to endoglucanases, they can act in the

crystalline region and produce cracks by oxidizing single carbon atoms in the cellulose backbone. Type 1 is specific for the C1 position, type 2 for C4 and type three is less specific for a certain position though they require an electron donor, which can be an enzyme too, like cellobiose dehydrogenase [69]. Figure 13 illustrates the synergistic concept of enzymatic cellulose degradation to glucose. Considering the high crystallinity of cellulose and its strong association to other cell wall components the above described mechanisms cannot be solely responsible for the comparably fast degradation of lignocellulose in nature.

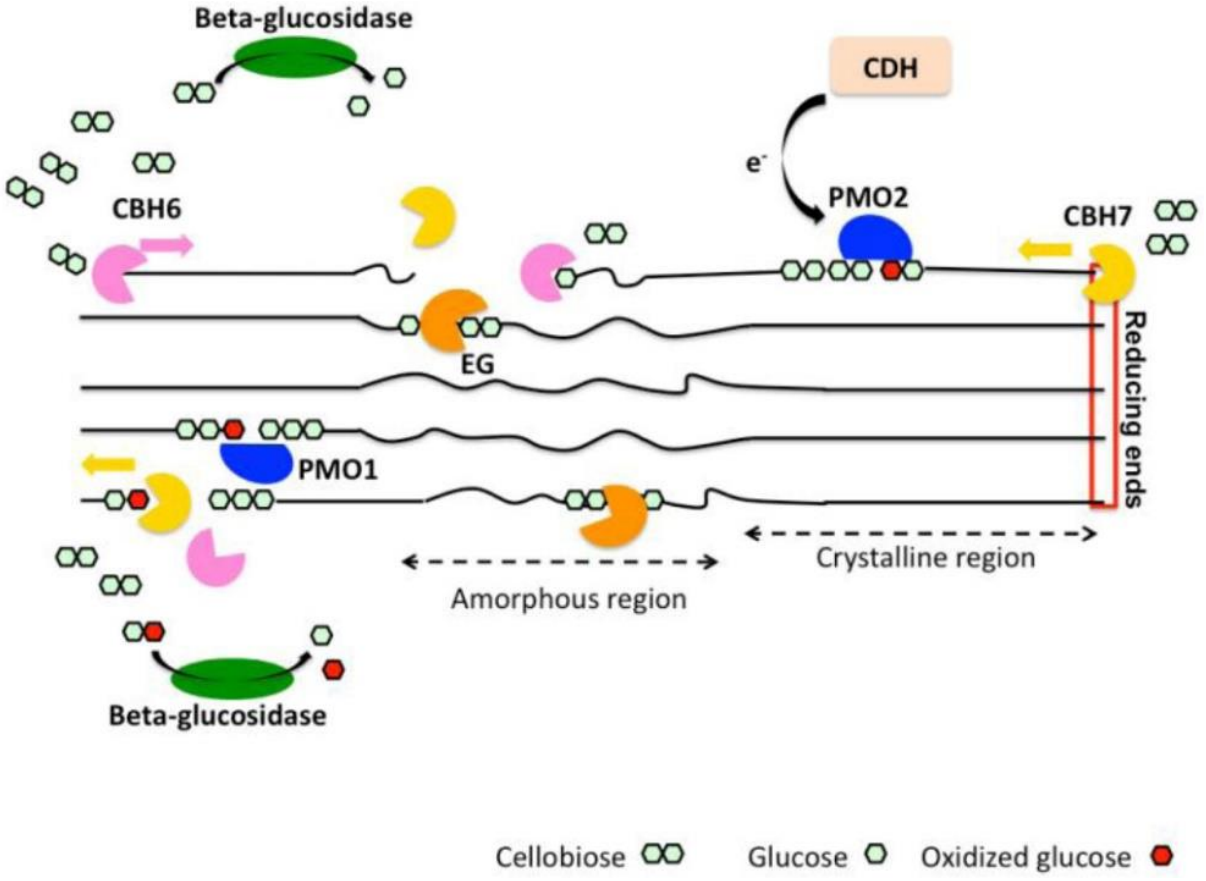


Figure 13. Synergistic cellulose degradation by enzymes. Polysaccharide monooxygenases (PMOs) oxidize single glucose units in the crystalline region. Therefore a coenzyme providing electrons is necessary, here Cellobiose dehydrogenase (CDH). Endoglucanases (EG) act in the amorphous region. Exoglucanases (e.g. CBH) work in both directions, releasing cellobiose. Cellobiose is hydrolyzed by β-glucosidases to glucose [69].

A group of non-catalytic enzymes active in cellulose degradation are expansins and swollenins. Expansins are present in bacteria and plant cells and are able to disrupt the hydrogen bonding in the cellulose polymer. Swollenins are expansin like proteins and can be found in fungi. They open the polymer without degrading it and expand thereby the accessibility for the hydrolytic and oxidative acting enzymes [70]. It was found that the expression of swollenin in *Trichoderma reesei* is in a largely similar

manner as the gens for cellulases. Further, a disruption of cotton fibers without an increase in sugar concentration was observed, when swollenin was present. Also a weakening effect on filter paper was observed [71] and a reduced crystallinity index [72]. Seven cysteines and eight aromatic amino acids seem to be essential for interaction with carbohydrates. Interestingly, a catalytic activity of swollenin on soluble substances containing b-1,4 glycosidic bonds, i.e. carboxymethyl cellulose was observed. A crucial feature for cellulose interacting enzymes as swollenins is the so called cellulose binding module (CBM). For *Trichoderma reesei*, this domain is N-terminal and between 4 – 10 kDa in size [72]. CBMs are a domain present in many different cellulose related enzymes. This means enzymes like cellulases are composed out of two distinct modules, the catalytic module and the binding module. They fulfil several non-catalytic functions: a concentration of enzymes at a specific position on the substrate (proximity effect), selecting the target within the lignocellulosic material and non-hydrolytic disruption of cellulose. CBMs that are specific to insoluble cellulose can be divided into two types. Type A binds crystalline cellulose whereas type B binds non crystalline cellulose. They adsorb to accessible sites on cellulose forming a complex held together by specific but non-covalent interaction. It was reported, that the interaction can be extremely strong even causing physical disrupting cellulose and releasing small particles [73]. A symbolized structure of a cellulose active enzyme with a linker connecting the catalytic domain and the CBM is visible in Figure 14. Additionally, the process of disrupting, called amorphogenesis, is given in Figure 14 too. First, the CBM adsorbs to cellulose defects such as micro cracks, followed by a penetration into the interfibrilar space. This penetration causes an increase in the mechanical pressure pushing the fibers apart. The so formed capillary space gets filled with water, which penetrates deeper and deeper in parallel to the cellulose fiber orientation, destabilizing the hydrogen bonds leading to a disassociation of the individual micro fibrils.

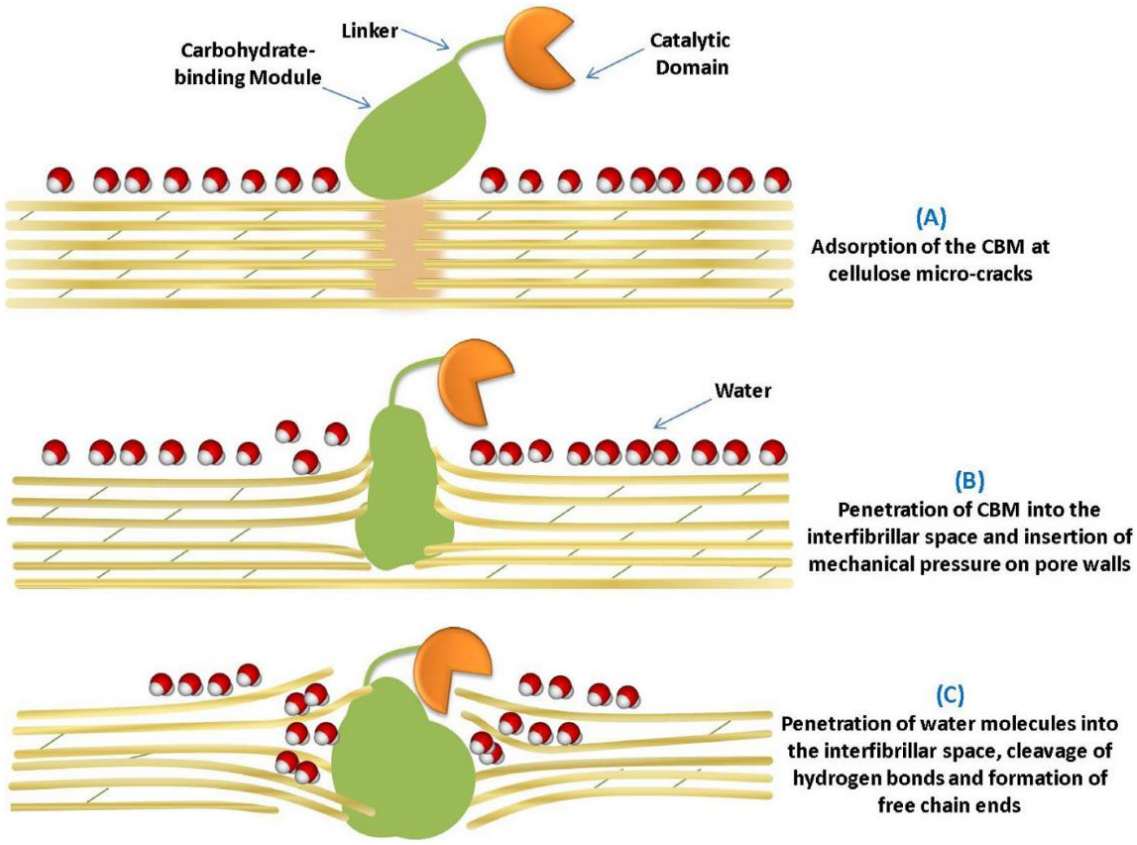


Figure 14. Schematic representation of an enzyme active on cellulose, carrying a cellulose binding module (CBM) linked to the catalytic domain. Further, the three steps of amorphogenesis of cellulose which are adsorption (A), penetration of CBM (B) and cleavage of hydrogen bonds by penetrating water (C) [73].

Latest research mentions 80 different CBM families with a wide range in affinity to either crystalline or amorphous cellulose. Monitoring the degradation process over time of CBMs with different affinities reveals an accelerated enzymatic degradation for zones of lower structural organization and increased cellulose accessibility. This caused fast fiber fragmentation and an increasing crystallinity of the surface of the cellulose. Monitoring the hydrolysis showed paradoxically an increase in the mean fiber length in the beginning. An explanation for this is the inhomogeneity of cellulose. Cracks are not distributed equally within a material. Therefore hydrolysis starts concentrated in some regions releasing many short fragments, whereas long stable regions remain. This shifts the fiber length distribution towards a higher mean length. The number of involved enzymes and the complexity of the lignocellulosic network makes it degradation complex to control, especially when the aim of a process step is not the complete degradation of a material [74].

2.5.2 Hemicellulases

As cellulase also hemicellulase is a collective term for different enzymes. Hemicellulose includes xylanase (EC 3.2.1.8), β-mannanase (EC 3.2.1.78), arabinofuranosidase (EC 3.2.1.55), β-xylosidase (EC 3.2. 1.37), and other glycoside hydrolases or carbohydrate esterases. Xylan constitutes approximately 70% of hemicellulose. As for cellulose the total degradation of hemicellulose is a stepwise process. Xylanases cleave their substrate into smaller oligosaccharides which are further hydrolyzed to xylose by β-xylosidase [75]. A mechanism including swollenins was reported for hemicellulose degradation in a similar manner as for cellulose. Gourlay et.al. reported a swollenin mainly disrupting hemicellulose in corn stover. They also found a pronounced synergism with xylanase monocomponents, but only little synergism with exoglucanase and endoglucanase for cellulose degradation. On the other hand the tested swollenin released small amounts of oligomeric arabinose galactose and mannose, indicating that the enzyme is not absolutely specific on releasing oligomeric xylose [76]. The lack of synergism was only found for the used swollenin. A synergistic degradation when the whole enzymatic cascade is used was seen. At which, it must be mentioned that synergism can be at least partly explained by increased accessibility of cellulose when hemicellulose is degraded and not by interactions of cellulosic enzymes on hemicellulose or vice versa. Synergistic effects of cellulases and hemicellulases where reported for enzymes produced by Clostridium cellulovorans. Interestingly, they reported about the contribution of a scaffolding protein called CbpA [77], which contains a cellulose binding domain, four hydrophilic domains and nine cohesin domains. This protein increased the cellulose degradation activity and enabled the physical proximity of the degradation process to the *Clostridium cellulovorans* cell wall [78]. Since the chemical composition of hemicellulose is more variable compared to cellulose and also differs from species to species and plant functional type comparisons are difficult [36]. Figure 15 gives an impression about ratios for different sugars in different plant types and parts. Therefore, enzymatic processes aiming on hemicellulose have to consider this sugar variety. For some plant parts and plant types xylose is not even the main constituent (e.g. needles of conifers).

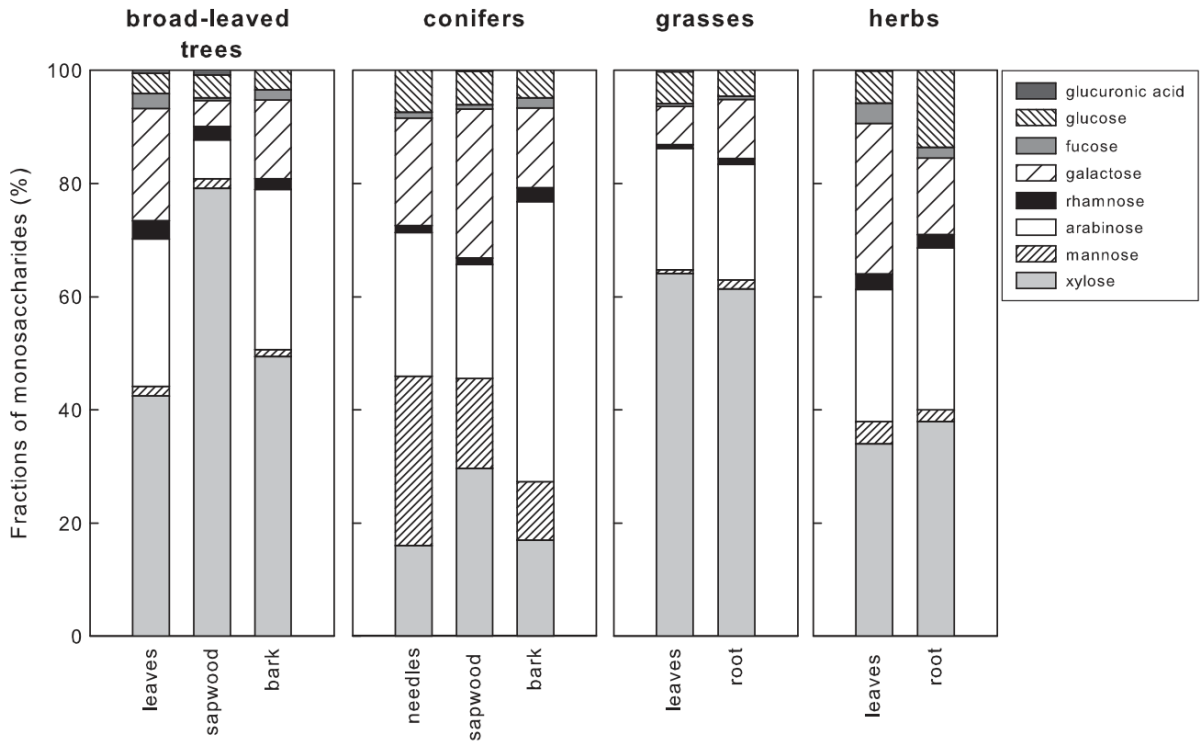


Figure 15. Ratios of different sugars in hemicelluloses of different plant types and parts [36].

The fields of application for hemicellulases in pulp processing are manifold. Xylanases can be used in pulp bleaching of kraft pulp. Substantial savings in ClO_2 could be obtained. Incubating pulp with xylanase followed by a treatment with a so called laccase mediator system saved 35% chlorine dioxide [79]. Deinking of wastepaper is another promising and sustainable application with xylanases playing an important role [80].

2.5.3 Laccases

In contrast to hydrolytic enzymes as cellobiohydrolase and endoglucanase mentioned above, laccases are oxidative enzymes. They do not act on cellulose or hemicellulose but play an important role in delignification, what makes their consideration in lignocellulose processing important [81]. Lignin, produced as residue material during delignification in pulp producing, is a highly heterogeneous polymer, with a great number of potential applications especially in combination with laccases and corresponding mediators. Lignin based dispersants or adhesives are an interesting application of lignin [82] as well as lignin hydrogels in agriculture to maintain soils humid [83]. Remaining lignin in pulp influences its properties, which makes laccases interesting in pulp related applications and processing of residue materials of a paper mill. Remaining lignin can influence the color of a paper sheet by the formation of so

called lignin derived chromophores during bleaching [84]. Further, a study using laccases to alter lignin in pulp showed an increase in burst, tensile and wet tensile strength [85]. Laccases are multi cooper enzymes oxidizing different phenolic compounds as lignin while reducing molecular oxygen to water [82]. Laccases can be found in white rot fungi like *Trametes versicolor* where they use laccases for delignification. *Trametes versicolor* showed also some delignification when incubated with kraft pulp. Laccases can polymerize and un-polymerize lignin. Understanding this mechanism of laccases on lignin is essential, especially from an industrial point of view [86]. The presence of oxygen to polymerize lignin has to be considered [49]. Laccase gens were also found in plants, more precise, 84 laccase gens were identified in *Gossypium hirsutum* a globally used source for cotton fiber production [87]. As for cellulases, also laccases can be equipped with a carbohydrate binding module (CBM). Positive effects for such an enzyme (CBM fused to *Pycnoporus cinnabarinus* laccase) in bioleaching of pulp was reported [88]. Another pulp and paper related application of laccases is their use in deinking old newsprint paper in combination with a mediator. The mediator thereby acts as supporting agent, oxidized by the laccase, either removing the ink by removing the lignin the ink is attached to, or direct decolonization of the ink [89]. The effect of such a laccase mediator system on lignin in pulp can be seen in Figure 16. After the treatment the SEM image is lignin free.

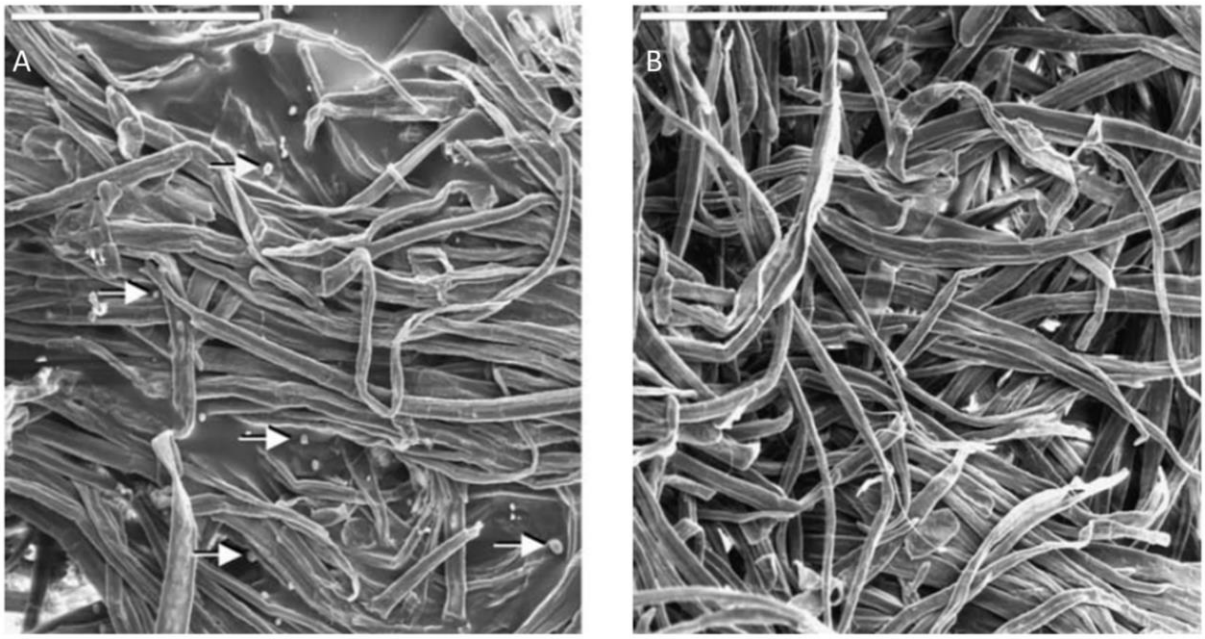


Figure 16. Scanning electron microscopy of eucalypt kraft pulp before the treatment with a laccase mediator system. Spherical structures (arrows) represent remaining lignin in the pulp (A). After the treatment no spherical structures were present (B). Bars: 200 µm. Altered from [90].

2.5.4 Industrial requirements on enzymes

So far, the structure and abundance of lignocellulose, derivatives of cellulose, traditional processing of lignocellulose and enzymes active on lignocellulose were discussed. To successfully apply an enzyme in an industrial application all these background information are needed, but further parameters and properties have to be considered. Analyzing potential advantages of a proposed process alteration is a complex scientific field. First of all, enzymes must fit the process conditions according to basic parameters as pH, temperature and stability. Considering the fact that pulping processes can operate at high temperatures and pH (pH > 12, temp. up to 180 °C) the application of enzymes during a process at this conditions is futile. Since an industrial process is not the natural habitat for an enzyme and the wanted effects might be different from what an enzyme is supposed to do in the organism it derives from, intensive research is required. Biotechnological innovation lies between identification of enzymes and successful industrial application. For example, most fungal laccases have acidic pH optima. But, some bacterial laccases showed alkaline pH optima, making them more promising in biobleaching than others. [91]. Another exceptional example is the use of cellulases in refining pulp. Cellulases should beneficially modify characteristics of the product like increasing strength, tensile and burst [92]. Therefore, the enzymes should help to fibrillate the fibers [93]. Fibrillation is the introduction of micro cracks within a fiber (internal fibrillation) as well as the fraying of fiber material on the outside (external fibrillation), caused by mechanical force (Figure 9). Fibrillation also causes the complete detachment from so called fines from the main fiber. Internal and external fibrillation can be seen in

Figure 17 [94]. In nature, promoting fibrillation for paper production is not a main aim of enzymes. Cellulases are part of the total hydrolysis machinery to degrade cellulose to glucose [95].

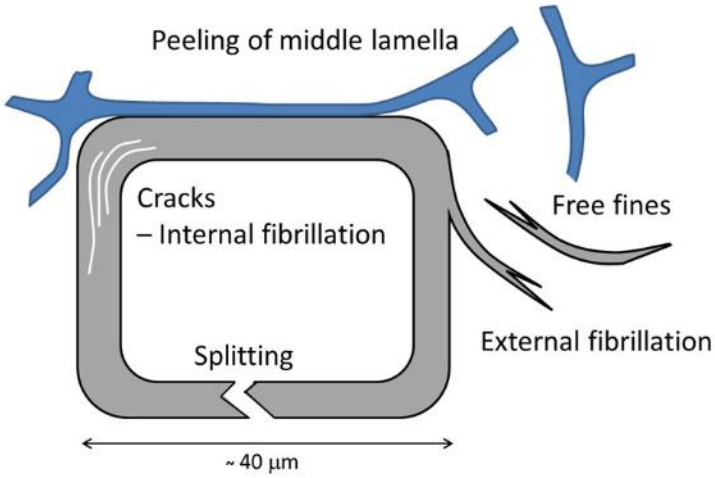


Figure 17. Internal and external fibrillation of cellulose caused by mechanical force. Internal fibrillation [94].

Therefore, biotechnology focuses on endoglucanases, since they operate within a fiber which is the preferred function for refining. Here, several factors require consideration. Endoglucanases can differ, several families exist with different properties. Further the fiber consistency has an enormous effect on the enzyme performance [96]. Additionally, purifying endoglucanases from the cellulose cocktails can be challenging. Already in 1985 six different endoglucanases three exoglucanases and one β-glucosidase were purified from a commercial cellulose preparation [97]. Further, isolating endoglucanases from the other components might influence performance since synergistic mechanisms between different cellulose active enzymes might be lost. For example, Valjamäe et. al. observed a strong synergy between Cellobiohydrolase I and Endoglucanase I on bacterial cellulose [98]. Even more difficult is the fact, that one enzyme can have more than one activity. Multiple substrate specifies for different endoglucanases was reported in literature [99]. All these circumstances have to be considered by biotechnologists when introducing enzymes to pulp and paper processes. Not least, economic factors, social aspects and production site properties need consideration when implementation of enzymes is considered. A life cycle assessment study performed on enzyme assisted processes in the pulp and paper industry defines bleach boosting, refining, pitch control, deinking, and stickies control as valuable fields for enzyme application. The impacts of enzymes production on global warming, acidification, nutrient enrichment and photochemical smog formation are significantly low compared to the savings achievable in the pulp and paper industry [100].

2.6 End of life of cellulose products

The lifespan of cellulose products as paper is not endless. After approximately seven recycling cycles the fiber damage is to strong and aging effects can cause yellow discoloration [101]. Further, even in regions with sophisticated recycling systems, the recycling rate of paper is not 100%. A declaration of the European paper industry and other stakeholders declares a recycling rate of 70% for 2015 [102]. Some paper-based products as money bills are not recyclable at all, with traditional systems, causing high cost for handling. In the United States, an amount of approximately 500 million dollars is associated with the management of waste banknotes. Alternative utilizations beyond incineration are needed. Ethanol production is a widely investigated concept. Different approaches exist, all following a basic route. The cellulosic material is enzymatically hydrolyzed to glucose with the process that is supported by different pretreatments e.g. with sulfuric acid. This process is followed by a fermentation under anaerobic conditions to produce ethanol using *Saccharomyces cerevisiae* [103]. Different products as newspaper [104] and cardboard were investigated according to their potential as substrate for bioethanol production [105]. A similar process performing the same reactions but combined in one vessel is simultaneous saccharification and fermentation (SCF). A study from 2010 obtained 78% of the theoretical maximum ethanol yield possible using kraft paper mill sludge as substrate [106]. A schematic overview about the two approaches, separated hydrolysis and fermentation (SHF) and SSF for lignocellulosic biomass is given in Figure 18. SSF requires thermotolerant yeasts that can operate at temperatures above 40 °C which is required by the enzymes for saccharification. Enzyme cost and end product inhibition of the enzyme by glucose are important factors, whereby SSF can overcome end product inhibition of the enzyme since the produced glucose is consumed immediately [107]. The bioethanol market is highly competitive and prices are under pressure. For bioethanol from sugar crane bagasse produced in Brazil, a minimum selling price of US$ 2.17 per gallon was projected [108]. Feedstock purchase, transport system, crude oil prices and environmental concerns are only some parameters effecting global bioethanol markets [109]. Therefore, more valuable products from cellulosic residue material deserve attention.

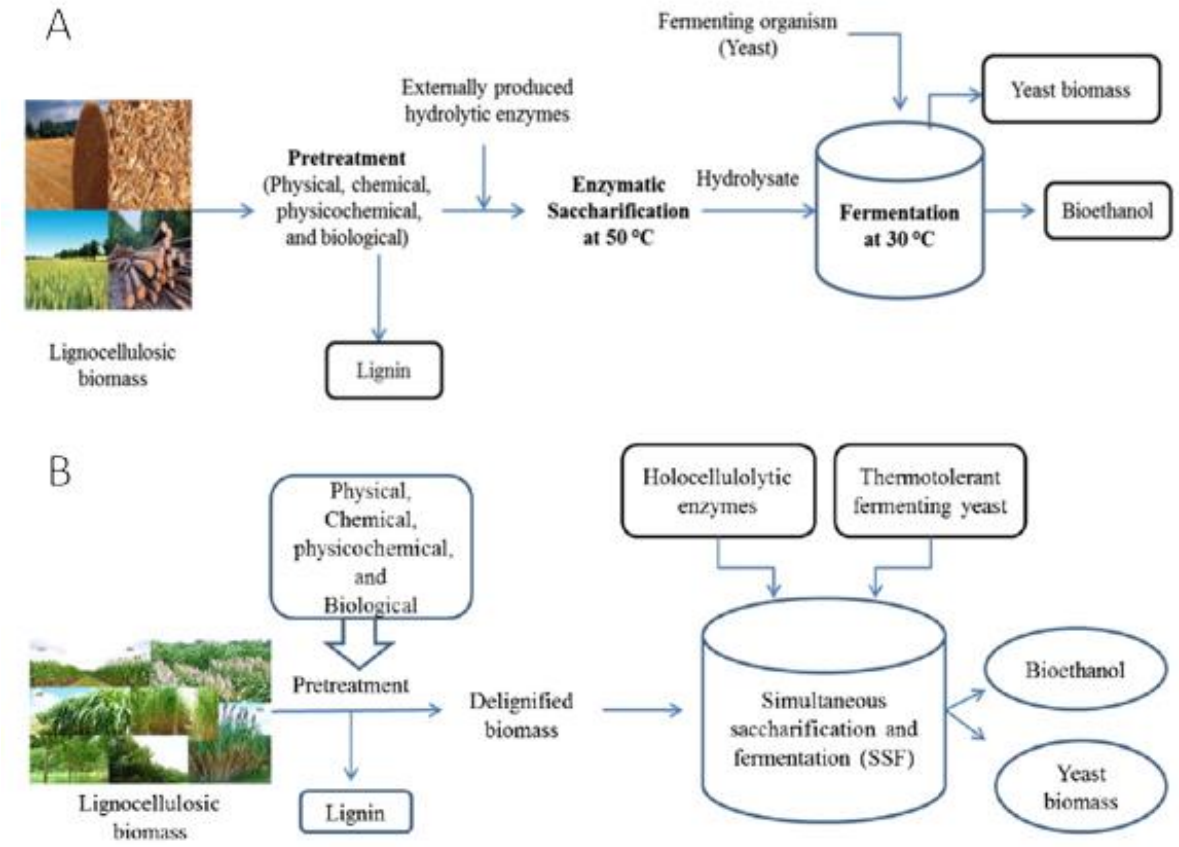


Figure 18. Process flow of separate hydrolysis and fermentation (SHF) (A) and simultaneous saccharification and fermentation (SSF)(B) [107].

Micro algae are a promising group of microorganisms, since cultivation is simple, growth rates are high, space requirement is low and they can consume contaminated water that is unsuitable for human consumption. Some of the potential products from microalgae are polyunsaturated fatty acids (PUFAs), pigments and proteins. Product flows and mass balances of an algae production in cooperated into an existing pulp and paper mill were simulated to convert secondary streams into value-added products. [110]. For many microalgae two different main cultivation strategies exist. All microalgae are photosynthetic, converting CO_2 to biomass and using light as energy source. Without light, some microalgae are able to replace the fixation of atmospheric CO_2 of autotrophic cultures with organic carbon sources such as glucose and acetate dissolved in the culture media. This process is called heterotrophy [111]. Glucose form different residue materials as molasses from sugar refinery [112] or food waste [113] was shown to be a sufficient medium for cultivation. Within 5 days biomass concentration of 17 g l^{-1} and 8 g l^{-1} could be obtained respectively. Microalgae have a

complex nutrient requirement, next to a carbon source nitrogen and phosphor are required for successful algae cultivation. Digestate is a waste material from anaerobic digestion rich in nutrients required by algae. Using the nitrogen from digestate for biomass production has the potential to avoid nitrogen loads in soil with all its negative influences [114]. Only a small number of micro algae strains is suitable and used on an industrial scale. One of the most promising microalgae genus is *Chlorella* with *Chlorella vulgaris* as most common used species. It is a fast growing round shaped single cell organism with a diameter of 2-10 µm reproducing asexually. During autosporulation, four new cells form in their mother cell. A schematic ultra-structure of a *C. vulgaris* cell and the different phases of daughter cell formation are given in Figure 19.

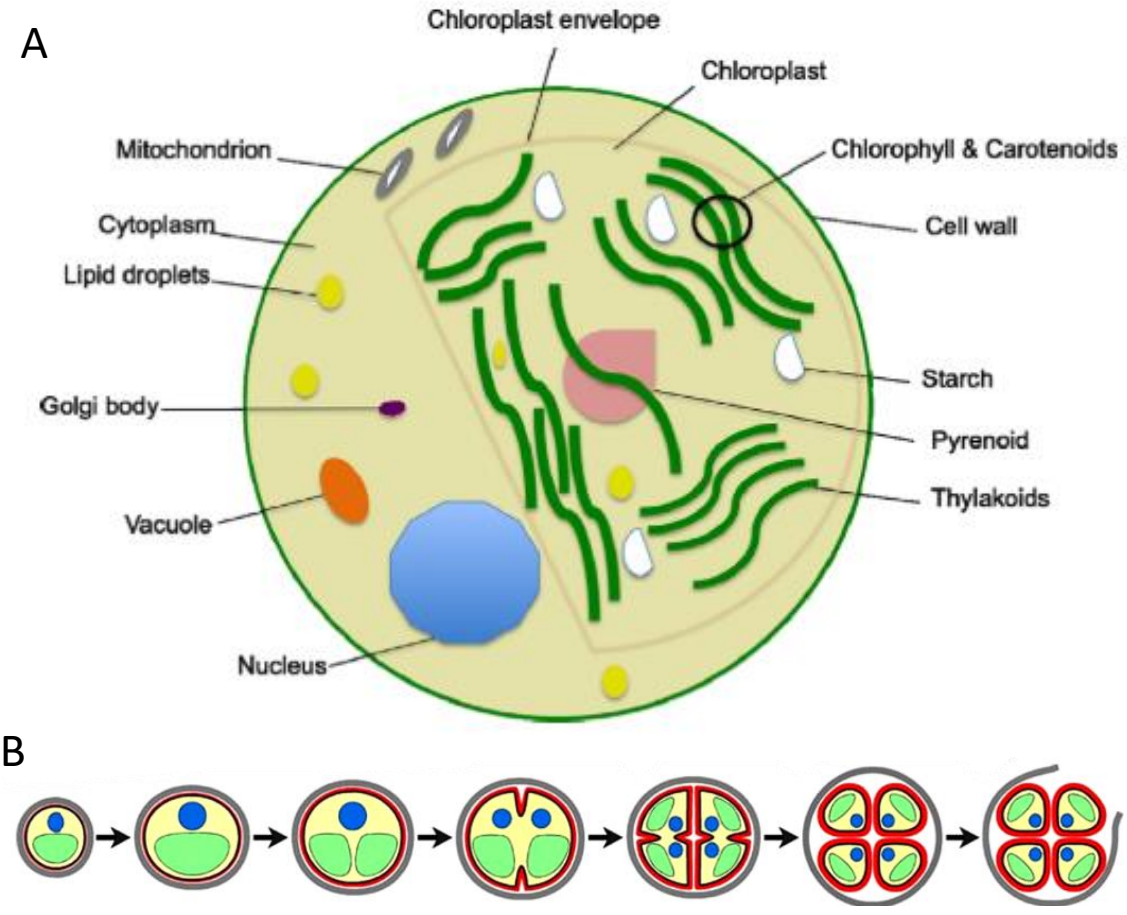


Figure 19. Schematic structure of C. vulgaris with its different cell organelles (A) and the seven stages of daughter cell formation during autosporulation (B). The seven phases are: early cell-growth phase, late cell-growth phase, chloroplast dividing phase, early protoplast dividing phase, late protoplast dividing phase, daughter cells maturation phase and hatching phase [115].

C. vulgaris is widely used in different sectors under both heterotrophic conditions and phototrophic conditions as well as hybrid forms of both techniques. Fields of application are human nutrition and due to a high protein concentration, animal feed, especially for fish. Other potential applications are third generation biofuels form the lipid fraction of the cell biomass, wastewater treatment systems for phosphorous and nitrogen depletion, CO_2 fixation from industry exhaust gases or as fertilizer in agriculture [115]. A system hydrolyzing residue paper materials for heterotrophic algae cultivation was not reported in literature so far.

2.7 References

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3

Effects of Enzymes on the Refining of Different Pulps

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3.1 Abstract

Comparative studies of the effects of two commercial enzyme formulations on fiber refining were conducted. Extensive basic characterization of the enzymes involved, assessment of their hydrolytic activities on different model substrates as well as on different pulps (softwood sulfate, softwood sulfite, and hardwood sulfate) were evaluated. Both enzyme formulations showed endoglucanase as well as some xylanase and β-glucosidase activity. In addition, Enzyme A reached a CMC end viscosity of 19.5 mPa compared to 11.1 mPa for Enzyme B. Reducing sugar release almost doubled from 695 µmol ml^{-1} for hardwood sulfate pulp to 1300 µmol ml^{-1} for softwood sulfite pulp with Enzyme B under the same conditions. Enzyme A increased the degree of refining even under non-ideal conditions from 23 °SR to up to 50 °SR. Further characterization of hand sheets, made from enzyme pre-treated and refined cellulose fibers with Enzyme A and B, showed that Enzyme A had the best effects leading to hand sheets with increased tensile strength and low air permeability. In summary, the increase in the degree of refining seen for Enzyme A correlated to higher xylanase and β-glucosidase activity and lower endoglucanase activity.

3.2 Introduction

Pulp and its products are one of the most important industrial materials in the world. With an annual worldwide production of 419,7 million metric tons of paper and paper board in 2017 [1], it represents an enormous resource having a massive environmental impact [2]. Pulp is produced using two main processes, namely the sulfate or kraft process, which is the more widespread, or the sulfite process. Sulfate pulp production is based on cooking the raw material with sodium hydroxide and sodium sulfide. Sulfate produced pulp is of high quality and stronger, particularly in tear strength, compared to other pulping processes [3] [4]. Chemically, hydrogen sulfide is responsible for delignifying while the high pH renders the lignin soluble [5]. The second common way of producing pulp is the sulfite process. Lignin is removed by sulfonation and hydrolysis (hydrogensulfite or bisulfite and SO_2), which has the advantage of producing pulps with higher brightness [6].

Papermaking is an energy intensive process, with the production of one ton of paper consuming 1.91 GJ of electricity and 5.5 GJ of fuel. The step consuming the most electrical energy in paper production is pulp refining, requiring 26.61% of the total electrical energy input [7]. Considering the enormous amounts of paper processed annually, even small reductions in energy demand would reduce the overall energy consumption and thus help to reduce CO_2 emissions on a global scale. Enzymes have the potential to reduce the energy required and thus the environmental impact of the pulping and papermaking process with some previous studies reporting electricity savings between 10% and 40%, depending on the enzyme used and the process type [7].

Enzymes are emerging as powerful tools that can support many process steps in pulp and paper production. They are, for example, used for pre-treating wood chips [7], processing of surface size starch [8], act in biofilm control [9] or more importantly used in refining [10]. Refining is a mechanical treatment of pulp fibers altering the fiber structure aimed at increasing fiber-fiber bonding in the paper sheet. The increased bonding is achieved through fibrillation, improved swelling, fiber shortening and straightening with internal fibrillation leading to flexibilization that is considered as the most important impact of refining since it causes network improvements [11]. Buzalla et. al. reported a reduction in refining energy input and improved tensile properties

when applying enzymes. They used, among others, xylanases from *Trichoderma longibrachiatum* (736.8 U ml^{-1}) and *Thermomyces lanuginosus* (7460 U ml^{-1}) to treat a bleached pine kraft pulp. At the same time, they observed that some enzyme formulations had a negative influence on the tear resistance [12]. This underlined the requirement for a broad characterization of potential enzymes for application on pulp. Considering cellulase activity as a combined effect of various individual enzymes (endoglucanase, cellobiohydrolase, polysaccharide monooxygenases, β-glucosidase etc.), detailed knowledge on the effect of these individual activities is important [13]. Cellulases derive from lignocellulolytic organisms, which use them to fully break down lignocellulose into monomers for energy and biomass generation. Some of these reactions, catalyzed by enzymes, are undesired when they should only improve fibrillation of fibers. Endoglucanases acting within the cellulose chain may cause beneficial effects such as fibrillation without liberating considerable amounts of sugars [14].

Despite some promising previous results on enzymatic refining, a better understanding of the effect of enzymes on different pulps is required. Therefore, in this study, the effect of two commercial refining enzyme preparations on bleached hardwood and softwood sulfite and sulfate pulps were systematically evaluated. The softwood pulps, (LF, long fiber) sulfite and (LF, long fiber) sulfate, derived from spruce, one produced by the sulfite process and the other by the sulfate process. The hardwood pulp was made from beech/birch wood and was produced using the sulfate process (SF, short fiber) sulfate). The effect of enzymes on these pulps was investigated under experimental conditions close to real industrial conditions. Enzymatically treated pulps and hand sheets formed from these pulps were further extensively characterized with regards to fiber properties, tensile strength, air permeability, curl, opacity and whiteness.

3.3 Materials and methods

3.3.1 Enzymes, pulps and chemicals

Two refining enzyme formulations, namely Enzyme A and Enzyme B, were supplied by manufacturers and used without further purifications steps according to the procedures reported below. Enzyme A and B derived both from genetically modified strains of *Trichoderma reesei*. Enzyme A was classified as cellulase whereas Enzyme B was classified as endo-1-4-β-D-glucanase. The enzyme formulations were stored in the dark at 4 °C when not in use to prevent time-related activity loss. The three different pulps, softwood sulfite pulp from spruce (LF Sulfite), softwood sulfate pulp (LF Sulfate) also from spruce and hardwood sulfate pulp from beech/birch (SF Sulfate), were provided by paper manufacturers. Their characteristics regarding lignin content and hemicellulose content are given in Table 11.Pulps were provided as dried pulp sheets and stored at room temperature until used. All used chemicals, purchased from Sigma-Aldrich (Austria), were of analytical grade and used without further purification if not stated otherwise.

Table 1. Pulps and their origin, lignin content and hemicellulose content.

Pulp	Origin	Lignin content [%][1]	Hemicellulose [%][2]
LF Sulfite	Spruce	0.9	10.4
LF Sulfate	Spruce	max. 0.3	15.2
SF Sulfate	Beech/Birch	below 0.5	24.4

[1] based on kappa number
[2] solid state NMR (^{13}C)

3.3.2 Protein determination via internal calibrated Bradford Method

Photometric protein content determination of enzyme formulations were performed using the principle published by Bradford [15]. For all dilution steps ultrapure water was used. Diluted enzyme solution (100 µl) was spiked with a BSA containing 0 – 40 µg BSA and filled up to a final volume of 300 µl. Samples were mixed by inverting and 5 ml of the Bradford reagent was added. Samples were then inverted and incubated for 5 min at 21 °C. Afterwards, 200 µl was pipetted into 96 well plates and the absorbance at 595 nm was determined using a spectrophotometer type U-2900 (Hitatchi, Japan). For analysis, the concentration of proteins was calculated via internal calibration using linear regression analysis. Measurements were carried out in duplicates (n=2). Error bars indicate the standard deviation.

3.3.3 FPU activity with the filter paper assay

The measurements of filter paper units (FPU) followed the standard assay procedure for enzyme systems, published by the International Union of Pure and Applied Chemistry (IUPAC) in 1987 with some modifications [16]. Tests were performed in a 50 mM citrate buffer, pH 4.8 at 45 °C. A 0.75 cm x 7.5 cm filter paper roll, Whatman grade No1, was added to 1 ml diluted enzyme solution to start the reaction. For each time point, 5, 10, 20, 40 and 60 min, duplicate samples were prepared (n=2). The reaction was stopped by adding 1 ml of dinitrosalicylic acid (DNS) solution, containing DNS, NaOH and Rochelle salt having concentrations of 10 g l^{-1}, 16 g l^{-1} and 300 g l^{-1}, respectively. After the reaction, samples were placed in a vigorously boiling water bath for 5 min and diluted 1:5 with ultra-pure water. Afterwards, 200 µl was pipetted into a 96 well plate and absorbance was measured at 540 nm using an Infinite M200Pro plate reader (Tecan, Switzerland). Calibration was done using a glucose solution in the 0 – 20 mM concentration range treated the same way as the samples. Results are expressed as µmol of reducing sugar liberated per ml of undiluted enzyme solution.

3.3.4 Xylanase activity assay

Xylanase activity measurement, at pH 6, 7 and 8 in 100 mM sodium phosphate buffer, was conducted according to Kample and Jadhav with slight modifications [17]. Tests were performed in triplicates (n=3), and error bars indicate the standard deviation. Xylan, 1% (w/v) from birch wood, in buffer, was used as the substrate solution. For testing, 800 µl substrate solution was mixed with 200 µl of enzyme solution, diluted in buffer and incubated at 40, 55 and 70 °C for 20 min. To stop the reaction, 1 ml DNS solution was added and the samples were placed for 5 min in a vigorous boiling water bath. After the addition of 8 ml ultrapure water samples were inverted followed by 200 µL being transferred into 96 well plates. For analysis, absorbance was recorded at 540 nm using an Infinite M200Pro plate reader (Tecan, Switzerland) and an activity calculation in nkat ml^{-1} was conducted. For that, a calibration curve with xylose in a concentration range between 0 – 25 mM was prepared and treated the same way as the samples. All tests were performed in duplicate.

3.3.5 Viscometry

For viscometry tests, a cone/plate rheometer CVO 50 (Bohlin Instruments, UK) was used. Single tests were performed. Viscosity decrease measurements, as a function of time, were conducted for approximately 20 min similar to experiments published by Lee et. al. [18]. As substrate, a 1% carboxymethyl cellulose (CMC) solution in 20 mM citrate buffer pH 4.8 was prepared. Molecular weight in g mol^{-1} (Mw) for the substrate was 700,000 and the degree of substitution (DS) was 0.9. For measuring, 2.2 ml of substrate solution was added on the tempered (45 °C) viscometer plate and a preliminary shear rate of 100 s^{-1} was applied. After stabilizing, 100 µl of properly diluted enzyme solution was added and the measurement was started. Conditions for data acquisition and measurement were: delay 1 s, integration 2 s, waiting period 3 s, repetitions 200 and a gap size of 150 µm. For better understanding, experiments with buffer instead of enzyme were conducted. A measurement with the substrate CMC solution (1%(w/v), Mw 700,000, DS 0.9) was set as the upper limit and a less viscous CMC solution (1%(w/v), Mw 250,000, DS 0.9) was set as the lower limit. All enzymes were diluted 1:5000, except the pure endoglucanase which required a 1:100 dilution. Next to Enzyme A and Enzyme B, an endoglucanase (Endoglucanase E1 from *Acidothermus cellulolyticus*, Sigma-Aldrich, Vienna, Austria) and a commercial enzyme preparation for saccharification (Cellulase sacch.) were used for total hydrolysis.

3.3.6 β-Glucosidase activity

The assay was performed following the method described by Parry et. al., with some modifications [19]. Enzyme activity was measured at pH 4.8 in a 20 mM citrate buffer. As substrate, 400 µl 2 mM 4-Nitrophenyl β-D-glucopyranoside in buffer was equilibrated at 45 °C and 100 µl diluted enzyme solution was added to start the reaction. Runs with different durations were performed, whereby the reaction was stopped by the addition of 1 ml methanol after 10, 20, 30, 40, 50 and 60 min. An aliquot of 200 µl of each reaction was pipetted into new tubes. To perform the colorimetric reaction, 200 µl of a 500 mM sodium phosphate buffer pH 7 was added. Samples were vortexed and 200 µl of the coloured solution, in 96 well plates, was used for absorbance determination at 410 nm using an Infinite M200Pro (Tecan, Switzerland). Enzyme activity was calculated from the slope when absorbance (y-axis) was plotted against time (x-axis) and expressed in katal (kat) per ml undiluted enzyme. Kat is

defined as the amount of substrate in mol cleaved per second. All tests were performed in duplicates (n=2) and a regression for the linear correlation over the test duration was calculated. Standard deviations were calculated and displayed as error bars.

3.3.7 Reducing sugar release from different carboxymethyl cellulose

A method measuring the release of reducing sugar from CMC was first published by Mandels et. al. [20]. For each enzyme two passages with different durations were performed, one short (0-12 min), and one long (0 – 180 min). All measurements were carried out in duplicates (n=2) and error bars indicate standard deviation. Four different CMC substrates were used, differing in Mw (g mol^{-1}) and DS. Lowest DS was 0.7 with an Mw of 90,000, followed by the next longer substrate with the same DS but an Mw of 250,000. The third substrate also had an Mw of 250,000; however, its DS was 0.9. The longest substrate had a Mw of 700,000 and a DS of 0.9. Substrate solution (400 µl) was placed in glass tubes and equilibrated to the right temperature (50 °C). To start the reaction, 100 µl of diluted enzyme was added and for each time point a set of duplicates was prepared. To stop the reaction at the defined time, 500 µl of DNS solution was added. The colorimetric reaction was conducted by incubating the samples for 5 min in boiling water. Ultra-pure water (1 ml) was added, the samples vortexed and 200 ml of the solution was pipetted into 96 well plates. Absorbance was measured at 540 nm in an Infinite M200Pro plate reader (Tecan, Switzerland). An absorbance increase in reducing sugar caused per µl of undiluted enzyme volume was calculated.

3.3.8 Reducing sugar release from different pulps

Three different pulps were used for reducing sugar release: softwood sulfite pulp from spruce (LF Sulfite), softwood sulfate pulp (LF Sulfate) also from spruce and hardwood sulfate pulp from beech/birch (SF Sulfate). Behavior on the pulps at three pH values (4.8, 7 and 8) and two temperature levels (45°C and 75°C) were investigated. Of the pulp, 750 mg was disintegrated in 50 ml process water and adjusted to the correct pH. Process water was a mixture of 50% tap water and 50% deionized water. After the pulp was fully disintegrated and the target temperature was reached, 300 µl enzyme was added and the reaction started. A sampling of 50 µL was done after 5, 10, 20, 40 and 60 min. 50 µl DNS solution was placed into tubes and 50 µL sample added. After boiling the samples for 5 min, 400 µl ultra-pure water was added and the absorbance measured at 540 nm using an Infinite M200Pro plate reader (Tecan, Switzerland).

Calibration was carried out with a 20 mM glucose standard as the highest concentration level. Results are expressed as reducing sugar release in µmol per ml of enzyme. From the values obtained after 5 min an enzyme activity was calculated, given in nkat ml^{-1}. The unit nkat ml^{-1} is defined as the amount of reducing sugar in nmol liberated per second by 1 ml undiluted enzyme solution. Standard deviations were calculated from the performed duplicates (n=2) and displayed as error bars in the figure.

3.3.9 Refining trials and determination of the degree of refining

Laboratory refining trials were performed as single tests in process water (50% tap water, 50% deionized water). Pulp was disintegrated (90 g) in process water for 10 min. Water was removed and the pulp cake was set to a consistency of 5%. The pH was adjusted with $Al(SO_4)_2$ resp. NaOH to the target value and the temperature was set and kept constant during the enzyme incubation. Enzymes were dosed in ppm according to the reducing sugar activity measurement performed on pulp and added after the required temperature was reached. Enzyme B was dosed at three different levels: 100, 550 and 1000 ppm. Enzyme A was dosed to add the same activity as that of Enzyme B. Dosage followed the result obtained from the reducing sugar release from the different pulps. Reactions were performed for 10, 35 or 60 min under constant agitation. To stop the reaction, 1000 ppm (according to reaction volume) of a 30% H_2O_2 solution was added. After enzymatic treatment, pulps were refined using a PFI mill (ISO 5264-2:2011), type Mark IV (Hamjem Maskin A.G, Norway). Revolutions of the PFI mill were different for each pulp. The aim was to reach an equal degree of refining [°SR] of 23 °SR, without enzyme treatment, for all pulps. To reach this, softwood sulfate pulp was refined for 4300 revolutions, softwood sulfite pulp for 2700 revolutions and hardwood sulfite pulp for 1500 revolutions. The degree of refining was determined according to the Schopper-Riegler method (ISO 5267-1:1999)) and expressed in degree Schopper-Riegler (°SR). Water retention value as a measure for fiber swelling and fibrillation was measured according to ISO 23714:2014. Zeta-Potential was measured with the VAP-method.

3.3.10 Hand sheet formation and analysis

Hand sheet formation was performed as single test according to the Rapid – Köthen procedure (ISO 5269-2:2004) using a device type DND (Austria) from Paper Testing Instruments (PTI). Standards for the tests performed on hand sheets are shown in Table 2.

Table 2. Parameters and details of hand sheet tests after enzymatically-assisted pulp refining and hand sheet formation.

Parameter	**ISO Standard**	**Device, type**	**Company (Country)**
Whiteness	ISO2470, ISO 11476	Touch II, CTH-ISO	Technidyne (USA)
Opacity	ISO 2471	Touch II, CTH-ISO	Technidyne (USA)
Thickness, density, specific volume	ISO 534	LDAL-03	Lehmann AG (CH)
Air permeability (Gurley)	ISO 5636/5	SE 212	Lorentzen & Wettre (SWE)
Air permeability (Bendsten)	ISO 5636/3	113-M	Messmer Buechel (NL)
Tearing resistance	EN 21974	4-1 Elmendorf	Lorenzen & Wettre (SWE)
Tensile strength, breaking force, breaking index, breaking length, stretching, work absorption	ISO 1924-2	SE 062	Lorenzen & Wettre (SWE)

3.4 Results and discussion

3.4.1 Basic characterization of the enzyme formulations

In a first step, the enzyme formulations were characterized regarding their individual enzyme activities and protein content. Enzyme A had a 2.6 times higher protein content compared to Enzyme B (Figure 1), tested using the Bradford method.

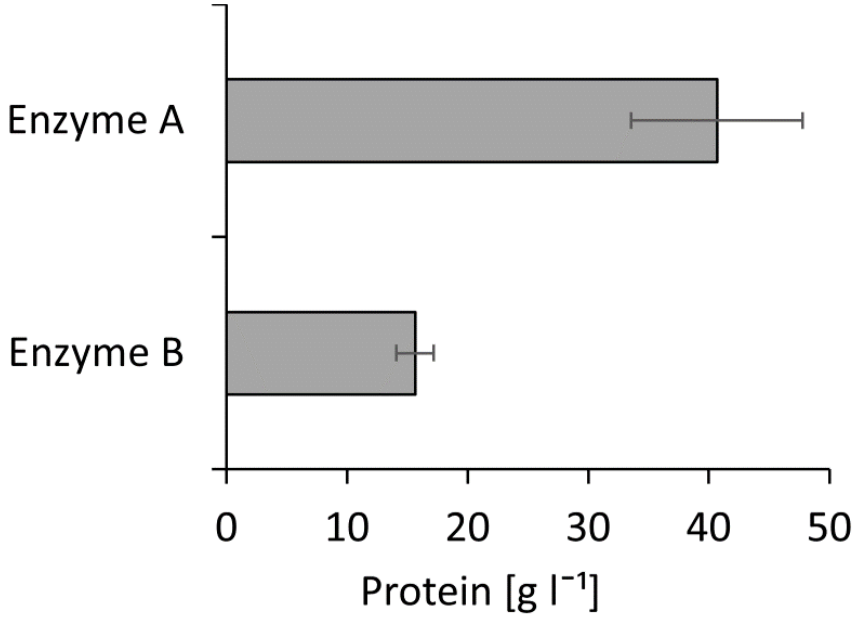

Figure 1. Protein content determination was part of the basic characterization of Enzyme A and Enzyme B. The Bradford method with internal BSA spikes was used for the analysis. Enzyme B contained only 38% of the amount of protein in Enzyme A. Error bars indicate the standard deviation from the duplicate measurements.

Further to this, β-glucosidase activity of Enzyme A was shown to be 4.2 times higher than that of Enzyme B (Figure 2). β-Glucosidase hydrolyses cellobiose into glucose in the last step of synergistic enzymatic cellulose degradation in nature (Singh, 2015; Dimarogona, Topakas and Christakopoulos, 2012; Ahmad Khairudin and Mazlan, 2013). However, high β-glucosidase activity in enzyme preparations causes the release of glucose and hence increases COD. On the other hand, high concentrations of cellobiose, produced by cellobiohydrolases, can lead to inhibition of e.g. endoglucanases which can thus be reduced by β-glucosidase activity. Ishihara et al. (2005) reported differences in behavior of three different CMCases from *Polyporus arcularius* when cellobiose was present. One enzyme showed a five-times increase at the higher cellobiose level (CMCase IIIa at 20 mM cellobiose), another one lost more than 2/3 of activity at the same cellobiose concentration (CMCase I). Similar effects were seen for longer cello-oligosaccharides. For cellohexaose, losses in activity from 31 to 97% were recorded for CMCases [23]. Another group reported the inhibition of

an endoglucanase II originating from *T. aurantiacus* when xylo-oligosaccharides and xylose were present [24]. Since pulps still contain hemicellulose and oligo- and monomers such influences are obvious. In summary, the presence and enzymatic hydrolysis of small oligosaccharides can influence the actions of those enzymes expected to be important in fiber modification, e.g. endoglucanases. Therefore, it is important to consider potential β-glucosidase activity present in enzyme preparations for refining, even if it does not directly contribute to fibrillation events.

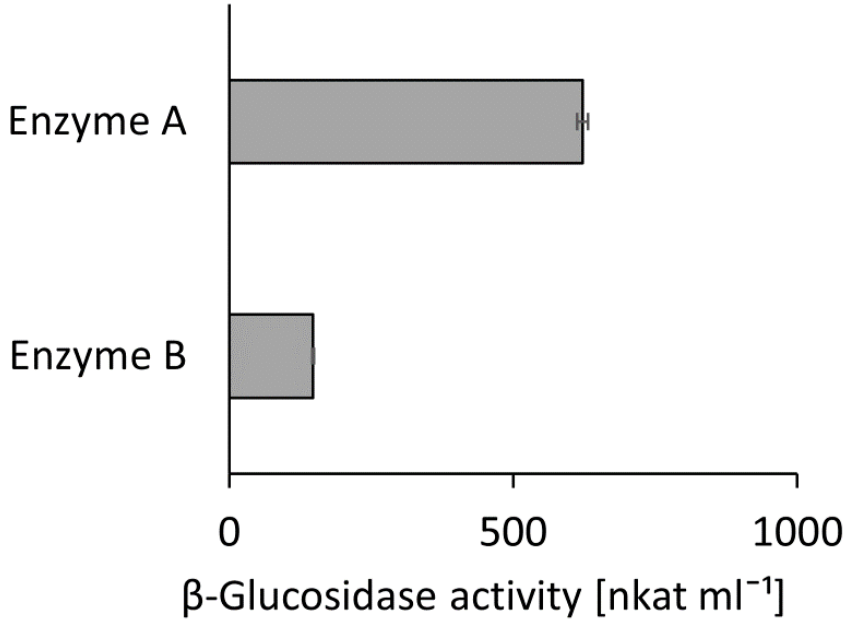

Figure 2. β-Glucosidase activity for enzyme A and B. 4-Nitrophenyl β-D-glucopyranoside was used as substrate. The activity of Enzyme A is four times higher than for Enzyme B. Error bars indicate standard deviations of the duplicates.

Xylanase activity was measured at different pH values and temperatures (Figure 3). Quite expectedly, a general trend for both enzymes was observed with lower activity occurring at increasing pH and temperatures. For example, for Enzyme A the activity decreased to 28% when increasing the temperature from 55 °C to 70°C at a pH of 6.0. Regarding temperature, 55 °C reflects the ideal value for both formulations. Comparing the differences in pH for this temperature showed a higher pH stability of Enzyme A, which retained 42% of its activity at pH 8.0 when compared to pH 6.0. For Enzyme B only 21% of activity was measured for the same pH shift at 55°C. Comparing both enzymes, Enzyme A seemed to be slightly more active than Enzyme B and the pH and temperature stability was significantly higher.

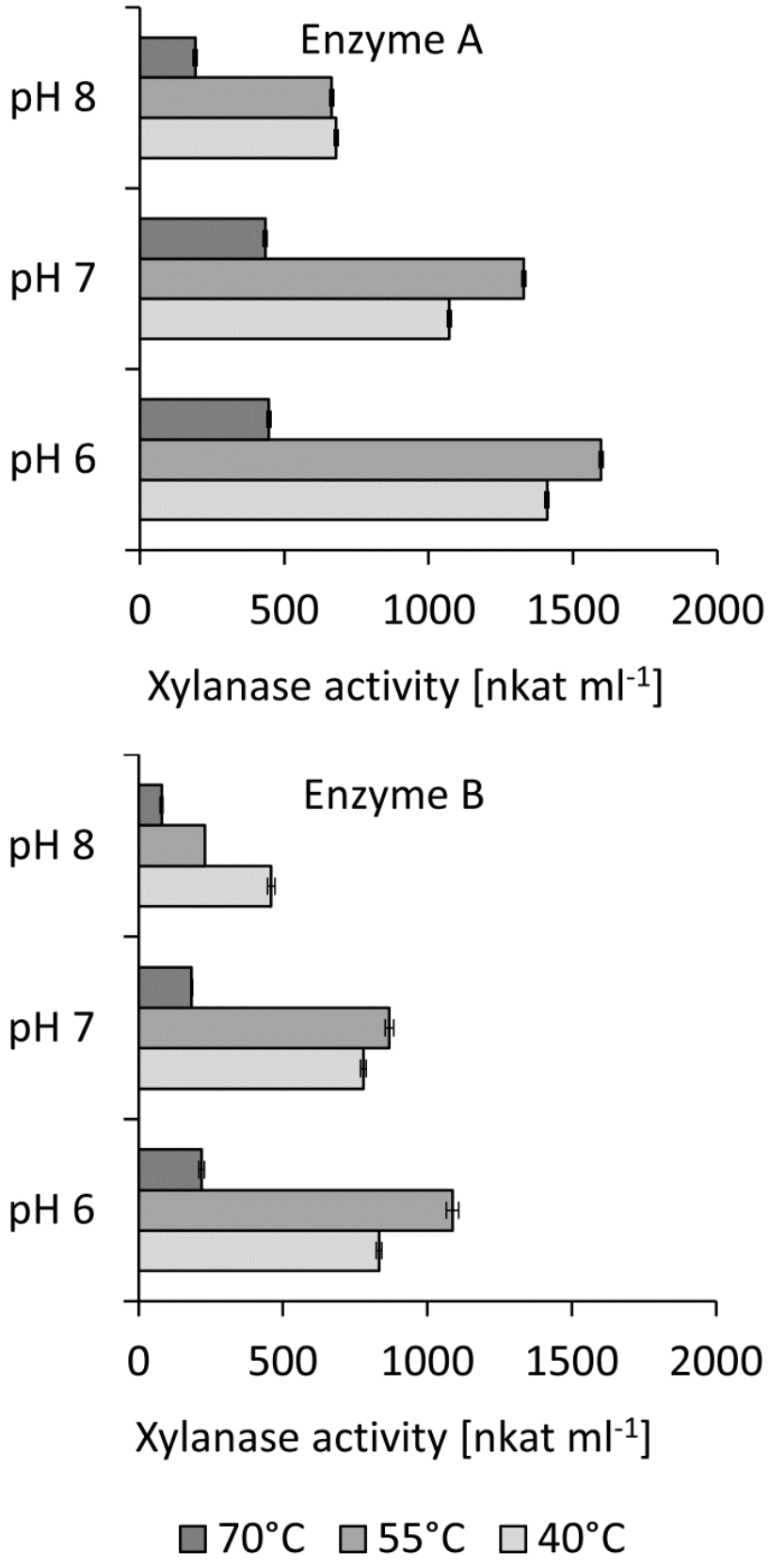

Figure 3. Xylanase activity for Enzyme A and B at a pH range from 6-8 for three different temperatures (40, 55 and 70 °C). Highest xylanase activity was visible for pH 6 and 55°C. Error bars indicate standard deviation of the performed triplicate.

Viscosity tests for further characterization of Enzyme A and B, on a 1% CMC solution in buffer, indicated endoglucanase activity for all tested enzymes. As visible in Figure 4, a typical decreasing curve progression, comparable to literature, was obtained for all tests, demonstrating endoglucanase activity [25]. Two commercial enzyme formulations “CTec3”, designed for complete hydrolysis of cellulose (CTec3), and a pure endoglucanase (Endoglucanase, Figure 4) were also included in the experiments. The action of the enzymes was measured between an upper limit (1% CMC, Mw: 7.0 x 10^5, DS: 0.9) and a lower limit (1% CMC, Mw: 2.5 x 10^5, DS: 0.9). Figure 4A showed a decrease in viscosity for the time the enzyme was added (buffer for upper limit). All enzymes showed activity, whereby the endoglucanases depolymerized CMC faster, followed by the cellulase for saccharification. Figure 4B elucidates the end viscosity reached from the data in Figure 4A. The highest remaining viscosity was measured for Enzyme A at 19.5 mPa while Enzyme B reached 11.1 mPa (Figure 4B). Since the final viscosity of Enzyme A was close to the lower limit, it can be stated that the viscosity properties of the solution containing Enzyme A were comparable to a CMC solution with a chain length (Mw) of 2.5x10^5 glucose subunits, supposing the cleavage was uniform across the polymer. Since viscosity properties rely on the average chain length and polydispersity, experiments clarifying the homogeneity of the produced fragments would help characterizing the cleavage pattern. However, a lower end viscosity result indicates shorter chain lengths for the fragments an enzyme formulation produced. It can therefore be stated that fragments produced by Enzyme B are smaller than fragments form Enzyme A. Figure 4C indicates how long one ml undiluted enzyme formulation would need to degrade the used CMC solution from the upper viscosity limit to the lower viscosity limit. The slightly faster reaction of Enzyme B towards Enzyme A can be explained by the fact that Enzyme B is classified as endoglucanase.

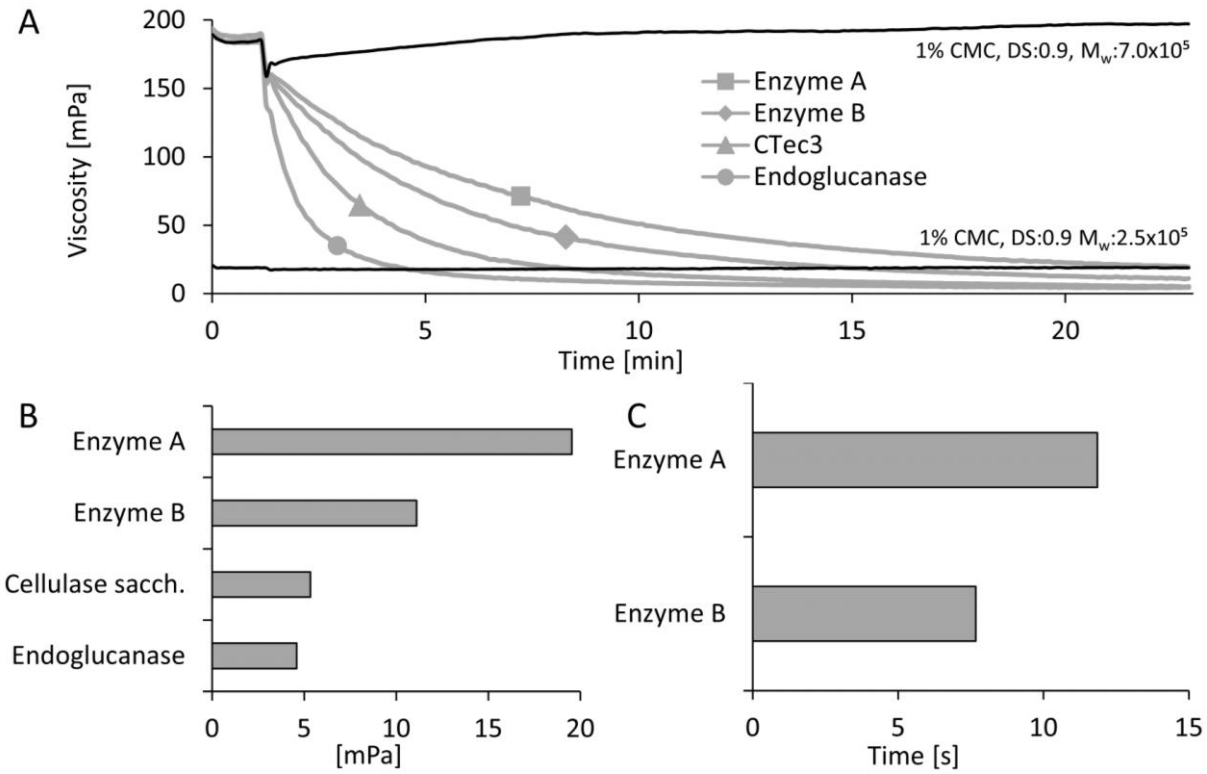

Figure 4. Viscosity profile of enzymes on a 1% CMC solution (DS 0.9, Mw 7.0x10⁵) recorded for 24 min (A). end viscosity in mPa of Enzyme A, Enzyme B, Endoglucanase and CTec3 (B). Time one ml undiluted enzyme needed to degrade a 1% CMC with a DS0.9 & Mw 7.0x10⁵ down to a viscosity comparable to a 1% CMC with a DS0.9 & Mw 2.5x10⁵ (C).

In a next step, hydrolysis of CMC with different degrees of substitution and degrees of polymerization by the two enzymes was studied (Figure5). For longer CMC chains, slower hydrolysis was detected for both enzymes. Secondly, a high degree of substitution had a negative influence on hydrolysis. Additionally, there was a lag phase of 1 to 6 min in the beginning of the reaction. Enzyme A showed a longer lag phase and lower hydrolysis than Enzyme B. A reason for the lag phase may lie in the assay itself. In the beginning, endoglucanases act within the CMC chain without significant amounts of reducing sugars liberated by cellobiohydrolases. Later, when enough attacking points are available, reducing sugar release enhances. Enzyme A showed less reducing sugar release. This accords with a slower viscosity decrease (Figure 4A) and the longer lag phase. Effects of the CMC properties on hydrolysis are known. Breuil and Saddler detected a lower reducing sugar release for CMC of medium viscosity (higher Mw) compared to CMC with low viscosity (lower Mw), however, no such lag phase was reported [26]. Reducing sugar levels do not align for long process duration of 180 min. A general critical point when using CMC is its chemical differences due to the substitution. To circumvent the degree of substitution of CMC, literature

recommends only including the first 2% of released reducing sugar calculations, when CMC (Mw 700,000) is used. This should ensure all reducing sugar derives from non-substituted linkages [27]. It can be stated from reducing sugar release with CMC that the higher the increase after the lag phase is, the higher the cellobiohydrolase activity is. Comparing Figure 4C confirms this: a longer time is needed to reduce viscosity with Enzyme A correlating with a longer lag phase. In contrast to reducing sugar measurements with CMC, no lag phase is detectable when the viscosity decrease of CMC is determined. Viscometry measurements only aim on the endoglucanase activity while reducing sugar measurements record the whole hydrolysis process.

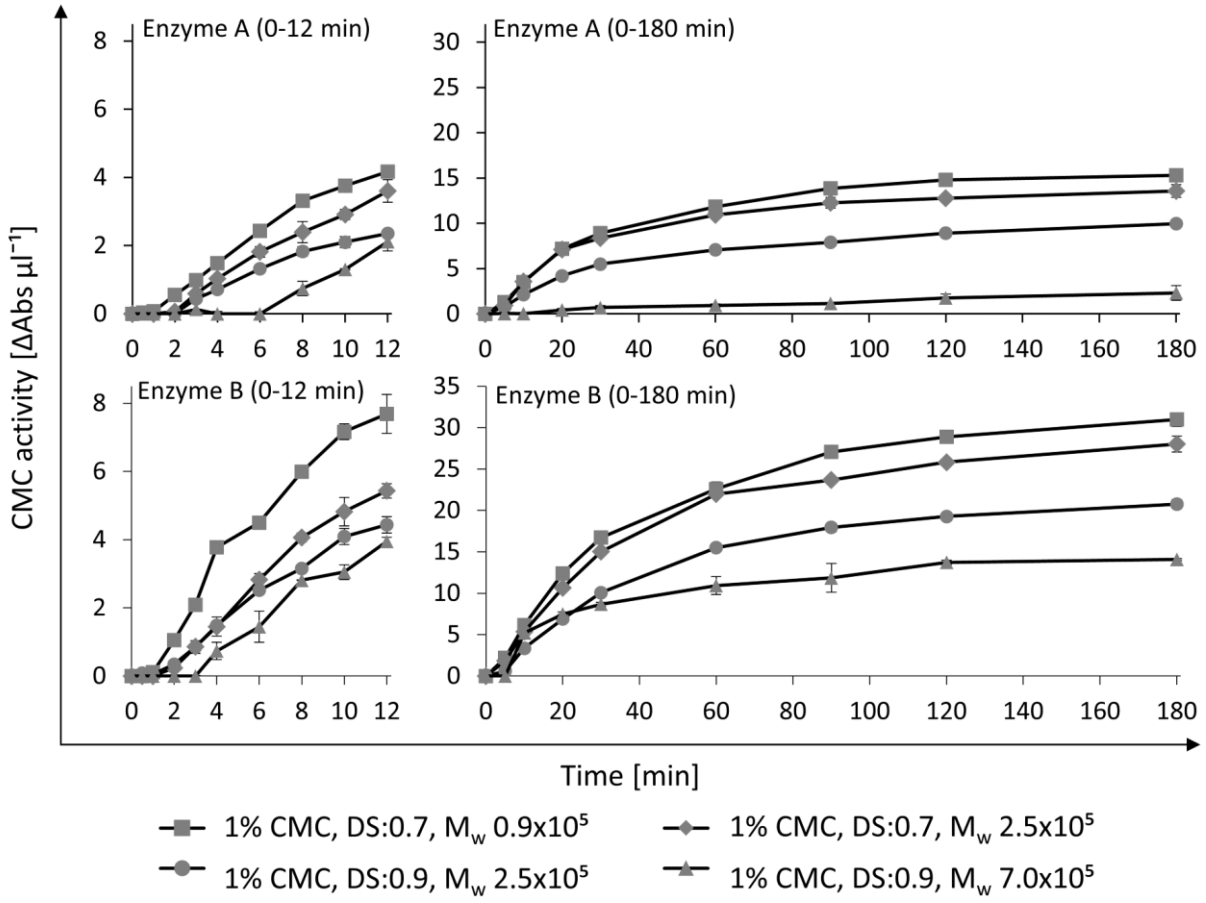

Figure 5. Enzymatic hydrolysis of CMC with different degree of substitution (DS) and polymerization (Mw), for two different timeframes by refining enzyme preparations A and B. Longer chain length and higher degree of substitution caused less hydrolysis. Enzyme B was slightly more active than Enzyme A.

3.4.2 Enzymatic treatment of pulps

Data from different assays with standard model substrates provide important data for enzyme comparison, while for their application in refining, it is essential to elucidate their specificities on real pulps. Hence, in a next step, hydrolysis of different industrial pulps based on quantification of reducing sugar was investigated (Figure 6). A major factor influencing hydrolysis is the crystallinity of cellulose, therefore, depending on the type of enzyme and pulp, hydrolysis performance can vary enormously. Szijártó et.al. reported higher activity for cellobiohydrolases on highly crystalline cellulose (Avicell, crystallinity ≈80%) than for endoglucanases. When amorphous cellulose was used, the contrary was found. A general increase in hydrolysis was observed for enzymes carrying cellulose binding modules [28]. Similar work by Suurnäkki et. al., who compared crystalline and amorphous cellulose, found higher activity of mono-component endoglucanase on amorphous cellulose. Further, they reported in the same work a cellobiohydrolase with a cellulose binding domain hydrolysing mainly crystalline cellulose [29]. For crystalline cellulose hydrolysis binding processes are important. Nakamura et.at. reported that a glycosylated linker between the catalytic domain and the binding module of a cellobiohydrolase interacts with the substrate chain and thereby promotes hydrolysis in comparison to the catalytic domain only [30].

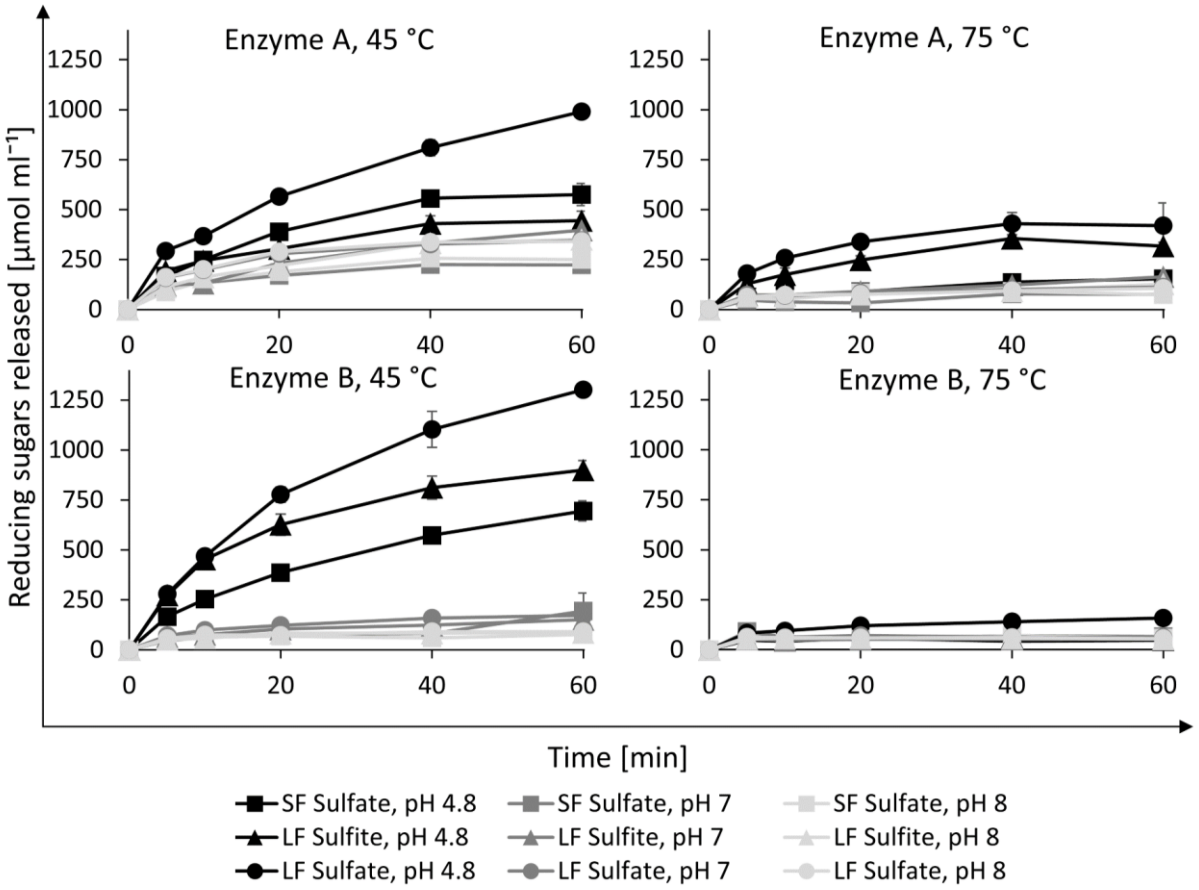

Figure 6. Reducing sugar released per volume of Enzyme A and Enzyme B from three different pulps and at three different pH values (4.8, 7.0 and 8.0) at 45 and 75 °C. The higher the pH, the lower the activity was. Activity for 45 °C was higher for both enzymes than for 75 °C. Highest activity was measured for LF Sulfate pulp. Enzyme B shows higher activity at 45 °C, Enzyme A shows higher temperature stability. Values for 5 minutes were used to calculate activity values, which were used for enzyme dosage in refining trials and hand sheet formation.

Enzymatic hydrolysis of pulps was carried out under conditions used in industrial refining related to pH, temperature and water conditions (50% distilled water, 50% tap water). Long-fiber pulps derived from softwood and short fiber pulp from hard wood were used. Differences in enzyme action for the different cellulose materials were reported before. For pulp related experiments, a duration of 60 min was defined as the optimal timeframe to see effects on pulp and paper. For pulp, previous studies showed that hardwood-based substrates, due to higher pore volume, were better hydrolyzed than softwood materials. Further the lignin distribution is crucial, since cellulose covered with lignin is not accessible to enzymes [31], [32] & [33]. On the other hand, Ibarra et. al. reported a higher reactivity for softwood pulps when compared to hardwood pulps. An effect they reported was the stronger decrease in viscosity for softwood pulps when compared to hardwood pulps [34]. However, apart from the

source of the cellulose, pulping processes have major impacts on the susceptibility of pulps to enzymatic hydrolysis.

In this study, both enzyme preparations showed higher stability at 45 °C than a 75 °C. This may be due to denaturation of enzymes as was previously shown. The half-life of endoglucanases decreased from days at low temperatures (28 °C) to only minutes at 65 °C [35]. For both enzyme preparations, highest activity was measured at pH 4.8 followed by 7.0 and 8.0. Enzyme B was more active at 45 °C than Enzyme A, but Enzyme A seemed to be more temperature tolerant compared to Enzyme B. At 75 °C Enzyme B was mostly inactive, whereas Enzyme A retained approximately 40% of its initial activity. A similar behavior was observed for xylanase activity (Figure 3A&B). Interestingly, significant different activities were seen on the different pulps. Both enzymes showed the highest sugar release for softwood sulfate pulp. The second best substrates were LF Sulfite pulp for Enzyme B and SF Sulfate pulp for Enzyme A. At ideal conditions (45°C, pH 4.8) differences in activity reach 50% when comparing the best with the worst pulp substrates. These findings indicate that reducing sugar measurements on pulps are an important step for the planning of process conditions, such as dosing or treatment times, in industrial based enzyme refining. Differences in Mw and DS for CMC (Figure 5) and differences in pulp affinity underline the need for both methods. Nevertheless, differences between the pulps were visible right from the beginning (Figure 6, 5 min).

3.4.3 Enzymatic refining and hand sheets properties

Refining trials were conducted on all pulps enzymatically treated with both enzyme formulations. Figure7 summarizes the degree of refining [°SR] for the three pulps used with and without enzyme treatment after refining in the PFI mill. Enzyme A showed the strongest effects on all three pulps, however, with significant differences between the pulps. Enzyme dosage should in principle ensure equal activity of the enzymes on each pulp. Enzyme dosage for refining trials were based on reducing sugar release from pulps for 5 min (compare Figure 6). Enzyme B was dosed at 1000 ppm for all three pulps (Figure 7B, light grey) from which the particular activity was calculated (Figure 88, Activity on pulp). Since reducing sugar release was higher for Enzyme A in the first 5 min, less enzyme needed to be dosed (Figure 7A, light grey), to ensure comparable activity of both enzymes on the pulps. For both long fiber pulps differences in dosages were small. Only for SF Sulfate pulp was 435.5 ppm enough to reach equal

activity. Overall, short term reducing sugar release for enzyme activity determination is not reflecting effects in refining, even when the real substrates are used. Also, long term measurements did not reflect refining results, since Enzyme B was more active at 60 min (Figure6) but showed less effectiveness in refining (Figure 7).

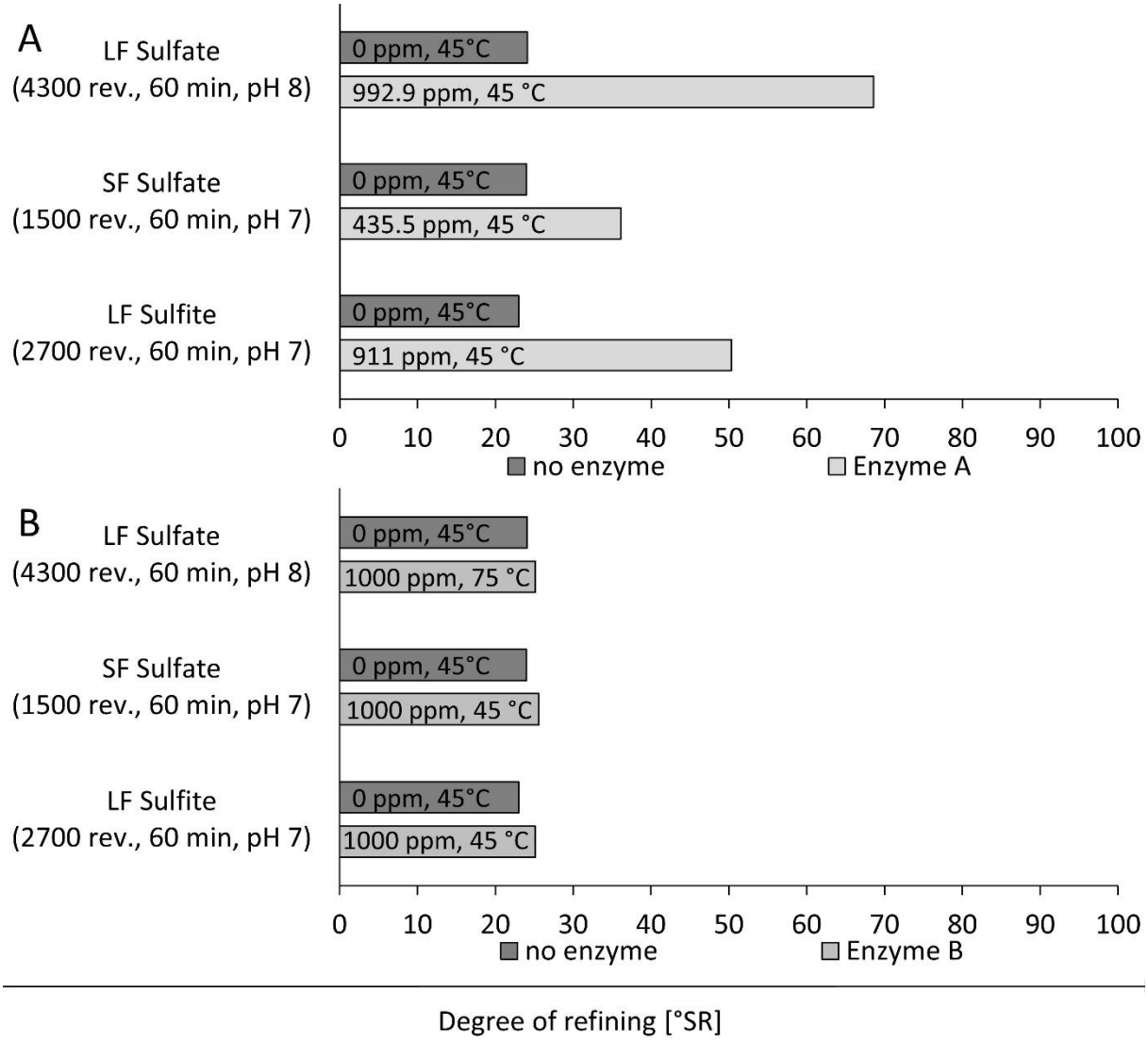

Figure 7. Comparison of refining degree for LF Sulfate, SF Sulfate and LF Sulfite pulp at different conditions. Strong effects are visible for Enzyme A (A), weak effects are visible for Enzyme B (B). Strongest effects are visible for LF Sulfate pulp for Enzyme A.

Under identical conditions, Enzyme A showed significant activity in refining of pulps, whereas, Enzyme B was mostly inactive. The higher degree of refining for LF Sulfate pulp may derive partly from higher PFI rotations compared to the other two pulps. LF Sulfate pulp was more accessible for Enzyme A than LF Sulfite pulp and SF Sulfate pulp. This is in agreement with a lower hydrolytic activity on this pulp as measured based on the release of reducing sugars. Findings in Figure 7 suggest that pulps with

higher acid group content are more susceptible to enzymatic attack. Schwikal et. al. used conductometric titration to determine the acid group content of a softwood sulfite pulp and birch kraft pulp. The sulfate pulp contained with 68 mmol kg^{-1} 70% more acid groups than the sulfite pulp [36]. Since pulp is chemically and structurally diverse, general assumptions about applicability of enzymes are difficult.

Quantitative comparison with data from literature is unreliable. Duan et. al. (2015) compared acid sulfite pulp with kraft pulp and found a higher xylose content in Kraft pulp. In this study Enzyme A showed higher xylanase activity per ml (Figure 3B) than Enzyme B. This may be an explanation for the strong increase in the degree of refining (Figure 7A). From this, it can be stated, that xylanase activity is a key parameter describing refining suitability of an enzyme. Another factor mentioned in the literature that should not be underestimated is the pore size. Duan et. al. reported higher accessibility due to pore size and volume of acidic sulfite pulp [37]. Pore size influences reducing sugar release, and as reported in Figure 6, it also seems to influence the degree of refining.

Further to the degree of refining, hand sheet properties (tensile strength, air permeability) were also measured with Figure 88 showing the comparison between LF Sulfite pulp with SF Sulfate pulp. LF Sulfite pulp treated with Enzyme A showed a higher degree of refining than SF Sulfate pulp (Figure 88) with an increase from 23 °SR without enzyme treatment to over 50.3 °SR. Therefore, when equally dosed based on hydrolysis activity on this particular pulp, xylanase activity of Enzyme A was higher than of Enzyme B while activities on CMC were lower (based on end viscosity and release of reducing sugars). Positive effects of xylanases on energy demand in refining processes were reported before [12]. For these conditions, the tensile strength was only marginally affected, decreasing from 4.75 to 4.40, which is in agreement with previous findings for well controlled enzyme treatments of pulps [38]. On the other hand, air permeability decreased drastically from 11,000.0 ml min^{-1} to 36.0 ml min^{-1}, indicating dense linking and packing of fibers. The strong impact on air permeability showed the effect of enzymes and underline the demand in control strategies for enzyme usage, thereby, helping to achieve target values according to the intended use of the pulp.

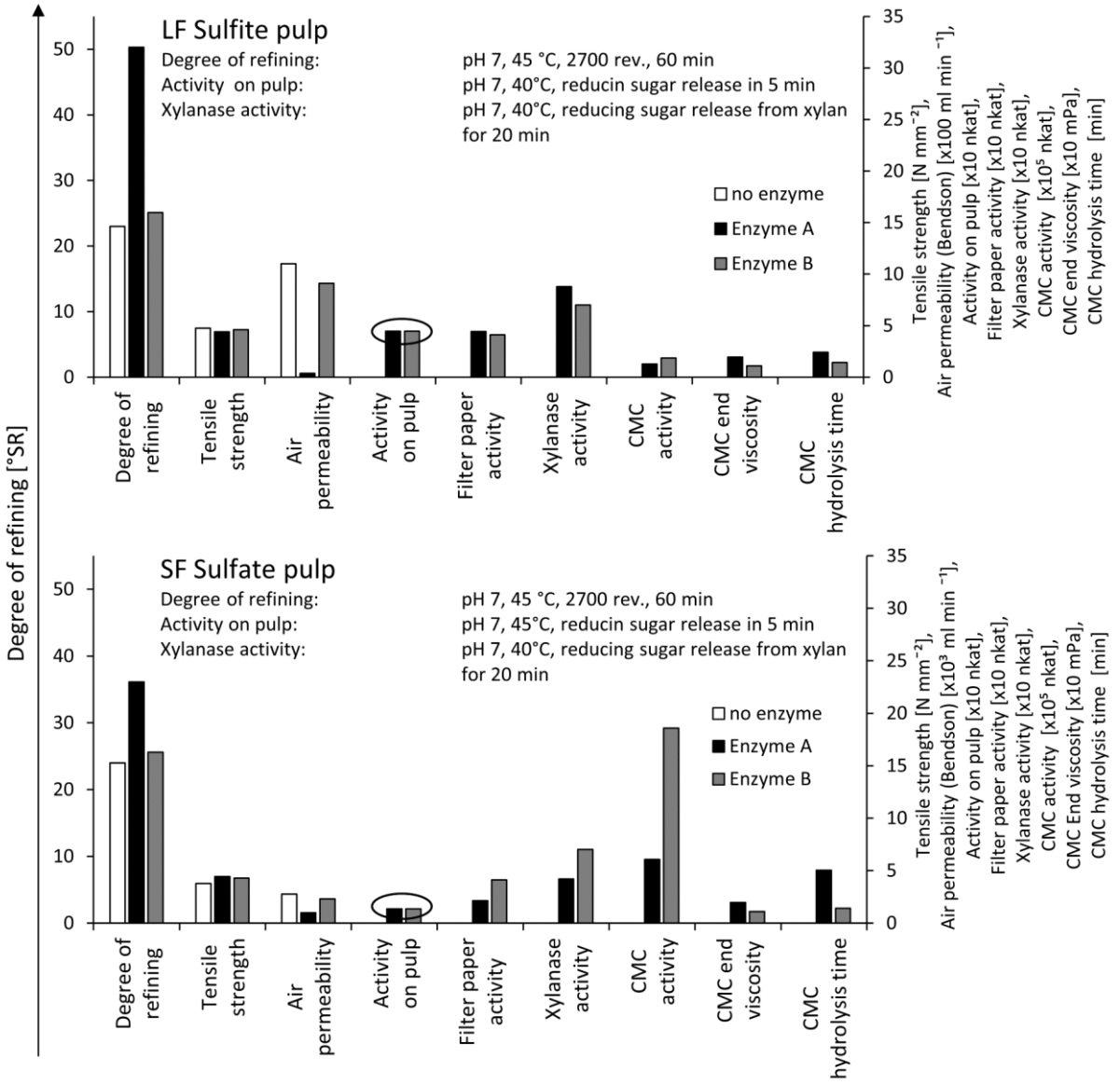

Figure 8. Comparison of pulp and paper parameters (degree of refining, tensile strength, air permeability) with enzyme characteristics (activity on pulp, filter paper activity, xylanase activity, CMC activity, CMC end viscosity, CMC hydrolysis time). The enzymes were dosed according to their activity on pulps (elliptic line, Activity on pulp). LF Sulfite pulp: 1000 ppm Enzyme B, 911 ppm Enzyme A. SF Sulfate pulp: 1000 ppm Enzyme B, 435.4 ppm Enzyme A.

Tensile strength:	*ISO 1924-2*
Air permeability:	*ISO 5636/3*
Filter paper activity:	*pH 4.8, 50 °C, reducing sugar release from filter paper in 5 min*
CMC activity:	*reducing sugar release from a CMC solution with a DS0.7 & Mw0.9x105*
CMC end viscosity:	*pH 4.8, 45°C, degradation of a 1% CMC solution*
CMC hydrolysis time:	*applied amount of enzyme needed to degrade a 1% CMC solution*

Interestingly, Enzyme B showed only a very moderate effect on the degree of refining even though it was even more active in hydrolysis of these pulps related to release of reducing sugars (compare Figure 5 and Figure 6). For both pulps, (Figure 88) Enzyme A dramatically increased the degree of refining. Based on an equal dosing of both enzymes according to hydrolysis activity on this particular pulp, again the resulting CMC activity of Enzyme A was lower than that of Enzyme B. However, in contrast to LF Sulfite pulp, for SF Sulfate pulp the resulting xylanase activity of Enzyme A was likewise lower than of Enzyme B. Interestingly, a higher degree of refining seen for Enzyme A also correlated to higher β-glucosidase activity (Figure 2). As elaborated above, the effect of β-glucosidase could potentially be a reduction of product inhibition well known for endoglucanases and cellobiohydrolases [39]. Next to this, another positive influence of cellobiohydrolases is conceivable. In refining, enzymes should locally weaken the outer fiber walls to achieve fibrillation with less mechanical force. Knowledge regarding the properties of these produced weak points would be important but is difficult to determine. Probably, weak points produced by endoglucanases alone are to small and weak to show effects in a mechanical treatment. The distance of weak spots caused by endoglucanases is essential. To make these weak points larger some cellobiohydrolase activity may be needed, and thus some β-Glucosidase activity is then needed too. This means there is no single enzyme activity that forecasts the refining behavior.

In addition to the pulp and paper related factors presented in Figure 88, further parameters were measured and summarized in supplemental material for LF Sulfite (Table S1). Effects followed expectations, e.g. increasing parameters: water retention, fines, density and elastic modulus. Others decreased due to enzyme treatment such as volume, air permeability (Bendtsen), bending stiffness and strain at break. Higher enzyme dosage for Enzyme A, 91.1 ppm and 911 ppm, showed stronger effects, resulting in overreaction regarding degree of refining. In addition, the need to refine the pulp after enzymatic treatment to make changes visible can be clearly seen (Figure S1). Buzala et. al. reported best paper strength properties for an enzymatic treatment with only xylanase activity, followed by a product showing both xylanase activity and cellulose hydrolysing activity. Worst paper strength properties were obtained with an enzyme showing only cellulose hydrolysis [40]. This is in accordance to findings for Enzyme A. Otherwise; Enzyme B showed no such effects although

measured activities were similar to Enzyme A. Therefore, summarizing, data from Figure 88 suggests that multiple factors influence the performance of enzymes.

Pulp properties may not be neglected. As reported by Ko et. al. refining and bleaching improved accessibility of endoglucanases to eucalyptus kraft pulps. Further to this, greater adsorption was visible for shorter and wider fibers. Moreover, higher lignin content negatively influences the adsorption of enzymes on unrefined pulps. However, refining negated these effects [41]. Additionally, as already mentioned, crystallinity plays an important role. Pulping removes less ordered fractions causing an increase in the crystalline fraction of the remaining pulp [42]. Comparing a sulfate pulp (Eucalyptus grandis) and a sulfite pulp (*Pinus taeda*) the crystallinity was higher for the sulfite pulp [43]. Therefore, further investigations require crystallinity measurements considering duration and intensity of the pulping process as well as the pulp type. Since crystallinity influences the different types of enzymes differently and for hydrolysis other enzymes are factorable, than for fibrillation, an enzyme formulation can show excellent hydrolysis, but limited performance in pulp fibrillation. Another factor might be the fibre geometry. It was found, that cellobiohydrolase I hydrolysis occurs only on the hydrophobic faces of a cellulose crystal and only reduces the width and not the height of a cellulose crystal. Increasing surface roughness after additions of the cellobiohydrolase I and a decrease in crystal size after 11 h, shows effects considerable on a macroscopic scale [44]. Thus, to summarize only reducing sugar release is not sufficient for prediction of enzyme performance, even though the same pulp is used. Detailed characterization of the pulps are critical.

Other factors such as incubation time, not shown above, can be found in the supplemental material. Figure S 1 showed stronger effects for Enzyme A when incubated for 60 min, compared to 10 min and as seen before, the effects of Enzyme B were rather limited, independent from the incubation time.

3.5 Conclusion

The beneficial effect of a cellulase enzyme preparation (Enzyme A) in refining was demonstrated based on increased degree of refining and hand sheet properties of two kraft and one sulfite pulp. The degree of refining for softwood sulfite pulps increased dramatically from 23 °SR to 50.3 °SR, at highest dosage (911 ppm) and treatment time (60 min), compared to pulps refined without enzyme addition. When compared to Enzyme B, this clear effect shows the potential of Enzyme A to reduce the energy demand in industrial refining processes. Air permeability decreased from 1100 ml min^{-1} to 3.6 ml min^{-1} indicating a drastic increase in fiber density, whereas other factors like the tensile strength of hand sheets were not affected as much any of the enzymes (range: 3.8 – 4.8 N mm^{-2}). Enzyme A overacted at high dosages, visible by a high degree of refining. Therefore, a sensible dosage and monitoring system is essential to regulate enzyme assisted refining on an industrial scale. In contrast, Enzyme B showed no significant improvement of refining behavior for the particular pulps tested, although it showed activity in various measurements including sugar release from pulps. This underlines the need of advanced preliminary lab-based screening strategies for a successful enzyme choice. Depending on the pulp, the refining effect of Enzyme A correlated with higher xylanase and β-glucosidase activities while endoglucanase activity did not correlate with the measured effects in the same manner. It seem that successful enzyme assisted pulp refining cannot be assigned to one single enzyme activity but requires the concerted action of various enzyme activities, considering effects such as reduction of enzyme inhibition in the presence of β-glucosidase activity. Moreover, distinct susceptibility to enzyme hydrolysis was found for the different pulps. Consequently, only one simple standard enzyme activity on a model substrate like filter paper cannot predict the effect of refining on different pulps. Hence, future mechanistic studies should focus on the analysis of fibers and pulps as on combinations of single enzymes with enzyme mixtures to elucidate potential inhibition or enhancement.

3.6 Author contributions

Oskar Haske-Cornelius, Alexandra Hartmann, and Florian Brunner performed the experiments. Oskar Haske-Cornelius, Alexandra Hartmann and Wolfgang Bauer planned the experiments and analyzed the data. Oskar Haske-Cornelius, Alessandro Pellis and Gibson S. Nyanhongo wrote the manuscript. Georg M. Guebitz and Wolfgang Bauer supervised the work. Georg M. Guebitz, Wolfgang Bauer and Alessandro Pellis corrected the manuscript. The presented data were discussed by all authors prior to submission.

3.7 Conflict of interest

The authors declare no conflict of interest.

3.8 Acknowledgments

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3.10 Supplementary information

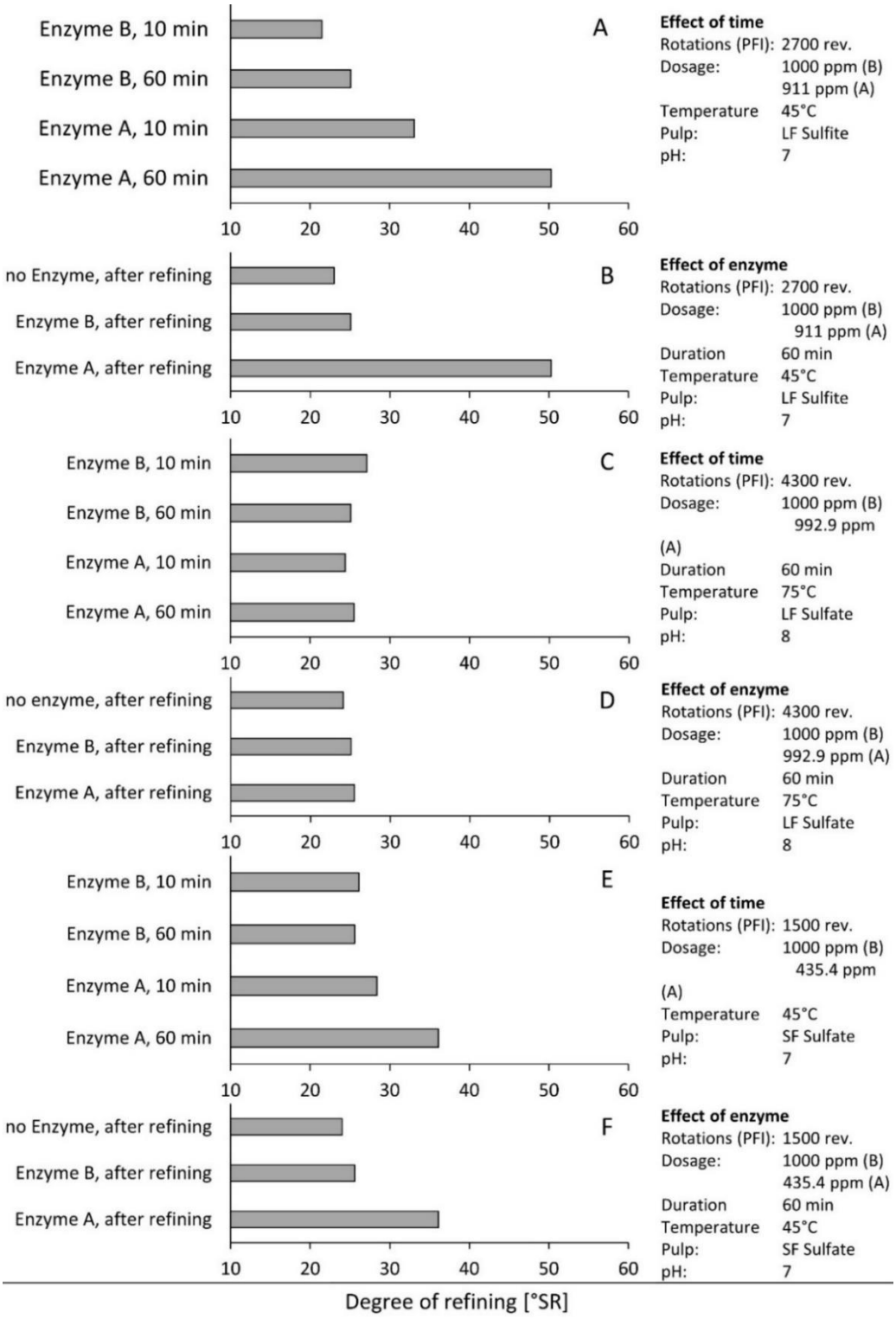

Figure S 1. Effect of time and enzyme on degree of refining of LF Sulfite, LF Sulfate and SF Sulfate. Effect of time for LF Sulfite at 45 °C (A), Effect of enzyme for LF Sulfite (B), Effect of time for LF Sulfate, at 75 °C enzyme denatured fast (C), Effect of enzyme for LF Sulfate, at 75 °C enzyme denatured fast (D), Effect of time for SF Sulfate (E), Effect of enzyme for SF Sulfate (F). For Enzyme A, a longer incubation time increases the degree of refining at pH 7. For pH 8 on LF Sulfate, no effects are visible for both enzymes and incubation time. When an effect is visible, Enzyme A is more active than Enzyme B.

Table S 1. Summary of pulp and hand sheet related parameters for LF Sulfite treated with different dosages of Enzyme A. Rotations (PFI): 2700 rev., Temperature: 45 °C, Enzyme A, Duration enzyme treatment: 60 min, Pulp: LF Sulfite, pH: 7

Parameter	No Enzyme	91.1 ppm	911 ppm	
Degree of refining	23.0	26.0	50.3	[°SR]
Water retention before refining	0.827	0.82	0.917	[g g^{-1}]
Water retention after refining	1.22	1.235	1.563	[g g^{-1}]
Zeta potential before refining	-24.33	-17.77	-14.73	[mV]
Zeta potential after refining	-15.73	-16.47	-16.37	[mV]
Fiber length after refining	1.593	1.485	0.6835	[mm]
Fiber width after refining	30.17	30.70	34.15	[µm]
Fines after refining	34.9	36.15	48.55	[%]
Grammage	80.44	79.68	81.35	[g m^{-2}]
Thickness	121	115	98	[µm]
Density	0.667	0.694	0.827	[g cm^{-3}]
Volume	1.504	1.441	1.205	[cm^3 g^{-1}]
Air permeability (Bendtsen)	1100	702	36	[ml min^{-1}]
Air permeability (Gurley)	11.64	17.96	338.49	[s]
Whiteness	80.96	80.61	79.10	[%]
Opacity	74.18	73.06	70.32	[%]
Bending stiffness	106	102	80	[mN]
Tensile force	71.18	75.52	65.98	[N]
Tensile strength	4.75	5.03	4.40	[kN m^{-1}]
Tensile index	58.99	63.18	54.07	[Nm g^{-1}]
Strain at break	2.88	2.83	1.93	[%]
Elastic modulus	5.03	5.57	7.09	[N mm^{-2}]
Tensile Energy Absorption	97.46	100.87	60.23	[J m^{-2}]
Tear index	9.63	7.75	2.75	[mN g^{-1}]
Internal Bond (Scott-Bond)	334	352	N/A	[J m^{-2}]

4

Environmentally friendly Covalent Coupling of Proteins onto Oxidized cellulosic Materials

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4.1 Abstract

Cellulose is a biodegradable and renewable material that is one of the most abundant biopolymers with many different applications from low value newsprint products to high value biomedical sensor devices. In the last years, the demand of functionalized cellulose for the development of new packaging materials was constantly rising. In this study, a new two-step method for surface functionalization of cellulose sheets and fibers involving oxidation by 2,2,6,6-Tetramethylpiperidinyloxyl (TEMPO) followed by coupling of different proteins was investigated. The cellulose oxidation was monitored via FT-IR at 1610 cm^{-1}, photometrically via toluidine blue staining and via titration for the determination of the -COOH group concentration. TEMPO oxidation increased the amount of -COOH groups from around 0.2 to more than 1.4 mmol g^{-1} when $NaClO_2$/NaClO regeneration was used. The TEMPO/laccase system instead led to 0.7 mmol g^{-1} of -COOH groups (determined via HCl titration). The oxidation was monitored over time and showed that 50% of the reaction were completed within the first 60 min of reaction time. Coupling of protein-based hydrophobins or bovine serum albumin using the EDAC/NHS system led to the desired increase in hydrophobicity and detection of protein on cellulose. Coupling was investigated using contact angle measurements and SEM microscopy paired with elemental analysis for oxygen and nitrogen. Hence, the coupled hydrophobins led to a significant increase of the initial contact angle by 33% with water drop stability of over 200 sec. In contrary, pure cellulose obtained no visible water drop and for surfaces with uncoupled hydrophobins no stable contact angle, with a soaking time dropping to 55 sec was achieved. As a result, the thorough study revealed that the new combinatorial approach of surface functionalization and protein coupling led to the successful increase of hydrophobicity.

4.2 Introduction

Cellulose is the most abundant biopolymer with a wide range of applications such as classical pulp and paper products, food packaging, cosmetics, coatings, laminates, optical films and sorption devices. Thus, cellulose is the most promising biopolymer for the production of sustainable and biocompatible products [1]. In 2014 135,024,000 Mt of printing, writing and newsprint paper were produced worldwide. Although pulp and paper products, especially printing and writing papers, are still an important worldwide consumer product, the increasing digitalization leads to an estimated reduction in demand of 76.7-87.1 million tons by 2030 [2]. New applications of paper will be needed to appease decline in classical application, therefore, new pulp and paper products are of great industrial interest. Conceivable alteration of cellulose properties could be surface functionalization or addition of catalytic functions to paper, resulting in high value products. Cellulose fibers and sheets are suitable materials for hydrophobization by protein coupling since they provide advantages like biocompatibility, porosity, flexibility or simple and economic production methods [3]. The addition of metal nanoparticles [4], single-stranded probe DNA coupling [5] or grafting/coating of different polymers [6],[7] are examples for paper surface functionalization strategies. These treatments result in antibacterial or bioactive paper [4] and diagnostic tests [5]. For example, Kuvruk et. al. developed a portable paper based sensor for detection of pesticides [8]. Furthermore, a higher hydrophobicity can be achieved by surface functionalization, making the cellulose more suitable for applications in packaging [9], protective clothing [10] and printing of paper-based analytical devices [11].

Other approaches providing new properties to cellulosic materials are wetting with ethyl-cyanoacrylate [12] or coupling of polysiloxanes [10]. Nevertheless, these methods are not environmentally friendly and sustainable since they rely on fossil-based polymers or use harmful solvents. Another systems to functionalize cellulose is the radiation induced grafting of polymers [13]. Most of these substances are not sustainable and biodegradable, especially when they appear grafted on cellulose. Proteins, as sustainable biopolymers with enormous heterogeneity, can be used to overcome drawbacks of these conventional copolymers. When successfully coupled to cellulose with a generally working approach, paper fibers and sheets can be equipped with the broad variety in functions different proteins have. Hydrophobins, a class of protein with unique characteristics, are small and rich in cysteine. Their ability

to form amphipathic films when confronted with a hydrophilic-hydrophobic interface [14], making them promising candidates for permanent surface modifications via covalent bonding to the material, while maintaining their amphipathic character. In this context, Scholtmeijer et. al. reported the successful coupling of engineered hydrophobins on Teflon for increased fibroblasts surface growth. They also constructed N-terminal modified hydrophobins, changing the physiochemical properties of the hydrophilic side of the assembled protein without altering its crude properties [15]. Such modifications enable the coupling of the N-terminus or amino residues of the protein to a surface, using the 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimid/N-Hydroxysuccinimid (EDAC/NHS) system. This system is widely used to crosslink carboxylic groups and amide groups in macromolecules like collagens [16], poly(acrylic acid) on silicon [17] and chitosan on triblock copolymers [18]. Hence, this method is considered as a promising approach to couple amide groups of various proteins to the carboxylic groups in paper sheets or cellulose-containing materials.

For the creation of new carboxylic coupling points, previous oxidation of hydroxyl groups of the glucose subunits is necessary, avoiding the destruction of the cellulose backbone. The ability of 2,2,6,6-tetramethylpiperidinyloxyl (TEMPO) to oxidize different cellulosic materials in presence of a strong oxidizing agent such as sodium hypochlorite (NaClO) is well known [19], [20], [21]. Sodium hypochlorite generates the oxoammonium salt, which catalyzes the oxidation of hydroxyl groups to carbonyl and carboxyl groups. Already small amounts of TEMPO can perform the reaction under mild conditions (0 °C, pH 8.6, 350 mM CH_2Cl_2) within a few minutes [22]. A modification of this system is using TEMPO in combination with sodium chlorite ($NaClO_2$) and NaClO , where the latter initiates the reaction in first place, and $NaClO_2$ acts as primary oxidant. Additionally, $NaClO_2$ oxidizes the aldehydes at the C6 position in cellulose to -COOH, while the degree of depolymerization of the cellulose is reduced, compared to other oxidizing agents used in combination with TEMPO [23]. The system is currently used for several industrial applications such as production of pharmaceuticals, flavors, fragrances, agrochemicals and other specialty chemicals [24]. TEMPO is not only able to perform oxidation reactions in the presence of chemical oxidants but has also the big advantage to be regenerable by a class of biocatalysts, namely laccases. These multi-copper oxidases naturally catalyze the oxidation of phenolic compounds to the

corresponding radical [25]. In combination with a mediating substance such as TEMPO, the oxidation ability of laccases can be extended to non-phenolic lignin moieties like veratryl alcohol [26] and cellulose [27]. For cellulosic pulps, the mild reaction system, containing laccase and TEMPO resulted in uniform oxidation of the high and low molecular weight areas of the material [27]. The cellulose oxidation with both TEMPO recycling systems (laccase and NaClO/$NaClO_2$) is reported in Figure1.

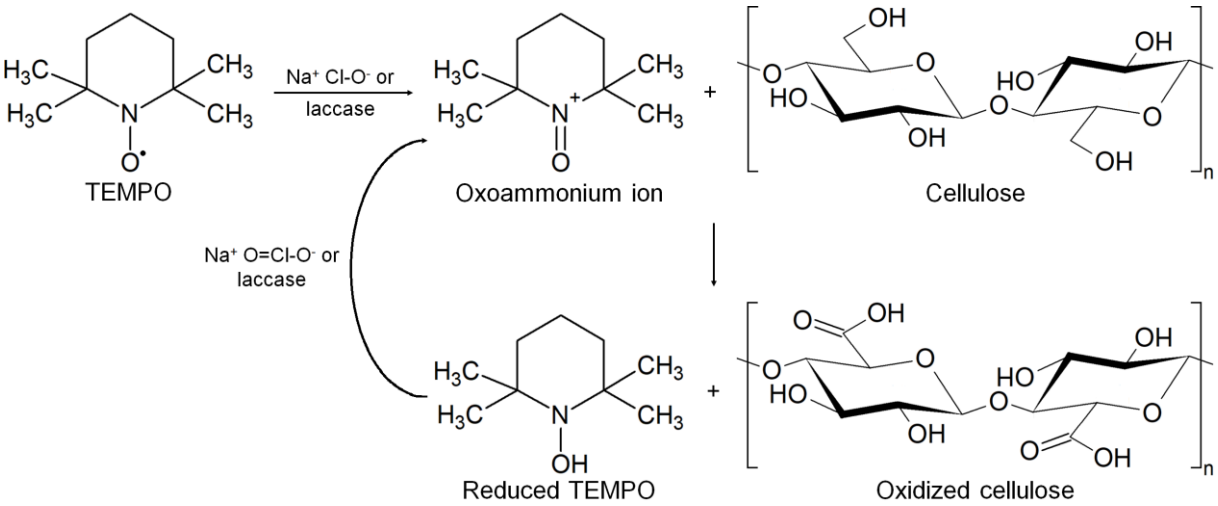

Figure 1. Oxidation of cellulose at the C6 position. Formation of the oxoammonium ion from TEMPO with NaClO (chemical) or laccase (enzymatic). The oxoammonium ion oxidizes cellulose and is than recycled by $NaClO_2$ (chemical) or laccase (enzymatically).

Oxidized cellulose, either in sheets or as fibers, is the starting material for covalent protein coupling performed in this work, using the EDC/NHS system. In Figure 2, the covalent coupling of proteins to oxidized cellulose is described. EDC forms a reactive ester, enabling the covalent binding of proteins. To reduce back reactions, the reaction equilibrium is shifted towards the coupling by NHS, which replaces the coupled EDC and forms amine esters. These amine esters result in covalent coupled NH_2 groups afterwards. Therefore, the EDC/NHS system provides two pathways to couple proteins covalently to oxidized cellulose [28].

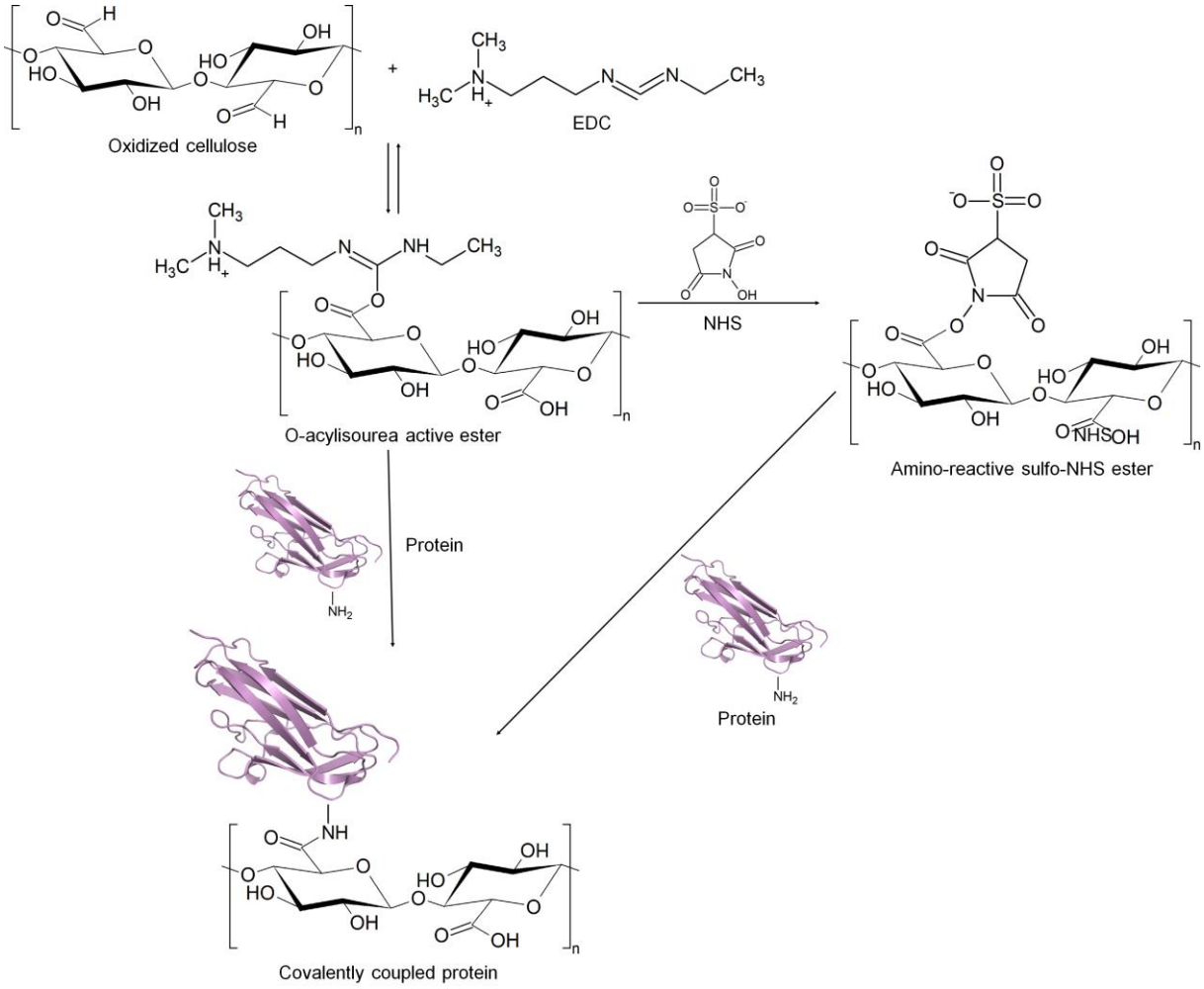

Figure 2. EDC/NHS coupling of proteins to oxidized cellulose. EDC couples to COOH groups of oxidized cellulose resulting in an active ester. In the presence of NH_2-groups a covalent amide bond with a protein, or hydrolysis back to oxidized cellulose is possible. With NHS the active ester can form an Amin-reactive sulfo-NHS ester, which results in covalent bonds to NH_2-groups of proteins.

In the present work, both, chemical and enzymatic oxidation were applied on cellulose fibers. Enzymatic oxidation was conducted with different TEMPO concentrations, to find the optimal conditions. Additionally, the chemical oxidation reaction was carried out on paper sheets. Hereby, TEMPO, NaClO and $NaClO_2$ were directly applied on paper sheet surfaces, as well as on cellulose fibers to allow the subsequent coupling of hydrophobins or BSA.

4.3 Materials and methods

4.3.1 Cellulose materials and chemicals

Three different cellulose sheet materials were used as raw material for oxidation and coupling experiments: commercially available grade 1 filter paper (Whatman, United Kingdom), Kraft White (100% Cellulose) and Kraft brown (93-94% Cellulose 6-7% Lignin). Kraft White and Kraft Brown were provided by Mondi Frantschach (Austria). Additionally, long fiber sulphite cellulose normally used for paper production was also used as raw material for oxidation and coupling. Commercially-available hydrophobins (BASF, Germany) were used for conducting the coupling experiments. Production details and properties of the used hydrophobins were previously reported by Wohlleben et al. [29]. Other chemicals and reagents were of analytical grade and purchased from Sigma-Aldrich (Austria) and used without further purification if not stated. Laccase from Myceliophthora thermophila (MtL) was supplied by Novozymes (Denmark) and used without any further purification.

4.3.2 Chemical oxidation of paper sheets

Chemical oxidation was conducted in 100 mM sodium phosphate buffer pH 7. A 36 cm^2 area of paper was cut into squares and placed into glass bottles. 45 ml of 10 mM TEMPO and 100 mM $NaClO_2$ in buffer were added. Oxidation started after addition of 5 ml of 1% NaClO solution diluted in buffer. Blank samples were performed with buffer instead of NaClO solution. Samples were incubated at 21 ºC and 100 rpm. Paper pieces were removed after 1, 3, 24, 48 and 72 h, washed three times with ultra-pure water and dried at 40 °C.

4.3.3 Chemical oxidation of cellulose fibers

600 mg paper fibers were mixed with 45 ml of a buffer solution (sodium phosphate 100 mM, pH 7) containing 10 mM TEMPO and 100 mM $NaClO_2$. Oxidation was started after addition of 5 ml of 1% NaClO diluted in buffer. After 24 h of reaction under agitation at 21 ºC, the oxidation was stopped by filtration of the fibers with a Whatman filter paper grade 1 (Ø 8.5 cm). Fibers were washed two times with ultra-pure water and dried overnight in an oven at 40 °C. Oxidized fibers, immobilized on a filter paper circle, were placed in petri dishes and used for coupling experiments.

4.3.4 Enzymatic oxidation of cellulose fibers

750 mg of dried pulp were dissolved in 83 ml of 100 mM sodium phosphate buffer pH 7 containing 0, 20 or 40 mM TEMPO. Different amounts of TEEMPO were used to check for linear correlation of fiber oxidation and TEMPO concentration. For regeneration of TEMPO, 1.5 ml of Myceliophthora thermophila laccase with an activity of 111 µkat ml^{-1} was added and all samples were supplied with 30 ml min^{-1} pure oxygen for 48 h under agitation. After oxidation, the fibers were filtered using a borosilicate filter (porosity 3) and washed two times with 100 ml of ultra-pure water for each washing step. The wet fibers were weighted, and 250 mg of the wet material was dried (40 °C) for FTIR analysis and to determine the amount of fibers for titration.

4.3.5 Carboxylic content determination of oxidized fibers by titration

To the wet fibers, 50 ml of 0.01 M HCl were added and stirred for 2 h. Fibers were filtered and washed again, before 50 ml ultra-pure water and 30 ml 250 mM Ca-Acetate solution were added. Samples were titrated with Phenolphthalein as indicator to the transition point with 0.01 M NaOH using a dosimeter. The number of carboxylic groups was calculated using Equation 1. All titration experiments were performed in triplicates.

Equation 1. Determination of carboxylic groups per g of cellulose fibers. V: Volume of NaOH [ml], c: concentration of NaOH [mol l^{-1}], m: weight of fibers [g], w: moisture content of fibers [%]

$$\mathrm{mmol}\,\frac{\mathrm{COOH}}{\mathrm{g}} = \frac{\frac{80}{30} * \mathrm{c(NaOH)} * \mathrm{V\ (NaOH)}}{\mathrm{m}\ (1 - \frac{\mathrm{w}}{100})}$$

4.3.6 Enzyme activity

Laccase activity was assayed according to the procedure reported by Prasetyo et. al. with some modifications [30]. ABTS was oxidized by laccase to its radical form and the change in absorbance was measured at 420 nm for 3 min using a U-2900 spectrophotometer (Hitachi, Japan). Laccase activity was measured at 21 °C in 100 mM sodium phosphate buffer pH 7 and expressed in katal (kat), which is the amount of enzyme that is necessary for the conversion of 1 mol ABTS sec^{-1}.

4.3.7 Photometric determination of oxidation with toluidine blue

Toluidine blue measurements, a method previously used for the detection of carboxylic groups on polymer surfaces was performed [31]. For this analysis, paper samples with an area of 2 cm^2 were treated with 3 ml 0.1% toluidine blue in TRIS-HCl buffer (0.1% TRIS, pH 8.6) for 15 min at 21 ºC. Afterwards, samples were washed two times with 5 ml ultra-pure water. The liquid phase of the second washing step was collected, centrifuged at 12500 rpm for 10 min and its absorbance was measured at 625 nm using an Infinite Pro 2000 photometer (Tecan, Switzerland). For FTIR analysis, a Spectrum 100 Photometer (Perkin Elmer, United States) was used.

4.3.8 FTIR to control the oxidation efficiency

FTIR Spectra were collected at a resolution of 2 cm^{-1} for 10 scans and normalized in the 2850-2950 cm^{-1} region before any data processing. The area between 2850-2950 cm^{-1} represents a typical band of cellulose and therefore was used for normalization to emphasize changes in carboxylic content [32]. Bands in the 1632-1604 cm^{-1} area (C=O) represent carboxylic groups [33]. All experiments and measurements were conducted in triplicates.

4.3.9 Coupling of hydrophobins on chemically oxidized cellulose fibers

160 mg of oxidized pulp fibers were mixed with 9 ml of a 10 g l^{-1} hydrophobins solution in 100 mM sodium phosphate buffer pH 6. Afterwards, 1 ml EDC and 0.5 ml NHS solutions were added (see details on concentration above). Samples were incubated at 21 ºC and 150 rpm for 10 h. A filtration step using a borosilicate filter (porosity of one) was conducted and the recovered fibers were washed two times with 100 ml ultra-pure water and dried overnight at 40 °C. The samples were fixed with double-sided tape on microscope slides to obtain a flat surface. These samples were further used for contact angle and microscopy measurements as described above.

4.3.10 Coupling of hydrophobins onto cellulose surfaces (paper sheets)

Hydrophobins were coupled on oxidized and dried paper sheets using the EDC/NHS system which is known to form amide bonds between primary amines and carboxyl groups [34]. The cellulose surfaces were placed in a petri dish and 3.6 ml of a 10 g l^{-1} hydrophobins solution in 100 mM sodium phosphate buffer pH 6 was added. EDAC and NHS solutions contained 4 and 22 g l^{-1}, respectively in 100 mM sodium phosphate buffer pH 6. 400 µl of EDAC and 200 µl of NHS solution were added to each sample. The final reaction volume for each sample was 4.1 ml. The samples were incubated

for 24 h at 21 °C under slight agitation. Thereafter, samples were washed three times with ultra-pure H_2O and dried at 40 °C until a constant weight was reached.

4.3.11 Coupling of BSA onto oxidized cellulose fibers

Each sample contained 150 mg oxidized fibers. 18 ml of a BSA solution containing 12 mg ml^{-1} enzyme in 100 mM sodium phosphate buffer pH 6 were added. EDC and NHS solutions contained 4 and 22 g l^{-1} respectively in buffer. 2 ml of EDC and 1 ml of NHS solution were added to each vessel. Samples were incubated overnight under slight agitation at 21 ºC. After coupling, the filter sheets were washed by rinsing them with ultra-pure water three times. The coupling reaction was also monitored using the elemental analysis function of the SEM.

4.3.12 Water contact angle

Samples were fixed with double-sided tape on microscopy slides and used for contact angle and scanning electron microscopy (SEM) analysis. Contact angle measurements were conducted with a DSA 100 Drop Shape analyzer (Krüss, Germany). For analysis, 5 µl of ddH_2O were deposited on the paper material. The water contact angle (WCA) was measured 1 s after the drop deposition and the soaking time was determined using the video recording mode. The soaking time was defined as the time that the water drop needs to disappear completely from the surface of the paper sheet.

4.3.13 Scanning electron microscopy

Cellulose surfaces and fibers morphology of the samples was qualitatively assessed through SEM analysis. Control cellulose materials without any oxidation or coupling treatment were also surface characterized. All SEM images were acquired collecting secondary electrons on a Hitachi 3030TM (Hitachi, Japan) table top microscope working at EDX acceleration voltage with the reduced vacuum mode. Elemental analysis for surfaces was performed for nitrogen and oxygen for the same picture frame, using the Quantax 70 software (Bruker, United States).

4.4 Results and Discussion

4.4.1 Oxidation strategies for cellulose fibers

4.4.1.1 FTIR analysis

As shown in Figure 3, the characteristic band for carboxylic groups and carbonyl groups at around 1600 cm^{-1} [35] is clearly increased in both, enzymatically and chemically treated fibers when compared to the blank reaction.

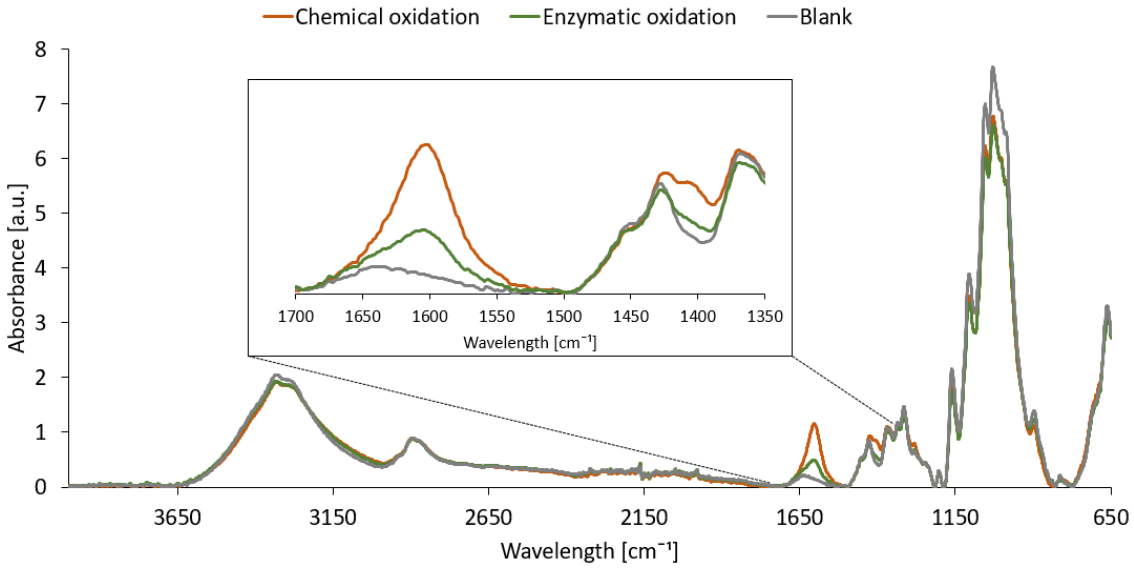

Figure 3. FT-IR monitoring of the chemical (A) and enzymatic (B) oxidation of cellulose fibers using TEMPO as mediator. Spectra were baseline corrected and normalized in the 2850-2950 cm^{-1} area and the determination of the carboxylic content was performed according to the absorbance at a wavelength of 1610 cm^{-1}. Chemical oxidation was conducted for 24 h and enzymatic oxidation for 48 h.

The oxidation of cellulose fibers treated with TEMPO regenerated by sodium hypochlorite or laccase was monitored using FTIR. Normalization was performed in the 2850-2950 cm^{-1} area, which represents a typical band of cellulose [33]. Additionally, spectra were baseline corrected as specified in the M&M Section relative to the FTIR analysis. It is possible to observe a clear band shift for TEMPO-mediated cellulose oxidation from 1630 cm^{-1} (blank) to 1600 cm^{-1} in both samples (Figure 3), regardless of which oxidation strategy was used. Nevertheless, the data showed that the chemical oxidation was more effective than enzymatic oxidation, since increase in band intensity is higher. FTIR analysis revealed not only changes in the typical region for carboxylic groups, but also the appearance of an additional band at 1405 cm^{-1}. Figure 3 and Table 1 confirm that the chemical structure of the sample, in relation to its crystallinity, was altered by the chemical oxidation. The additional band at 1405 cm^{-1} in the chemically oxidized samples is caused by a transformation of the

intramolecular hydrogen bonds of the polymer, resulting in increasing amount of cellulose II structure [32]. According to Figure 3 the changes in crystallinity only occur for chemical oxidized fibers. For the samples that underwent enzymatic oxidation crystallinity changes were not detected. The lower shift in enzymatically oxidized cellulose samples indicates that the enzymatic oxidation preserves the natural cellulose structure and is a less harsh modification approach than the chemical oxidation that despite being environmentally friendly also allows the preservation of the initial crystallinity of the material.

Table 1. Change in carboxyl content and crystallinity for chemically oxidized, enzymatic oxidized and non-oxidized cellulose fibers. Bands at 1610 cm⁻¹ for carboxyl groups and 1405 cm⁻1 for crystallinity.

Entry	Wavelength [cm^{-1}]	Chemical oxidation [a.u.]	Enzymatic oxidation [a.u.]	Blank sample [a.u.]
-COOH	1610	1.088±0.003	0.485±0.012	0.167±0.019
Crystallinity	1405	0.854±0.012	0.557±0.013	0.449±0.020

4.4.1.2 Titration of oxidized cellulose fibers

To optimize the enzymatic oxidation, the influence of TEMPO concentration on the reaction was determined by titration [36], [37]. A concentration of 20 mM TEMPO increases the amount of carboxylic groups in the fibers from 0.2 (without TEMPO) to 0.65 mmol g^{-1}, when laccase was used. Higher amounts of TEMPO (40 mM) showed only a small increase of -COOH groups yield with a 60 mM TEMPO concentration that showed the same oxidation effect as the 40 mM one. From Figure 4, it is possible to observe that the oxidation rate obtained using the chemical method is approximately double than the one obtained using the enzymatic approach (when the same amount of the TEMPO mediator is used). A comparison of carboxylic groups in samples with chemically and enzymatically regenerated TEMPO (20 mM) is also shown in Figure 4. The chemical-driven process resulted in 1.4 mmol g^{-1} of COOH groups, while the enzymatic treatment produced 0.65 mmol g^{-1}. These data confirm the results presented in Figure 3 and Table 1, where the FTIR spectra analysis indicated a higher effectivity of the chemical oxidation process, when compared to the enzymatic treatment. Variations in the oxidation behavior between the two systems are most

probably caused by differences in the redox potential of the enzyme and its chemical counterpart.

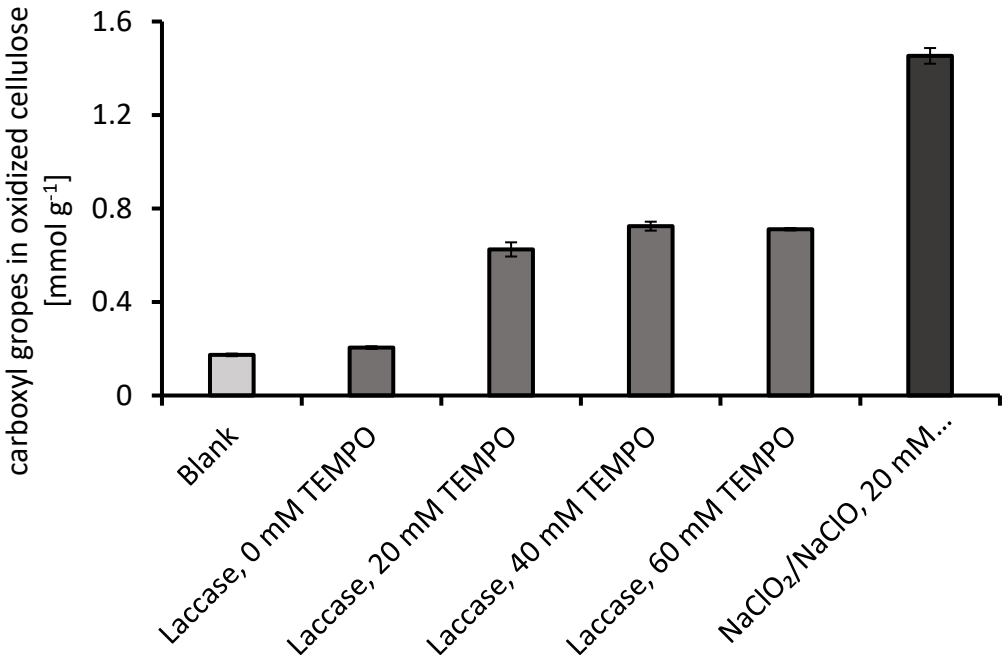

Figure 4. Influence of the TEMPO concentration on enzymatic oxidation of cellulose fibers in comparison to chemical oxidation. Blank is without TEMPO and without oxidation system (bright grey), enzymatic oxidation with laccase and different TEMPO concentration from 0 – 60 mM (middle grey) and chemical oxidation with $NaClO_2$/NaClO and 20 mM TEMPO (dark grey). All experiments were performed in triplicates.

4.4.1.3 Oxidation of different paper sheets

Since not only the oxidation of fibers as raw material, but also the treatment of finished products is of industrial interest, the chemical oxidation was applied on paper sheets. Compared to the enzymatic oxidation, which demands oxygen blown into the solution, the chemical oxidation can be performed under gentle agitation, preserving the sheets from disruption. Since sheets are a fragile material the -COOH groups for later coupling of hydrophobins should be detected without destroying the treated sheet. Therefore, the oxidation monitoring was performed by staining the carboxylic groups with toluidine blue on the oxidized sheets. The determination of -COOH groups on other materials such as poly(lactic acid) [38] and polyethylene terephthalate fabrics [39] using toluidine blue was previously reported and we adapted the existing protocols for our cellulosic materials. The data shown in Figure 5 indicated that, in the samples treated with TEMPO, sodium chlorite and sodium hypochlorite, the amount of -COOH groups increases 4 times when compared with the untreated sample when using paper sheets (Figure 5).

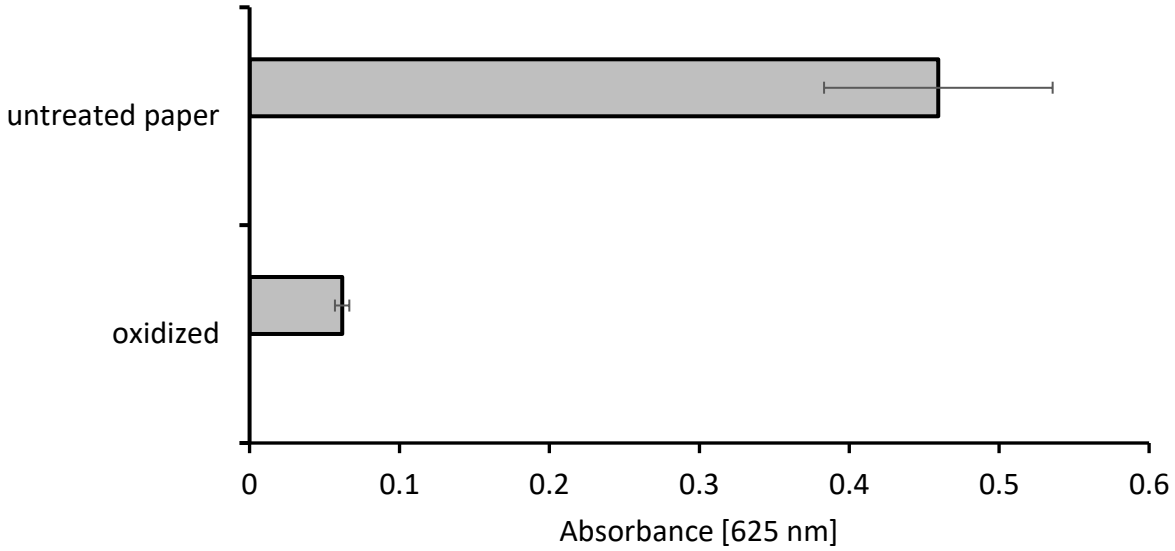

Figure 5. Qualitative analysis of carboxylic groups introduced into paper sheets via TEMPO/sodium hypochlorite. Unbound toluidine blue after staining was photometrically determined in the washing water after two rinsing steps.

For the characterization of the oxidation behavior of lignin-containing cellulose, FTIR measurements of chemically treated paper sheets were performed during a 72 h time course. The results plotted in Figure 6 show an increasing absorbance going from 1.5 a. u. to over 5 a. u. within 24 h. After that, only limited increases were observed till the last recorded point at 72 h. The observed trend was possible either because all accessible -OH groups were oxidized or because the whole sodium hypochlorite added in the beginning of the reaction was consumed within the first 24 h. In any case, the difference between the blank and the oxidized sample is remarkable, with the lignin content of the material that doesn’t affect the reaction efficiency.

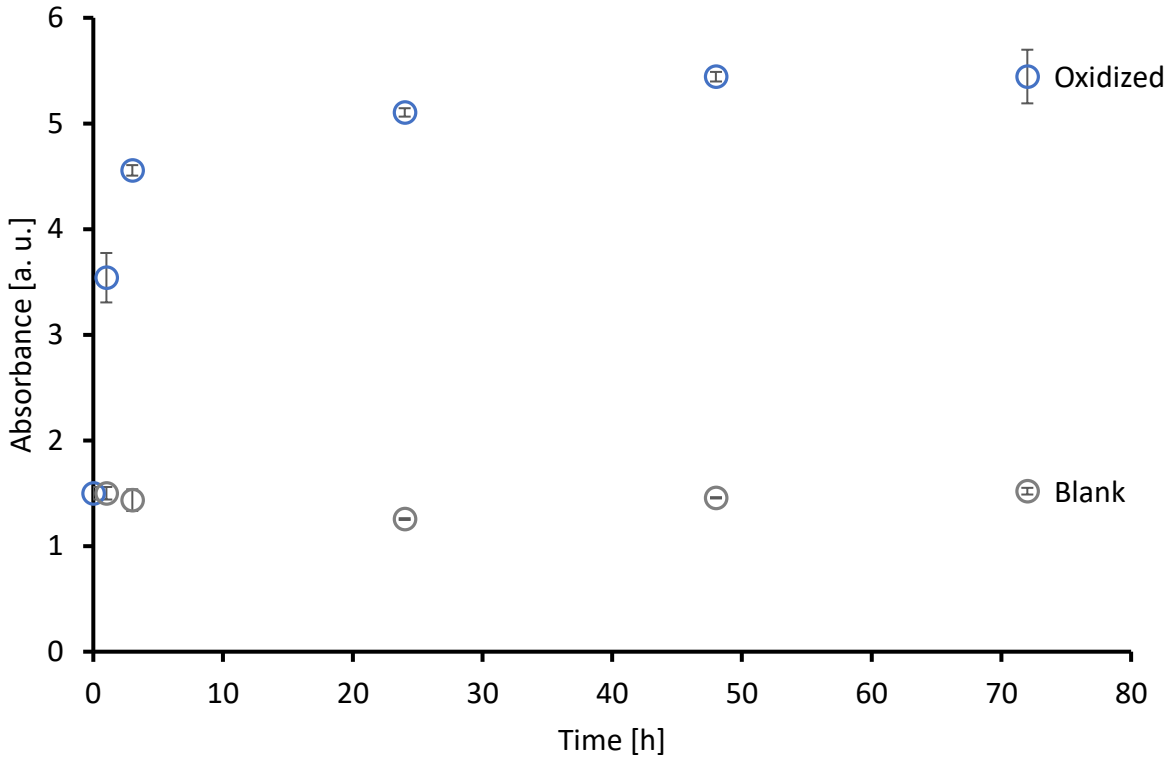

Figure 6. Chemical oxidation of a Kraft White cellulose surface with sodium hypochlorite and TEMPO for 72 h as quantified by FTIR at a wavelength of 1605 cm^{-1} (normalization from 2850-2950 cm^{-1}). All experiments were performed in triplicates.

4.4.2 Protein coupling

4.4.2.1 Hydrophobicity Analysis

To investigate the influence of oxidation on the coupling of hydrophobins to cellulose sheets, oxidized and non-oxidized filter papers sheets were compared according to their water uptake after hydrophobin attachment. In the first measurements oxidized and non oxidized samples were treated with Hydrophobins and EDC/NHS to investigate if oxidation is necessary for coupling (shown in the ESI, Figure S1). Chemically oxidized sheets treated with hydrophobins showed a four times higher soaking time than the blanks without any treatment.

In the second measurements, the protein attachment system, containing EDC/NHS was further investigated for its suitability for the coupling of hydrophobins on oxidized cellulose. The chemical coupling is used to prevent the possible formation of an independent hydrophobic protein layer, or a non-directional coupling to the surface (Figure 7).

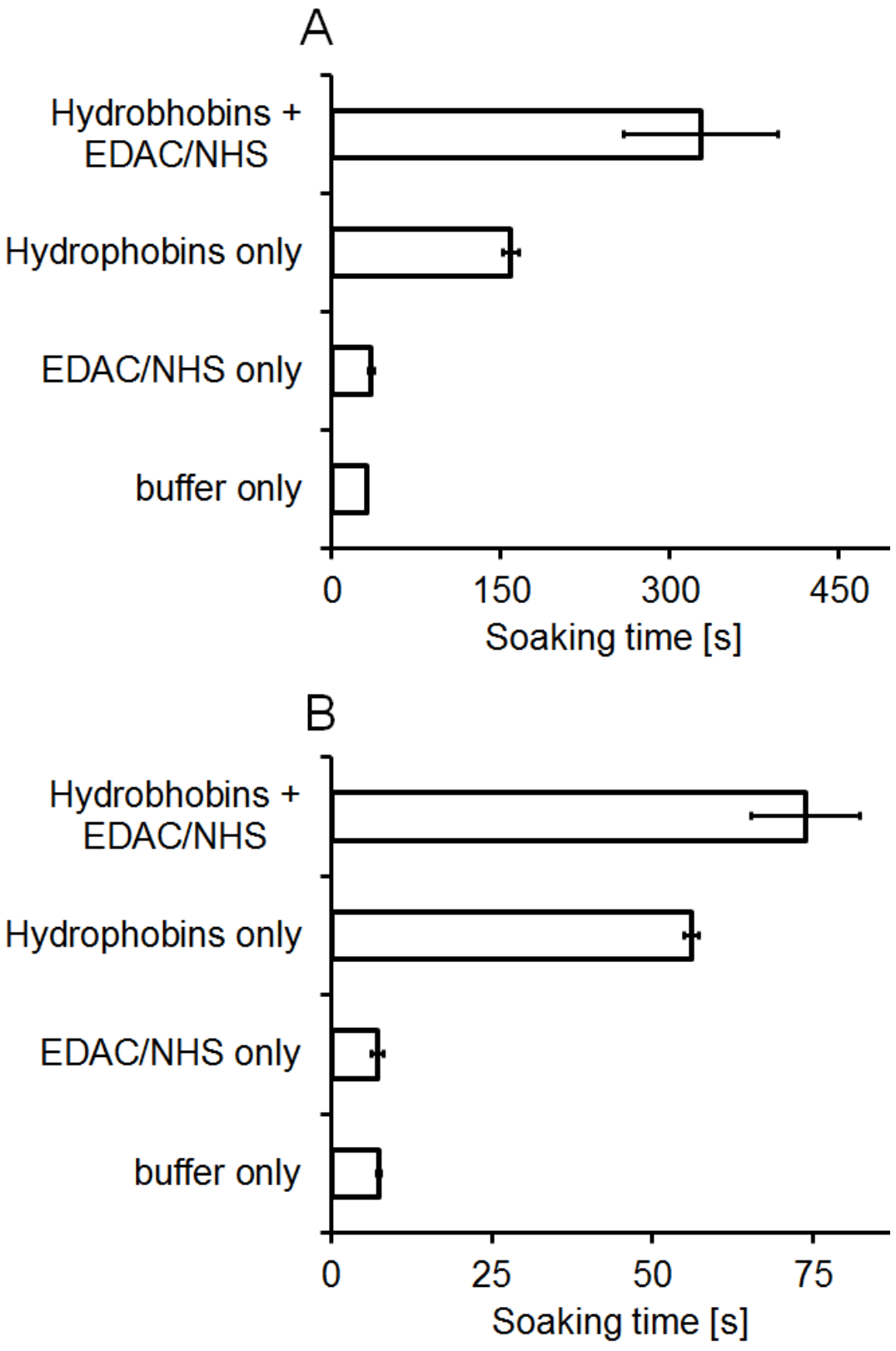

Figure 7. Hydrophobicity determination for cellulose surfaces covalently functionalized with hydrophobins subsequently to prior chemical oxidation of cellulose. Soaking time determination using contact angle measurements for various paper surfaces. Paper containing 6-7% lignin (A) and paper composed of 100% cellulose (B).

Two different paper materials, namely a brown paper containing residual lignin (Figure 7A) and a paper consisting of cellulose only (Figure 7B) were functionalized with hydrophobins, followed by surface soaking time measurements. Both materials showed a strong increase in hydrophobicity when compared to samples without proteins. Especially for the lignin containing paper, the covalent coupling using the EDAC/NHS system improves the hydrophobicity of the sheet from 31 s (when no hydrophobins are present) to 328 s when hydrophobins are covalently coupled with the EDAC/NHS system. The soaking time increases also when comparing the uncoupled hydrophobins (160 s) to the covalently coupled hydrophobins (328 s) proofing the importance of the covalent coupling that yields the highest hydrophobicity. A possible explanation for the differences between surfaces with coupled and non-coupled hydrophobins is that unbound hydrophobins can move across the material with the non-covalently coupled (adsorbed) hydrophobins that can be displaced by water drops causing a decrease of the soaking time if compared to the stable, covalently bounded proteins.

In the past, different approaches for cellulose surface functionalization have been reported in literature [40]. Bayer et al. presented ethyl-cyanoacrylate monomer solutions to functionalize cellulose and make it highly hydrophobic [12] while Vismara et al. obtained hydrophobic material by grafting of glycidyl methacrylate with the material's properties that were changed in a second step by ring opening of the grafted molecule, resulting in hydrophilicity [41]. Boufi et al. functionalized cellulose with silver and gold nanoparticles as a contribution for the production of electrically conducting substrates passed on paper [42]. All these methods, despite elegant and innovative, contain toxic or non-renewable materials and have the drawback of not being suitable for large-scale production due to price and limited to a narrow field of applications. The production of hydrophobins in industrial scale is feasible and they are already commercially available [29].

A further elucidation of the effects caused by hydrophobin coupling on the cellulose surfaces was conducted performing WCA measurements on the obtained materials. Figure 8A shows a water drop directly after deposition (t<5 sec) on an untreated paper sheet. The drop is flat and the WCA is >30°. In Figure 8B (coated paper), the water surface maintains in a round shape and an angle of around 90° was measured. The

hydrophobins, as expected, increase not only the soaking time but also the WCA of the material, clearly improving the hydrophobicity of the functionalized surface.

The additional elemental analysis, depicted in Figure 8C-E, shows the same section of a cellulose fiber in a treated sheet and its corresponding elemental analysis for oxygen (Figure 8D) and nitrogen (Figure 8E) while in the SEM image (Figure 8C) a cellulose fiber is visible surrounded by hydrophobin spheres. Figure 8D presents a remarked turquoise line, which reflects the high oxygen amount in the cellulose fibers whereas the surrounding area is darker and homogenously colored. In contrast to that, in Figure 8E the distribution of the orange color is the same in the whole area, also where the fiber is located. Hydrophobins were therefore coupled on the fiber since the nitrogen atoms can only derive from added proteins (since not present in the starting cellulosic material).

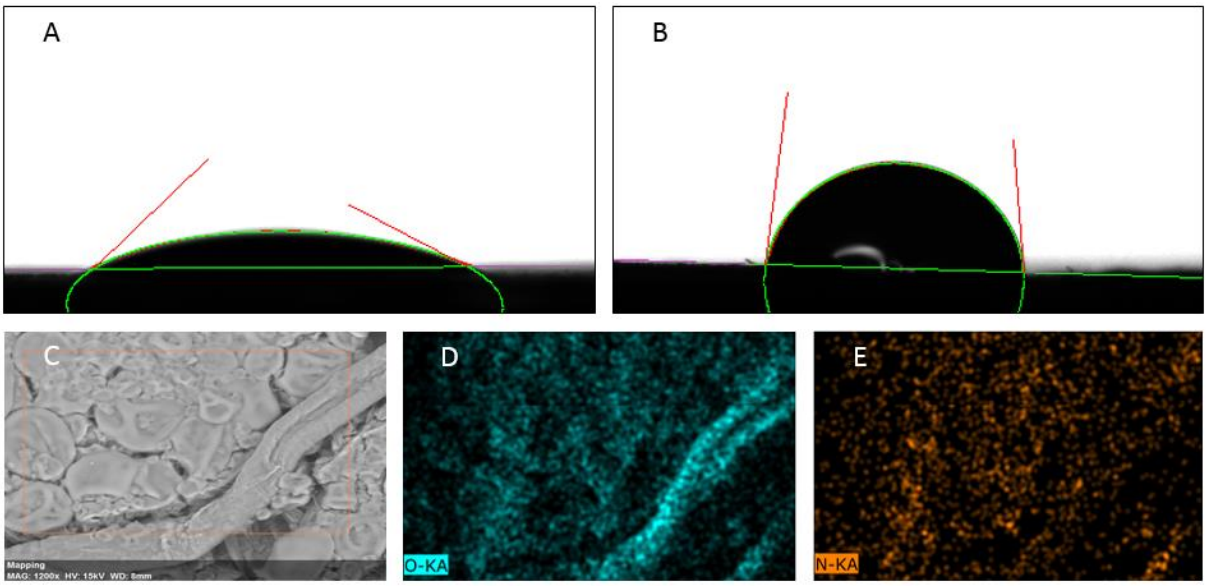

Figure 8. Coupling of hydrophobins to chemical oxidized Whatman No.1 filter paper. Contact angle measurement (A-B) and SEM imaging (C-E) for paper sheets oxidized and EDAC/NHS coupled hydrophobins, Contact angle for non-oxidized material treated with hydrophobins (A), contact angle for oxidized and coupled surface (B), SEM image of a paper fiber with coupled hydrophobins (C), distribution of oxygen for the fiber visible before (D), nitrogen distribution for the same fiber (E).

Using the unique properties of hydrophobins in functionalization of materials was shown before on several other substrates. Laaksonen et al. showed functionalization of graphene with hydrophobins [43] while Opwis and Gutmann thermally deposited hydrophobins on polyester, polyamide and cotton [44]. Moreover a functionalization with hydrophobins was also shown for metals, plastics, glassy carbon electrodes and carbon nanotubes [45]. In contrast to these previous reports, the here presented

process can be applied to temperature sensitive materials and the coupling is covalent to the substrate, and therefore stable for longer periods of time. Additionally, all reactions can be performed at the solid-liquid interface, therefore maximizing the mass-transfer of the process.

4.4.2.2 Coupling of proteins on cellulose fibers

Oxidation and functionalization were not only performed on cellulose surfaces like pre-oxidized paper sheets, but also demonstrated on oxidized pulp fibers. This approach shows that an oxidation and coupling is possible at different stages of the paper manufacturing process. According to the required paper properties, manufacturing conditions and cost factors it is of importance to have a system of broad applicability and flexibility. For this reason, it was of interest if oxidation can be performed on fibers and if next to hydrophobins other proteins can be coupled to fibers. BSA was chosen as a model protein. On a protein chain length of 583 amino acids, Hirayama et. al. reported 20 glutamines and 13 asparagine residues in BSA. These amino acids are distributed along the primary structure of the protein beginning at position 29 and ending at 597. Asparagine and glutamine offer a primary amino group in proteins, which can be used for coupling as long as the tertiary structure of the protein allows it [46]. Figure 9 shows the nitrogen content of fibers chemically and enzymatically oxidized (including blanks for chemical and enzymatic oxidation) and the immobilization success (including blanks for only BSA and only coupling reagents).

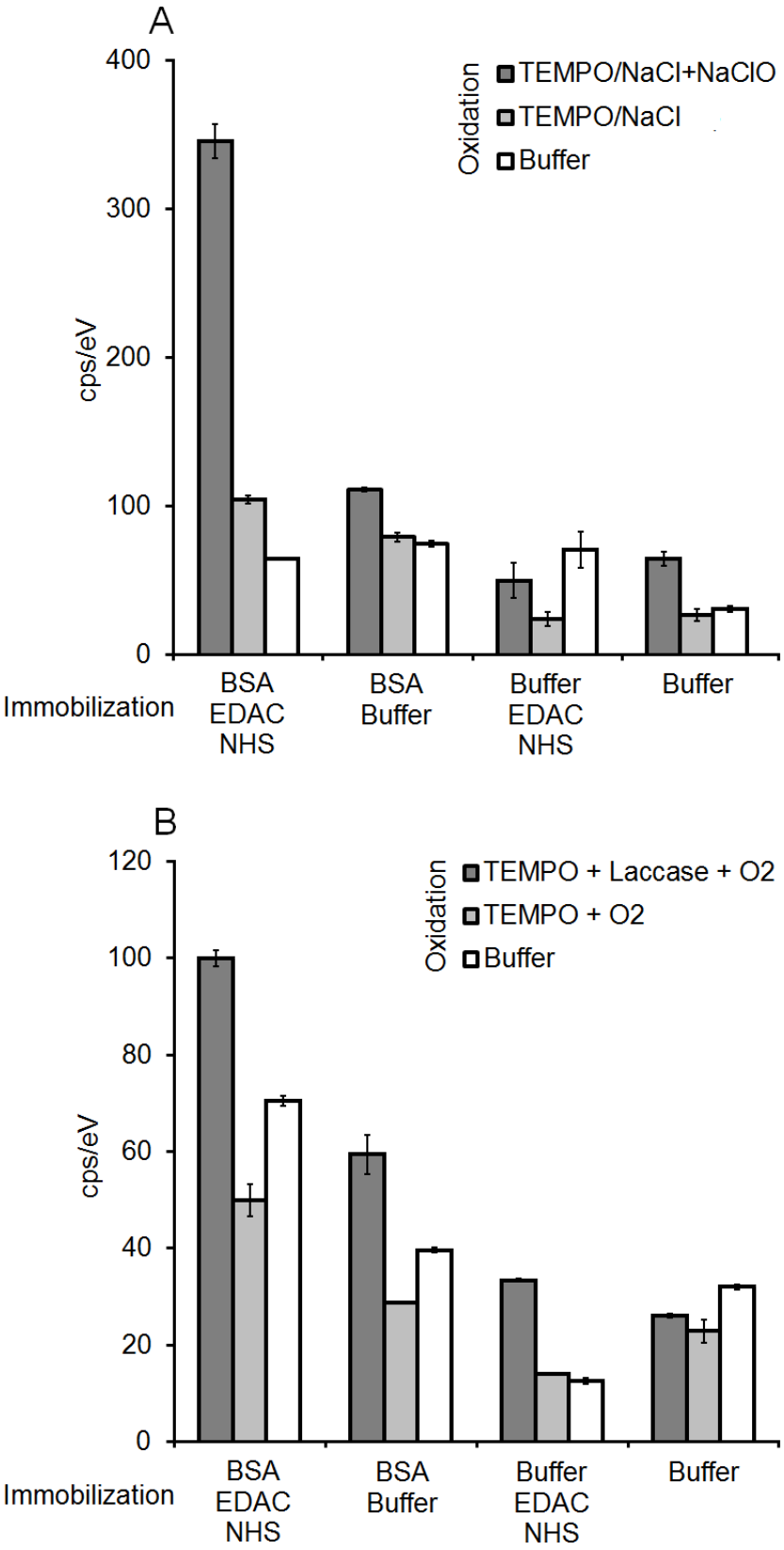

Figure 9. Variations in nitrogen concentration after EDAC/NHS covalent coupling of BSA to chemically oxidized (A) and enzymatically oxidized cellulose (B). Nitrogen concentration on the cellulose fibers was monitored using SEM (Hitachi 3030, Japan) and the software Quantax 70 (Bruker, United States).

Clearly, both oxidation strategies enable the coupling of BSA onto cellulose. Higher nitrogen contents were determined for samples first oxidized and then incubated with BSA in combination with EDAC/NHS. The low nitrogen concentrations detected in samples containing only cellulose and buffer can be explained by nitrogen impurities present in the industrial cellulose (Figure 10). Furthermore, the already observed trend that indicates that enzymatic oxidation is less effective than the chemical procedure can be also observed regarding the nitrogen content evaluation. Comparing data presented above shows that a lower oxidation results in less detectable nitrogen after coupling when enzymatic oxidation is performed. For chemical oxidized samples the coupling results clearly indicate the highest amount of bound protein. For enzymatically oxidized samples containing BSA but no coupling regents, BSA got coupled to the fibers only in limited amounts. These higher nitrogen concentrations (if compared to the chemical oxidation blank reactions) can be explained by the fact that laccase, the enzyme used for the oxidation, is a biocatalyst containing nitrogen and the unspecific adsorption/attachment of substances to the cellulosic material cannot be completely excluded. The results show also an increased degree of coupled enzymes when the coupling reagents are present in the system. Due to different surface properties of the inhomogeneous cellulose fibers material, results for protein detection can vary within a sample. For this reason, the detection of decreasing protein concentration during the coupling mechanism is of high interest.

For further proof and to compare processes for sheets and fibers, hydrophobin coupling was performed on oxidized fibers. In this context it is important to note that fibers oxidized and then coupled might have different sheet formation behavior and altered final product properties. These changes must be investigated by extensive papermaking studies. Here after oxidation and coupling of fibers a surface for WCA measurements was formed by drying the fibers after final washing.

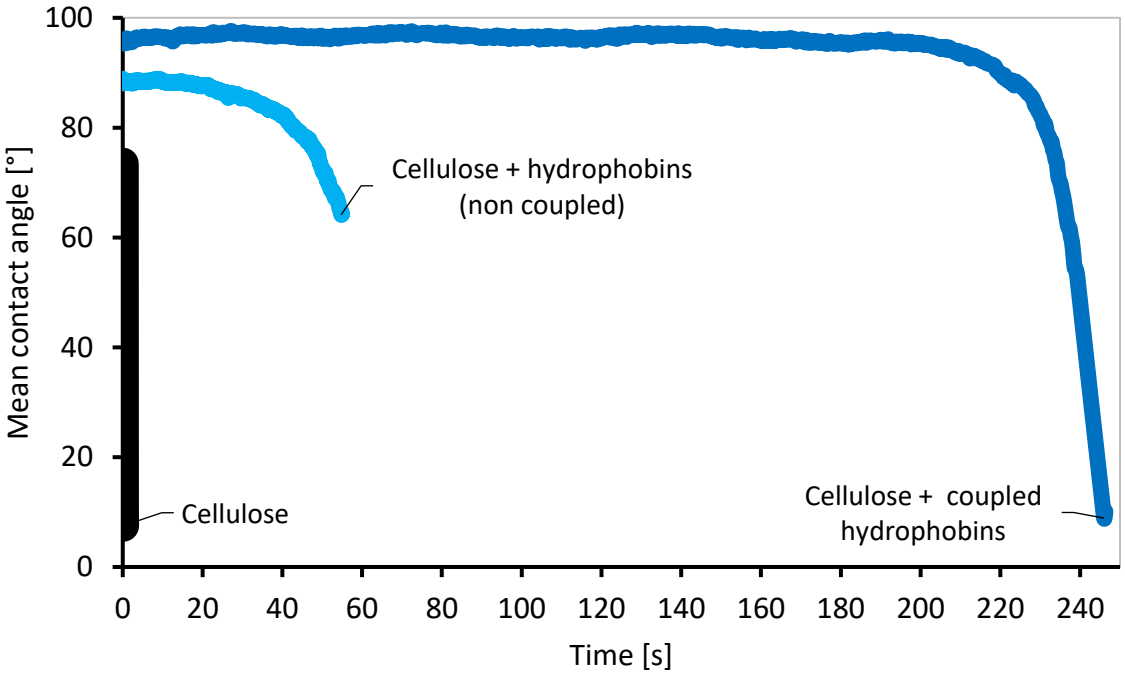

Figure 10. Hydrophobicity of fibers. Contact angle measurements of cellulose surfaces formed from fibers after chemical oxidation and hydrophobins coupling.

The fibers were chemically oxidized, washed and then functionalized similarly to the paper sheets discussed above. Initial contact angles for hydrophobins containing samples were similar for fibers and sheets (Figures 7A, 7B and 10). The limit of the achievable initial contact angle with the used hydrophobins and cellulose materials seems to be around 90°, independently if the coupling occurred on a paper sheet or on the fibers in liquid media. A better accessibility of the cellulose surface, as in the case of fibers, is not dramatically changing the hydrophobic properties of the end material because only the surface behavior is considered. However, coupling to fibers is preferable when compared to paper sheet coupling due to the larger contact zone of free fibers in a solution that improves the masstransfer and therefore the efficiency of the coupling reaction. Additionally, higher soaking times could be observed for functionalized fibers than for surfaces. The soaking time for a lignin free surface was around 70 s (compare Figure 7B), whereas ligninfree fibers had a soaking time of about 250 s. This makes coupled fibers 3.5 times more water repellent than surface sheets, according to the soaking time data, most probably because the bulk portion of the newly generated sheets also contains coupled hydrophobic proteins. For these samples, it did not make any differences if a paper sheet surface or the free fibers were treated. For both setups, the soaking time is around 55 s. The coupling reagents

therefore fulfil the important function of stabilize the water repellent layer of the fibers. Surface formation was not hindered due to hydrophobins coupling on the material. Besides this, the hydrophobic character of the proteins was maintained during the covalent coupling reaction. This makes protein coupling with the EDC/NHS system on oxidized samples a promising tool for the functionalization of other materials. In this context, it has also to be considered that a water repellent surface with hydrophobins can only be achieved if the orientation of the responsible protein domains is equal for all coupled proteins. This means, hydrophobic compartments must be orientated to the surface, whereas the hydrophilic part should face the fiber side. It can be stated that also this natural occurring effect is not influenced by the used coupling reagents.

Different fusion and coupling processes with hydrophobins were described in literature. Schulz et al. described protein fusion of a cytochrome monooxygenase and hydrophobins to increase the coupling efficiency between the monooxygenase and its redox partner [47] and Ribitsch et al. enhanced the ability of cutinase to enhance the degradation of poly(ethylene terephthalate) by fusion with hydrophobins [48]. These applications describe the expression of a fused protein, nevertheless they proof, that it is possible to couple covalently the hydrophobic proteins while maintaining their original properties.

4.5 Conclusion

TEMPO oxidation followed by covalent coupling of proteins to different cellulose based materials can drastically change the water repellent behavior. The presented method for functionalization of cellulose provides several improvements compared to existing approaches and development of new specialized materials for specific applications. Since cellulose is a cheap and common product produced in a million ton range, industrial capacities are available. The production of water replant products is interesting for packaging applications replacing plastic products. Individual characteristics of coupled proteins that cannot be investigated and imitated easily could be promising in the field of security paper products. A high value application could also be the development of flexible high selective medical devices, which nestle to wounds or can be used as surface mimicking tissue. Degradability and harmlessness of cellulose are other important advantages for these kinds of applications. Combination of different protein or enzyme properties on one surface or different surfaces in a layer by layer orientation makes totally new composite materials possible.

4.6 Author contributions

O.H.C., S.W., F.Q and F.B. performed the experiments. O.H.C., C.T. and A.P. planned the experiments and analyzed the data. O.H.C., C. T. and A.P. wrote the manuscript. G.M.G. and A.P. supervised the work. G.M.G. corrected the manuscript. The presented data were discussed by all authors prior to submission.

4.7 Conflict of interest

The authors declare no conflict of interest.

4.8 Acknowledgments

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5

Enzymatic Systems for Cellulose Acetate Degradation

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5.1 Abstract

Cellulose acetate (CA) based materials like cigarette filters contribute to landscape pollution challenging municipal authorities and manufacturers. This study investigates the potential of enzymes to degrade CA and to be potentially incorporated into the respective materials enhancing biodegradation. Deacetylation studies based on LC-MS-TOF, HPLC, and spectrophotometric analysis showed that the tested esterases were able to deacetylate the plasticizer triacetin (glycerol triacetate) and glucose pentaacetate (cellulose acetate model compound). The most effective esterases for deacetylation were of the enzyme family 2 (AXE55,AXE 53, GAE), they deacetylated CA with degree of acetylation of up to 1.8. Combination of esterases and cellulases showed synergistic effects, the absolute glucose recovery for CA 1.8 was increased from 15 to 28%, when an enzymatic deacetylation was performed. Lytic polysaccharide monooxygenase (LPMO), and cellobiohydrolase were able to cleave cellulose acetates with a degree of acetylation of up to 1.4, whereas chitinase showed no activity. In general, the degree of substitution, chain length and acetyl group distribution were found to affect CA degradation. This study shows that for successful enzyme based deacetylation system, a cocktail of enzymes, which will randomly cleave and generate shorter CA fragments, is the most suitable.

5.2 Introduction

Cellulose acetate (CA), produced from high quality cellulose materials obtained from cotton or wood dissolving pulp, is used in many consumer products. As blend material with different water soluble and insoluble polymers like poly(-lactic acid) or starch, CA can be found in several plastics and coating materials [1], [2] which, if handled improperly, can have a severe environmental impact. Other sources of CA pollution are cigarette filters and textiles, which end up as litter, causing multiple problems in the environment [3], [4]. This is particularly true for cigarette filters, which are inappropriately disposed by smokers. An investigation in the USA showed that 25-50% of all litter items collected on streets and roadways were cigarette butts [5]. Thus, developing biodegradable cigarette filters may help to overcome pollution and reduce effort in litter cleanup activities. For some regions these litter issues already influenced legislation: in 2012 the state of New York took a law into consideration, that will promote sales of biodegradable cigarette filters [6].

CA based filters are produced by chemical acetylation of cellulose (95% α-cellulose), achieved in a two-step process using an excess of acetic anhydride in the presence of sulfuric acid or perchloric acid as catalysts. This first step is followed by a partial deacetylation, which enables the control of acetylation across the molecule.

For CA degradation, several natural mechanisms are known. Microbial caused reduction in degree of substitution and photo degradation of CA products like fibers and films were described before. Depending on multiple factors like product configuration (film or fiber), DS and light exposure degradation times varies from days to weeks [7], [8]. Here it might be useful to introduce an enzymatic systems supporting the natural degradation of CA materials. Thereby strategies of incooperartion of enzymes into fibrous materials of CA [9] and cellulose [10] are known. An important aspect in enzymatic degradation is to monitor the influences of different substrate properties on the enzymatic degradation. CA can be characterized according to its chain length, which is described by the degree of polymerization (amount of acetylated glucose subunits in a chain) and the number of acetyl groups per monomer, which is given as degree of substitution (DS). A DS of 1.9 means that on ten glucose subunits 19 of 30 potential positions are acetylated. An overview about all used substrates is given Figure 1.

Cigarette filters for example have a degree of acetylation of 2.5 [3], [4]. This high degree of acetylation makes tobacco CA filters difficult to degrade by microorganisms [11]. In addition, high molecular weight, crystallinity, and the physical form influence the microbial degradation efficacy [12], [13]. However, the degradation ability of highly acetylated CA is still under investigation in the scientific community [4]. Saki et al. reported the ability of Neisseria sicca to produce extracellular enzymes able to degrade CA with a 2.3 degree of acetylation. The authors suggested a cooperative system of esterases deacetylating CA, followed by cellulases breaking the backbone into smaller fragments, which can be quickly taken up by the cells [13]. An approach using single enzymes or a combination of biocatalysts may lead to an industrial application for planned litter depletion. For cellulases, a decrease in degradation for acetylated pulps was reported compared to non-acetylated pulps [14]. Data published by Olaru et al. show a reduced action of cellulases, based on viscometry of CA, with increasing degree of substitution (DS) [15]. Apart from mechanistic insights, identification of more efficient CA degrading enzyme systems could also allow the incorporation in CA materials enhancing biodegradation upon contact with water. Thereby it could be sufficient to incooperate one of the functions into the material whereby the second step can be carried out by the natural biome in the surrounding of the litter item.

However, only a few representatives of esterases have been assessed so far for CA deacetylation. While the effect of oxidoreductases like lytic polysaccharide monooxygenase (LPMO), has not yet been investigated on CA as a substrate. Therefore, in this study CA cleavage was systematically investigated using a variety of enzymes belonging to different classes. All enzymes were tested on different substrates displaying different properties of CA materials.

In this study we evaluate the eligibility of different enzymes from different classes, which are involved either in the deacetylation of CA or in backbone cleavage of the polymer. Enzymes were tested extensively on different substrates (Figure 1) and in combination to enhance performance on CA.

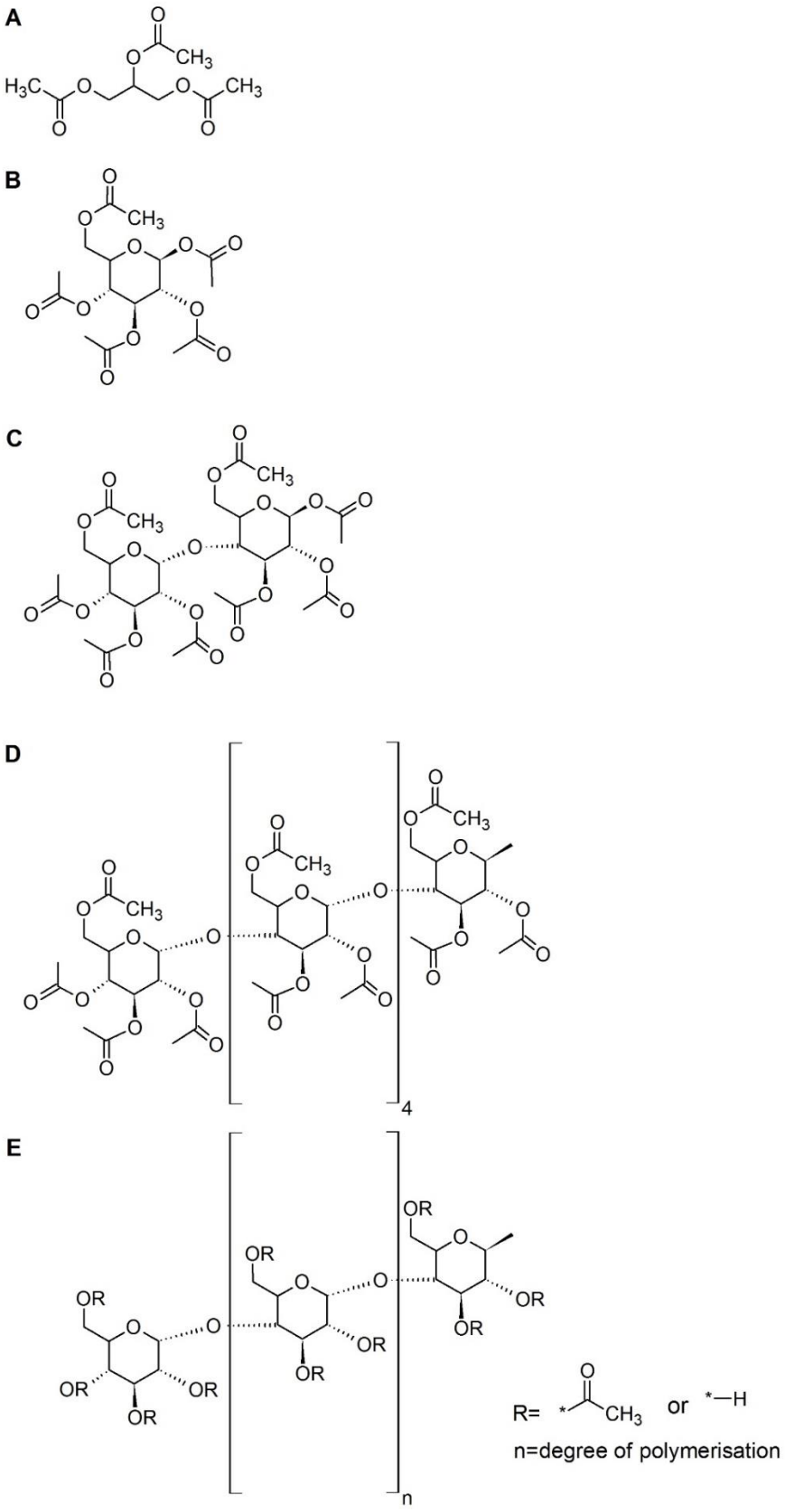

Figure 1. Substrates for enzymatic CA degradation. Triacetin (A), Glucose Pentaacetate (B), Cellobiose octaacetate (C), Hellohexose eicosacetate (D) and CA (E). For CA: R refers for the degree of substitution and n+2 is the degree of polymerization.

5.3 Materials and Methods

5.3.1 Enzymes, substrates and other chemicals

Enzymatic deacetylation and CA backbone cleavage was investigated using different model substrates like triacetin, glucose pentaacetate, cellobiose octaacetate, cellohexaose eicosaacetate, CA with different DS [0.9, 1.4, 1.8, 2.3 and 2.5] and CA cigarette filters were from R. J. Reynolds Tobacco Company (Winston-Salem, United States) with a DS of 2.5. CA model compounds with different DS (0.9, 1.4, 1.8, 2.3 and 2.5) and chain length were prepared by acid deacetylation of cellulose triacetate as previously described [16]. All other substrates were purchased by Sigma-Aldrich (Vienna, Austria) and used as received if not otherwise specified. Esterases were obtained from Nzytech (Lisbon, Portugal) (GAE, PAE), Megazyme (Wicklow, Ireland) (AXE O) and Prozomix (Haltwhistle, United Kingdom) and used without further purification, whereas cutinase from Thermobifida cellulosilytica was produced and purified as previously described [17]. Additionally, chemical hydrolysis with 0.1 M NaOH was carried out for 24 h using 100 mg of each substrate. Table 1 shows all used esterases for the screening, including optimal reaction conditions and enzyme family.

Table 1. Esterases and their optimal reaction conditions investigated for deacetylation of oligomeric model substrates and of CA. Family assignments correspond to the classification of carbohydrate esterases according to the Carbohydrate Active Enzymes database (www.cazy.org).

Enzyme	Origin	pH	Temp. [°C]	Family
Acetyl xylan esterase (AXE O)	*Orpinomyces sp.*	6.7 - 7	40	6
Acetyl xylan esterase (AXE 34)	*Clostridium thermocellum*	7	50	3
Acetyl xylan esterase (AXE 35)	*Clostridium thermocellum*	6.5	50	4
Acetyl xylan esterase (AXE 36)	*Clostridium thermocellum*	6.5	50	4
Acetyl xylan esterase (AXE 53)	*Cellvibrio japonicus*	8.5	25	2
Acetyl xylan esterase (AXE 55)	*Cellvibrio japonicus*	8.5	25	2
Carboxylesterase (CE 265)	*Bacillus subtilis*	7	37	N.A.
Cutinase (CUT)	*Thermobifida cellulosilytica*	7	50	N.A.
Glucomannan acetyl esterase (GAE)	*Clostridium thermocellum*	7	50	2
Pectin acetyl esterase (PAE)	*Clostridium thermocellum*	6.5	50	12

Cellulases used for combinatorial experiments with esterases were purchased from Megazyme, Ireland (E-CELAN, E-CELTR, E-CELTE, E-CELBA), Nzytech, Portugal (Cellulase 8A, Cellulase 5A) and Novozymes, Denmark (Cellic C Tec3). Table 2 shows all used cellulases for the screening, including optimal reaction conditions and enzyme family.

Table 2. Cellulases used for degradation of deacetylated cellulose acetate with their optimal conditions and origin. Family classification follows the Carbohydrate-Active Enzymes Database.

Enzyme	Origin	pH	Temp. [°C]	Family
Cellulase E-CELAN	*Aspergillus niger*	4.5	55	GH12
Cellulase E-CELTR	*Trichoderma longibrachiatum*	4.5	70	GH7
Cellulase E-CELTE	*Talaromyces emersonii.*	4.5	70	GH5
Cellulase E-CELBA	*Bacillus amyloliquefaciens*	6.5	55	GH5
Cellulase 8A	*Escherichia coli*	7	40	GH8
Cellulase 5A	*Bacillus subtilis*	7.5	55	GH5
Cellulase Cellic C Tec3	*N.A.*	5	55	N.A.

Chitinase and Cellobiohydrolase were supplied by Sigma-Aldrich (Vienna, Austria) and Nzytech (Lisbon, Portugal), respectively and were used without further purification. LPMO from Neurospora crassa PMO 2916 [18] was used for direct CA backbone cleavage.

5.3.2 Deacetylation of CA and acetylated oligomers

For the investigation of enzymatic deacetylation, 100 mg of each substrate were used, except for CA with a DS of 0.9, 1.4 and 1.8 of which only 50 mg were weighted. The enzymatic hydrolysis was carried out in 0.1 M sodium phosphate buffer using the optimum pH for each enzyme, using 20 (with 50 mg substrate) or 50 ml (with 100 mg substrate) as reaction volume. The dosage of each enzyme was 0.1 U mg^{-1} substrate (with 1 Unit defined as the µmol of substrate converted in 1 min by the enzyme). The samples were incubated at their respective optimum temperature and samples were taken at certain time points.

5.3.3 Combined deacetylation and hydrolysis of CA

For deacetylation, CA was pretreated with the best performing esterase, which was AXE 55. CA concentration was 7.54 mg ml^{-1} in 20 mM Tris-HCl buffer at pH 8.5 for the esterase treatment. Enzyme concentration for the pretreatment was 34 µg m^{-1}. Blanks were performed without esterase. All samples were incubated for 165 h. After the pretreatment, the pH was changed for all cellulase treatments. Therefore, 100 mM buffer with the right pH was added. Change of pH to 4.5 and 5 was done by the addition of citrate buffer, pH 6.5 and 7.5 were obtained using sodium phosphate buffer. Final CA concentration was 3.77 mg ml^{-1}. For the different cellulases, the protein content was determined and between 0.01 and 1 mg enzyme was added. All samples were incubated for 168 h at the optimal temperature.

5.3.4 Direct enzymatic hydrolysis of CA

For all experiments, 5 mg of the different substrates were used. Conversion of CA by LPMO was carried out in 975 µl sodium phosphate buffer (50 mM, pH6) with 20 µl of 10 mM gallic acid and 5 µl enzyme, (with an initial concentration of 60 mg ml^{-1}). Samples were incubated at 25 °C and 900 rpm in the dark. To ensure appropriate oxygen supply, samples were covered with an O_2 permeable foil. Experiments containing chitinase (EC 3.2.1.14) and cellobiohydrolase (EC 3.2.1.91) were performed in a 1.5 ml total reaction volume containing buffer (50 mM citrate) and 10 µl of the enzyme solution. Optimal pH values were 6.5 for chitinase and 5.0 for cellobiohydrolase. 10 µl of the enzyme were used (corresponding to 250 U for chitinase and 13 U for cellobiohydrolase). Samples were incubated for 145 h and 200 rpm at the temperature optimum (cellobiohydrolase: 50 °C, chitinase 60 °C).

5.3.5 Monitoring deacetylation of CA and oligomers

Deacetylation was monitored using a high performance liquid chromatography (HPLC) system equipped with a transgenomic ION-300 column (New Haven, United States). 0.01 N H_2SO_4 was used as mobile phase with a flow rate of 0.325 ml min^{-1} at 45 °C. 40 µl of the desired solution were injected at a runtime of 60 min. The method was calibrated using acetic acid standards within a 10-1,000 mg l^{-1} range. In order to render the deacetylation results for substrates with different degree of substitution comparable, all values were converted into percentage values.

5.3.6 Monitoring enzymatic cleavage of CA

Backbone cleavage resulting in the formation of reducing sugars was monitored using the DNS method as previously reported by Ghose [19] with slight modifications. Furthermore, released CA fragments were analyzed by liquid chromatography electronspray ionization time-of-flight mass spectroscopy (LC-ESI-TOF MS), injecting 30 µl samples, using a Poroshell 120 EC-C18 (Agilent) column at 40°C with a flow rate of 0.4 ml min^{-1}. The system was operated with a linear gradient of 100% water containing 0.1% formic acid to 100% acetonitrile with 0.1% formic acid in 45 min. For mass spectrometry, a Dual ESI G6230B TOF (Agilent) was used. The sample was ionized with a nebulizer at 40 psigin and positive ion mode with a gas temperature of 200 °C at 8 l min^{-1} gas flow. The fragmentor voltage in the system was set to 200 V and the skimmer voltage was 65 V for mass correction the masses 121.0509 m/z and 922.0098 m/z. Mass range for the analysis was from 50 to 3000 m/z. The acquired data were analyzed with the Agilent MassHunter software (Version B07.00).

Samples treated with a combination of esterases and cellulases were analyzed by HPLC monitoring the glucose release for pretreated and non-pretreated samples. 40 µl sample were injected and mobile phase was 0.01 N sulfuric acid with a flow rate of 0.325 ml min^{-1}. Column temperature was 45 °C using a transgenomic ION-300 column (New Haven, United States). Signal recording was achieved by refractive index measurement using an Agilent 1260 Infinity II detector. The method was calibrated using glucose standards between 10 and 1,000 mg l^{-1}.

5.4 Results and discussion

5.4.1 Deacetylation

In a first step, deacetylation of glucose pentaacetate (GPA) and triacetin (TA) by different esterases was investigated. Hydrolysis of triacetin was studied since this compound is used as plasticizer in many CA materials while hydrolysis may have an impact on biodegradation of CA [20]. Although several enzymes were able to completely deacetylate both compounds, there were significant differences between the individual enzymes (Figure 2). Major differences were seen for Acetyl Xylan Esterases (AXE 35) and (AXE O), which deacetylated GPA to 80% and 90%, respectively, but had only minor effects on triacetin. Classified as AXE, these enzymes clearly prefer sugar-bound esters compared to aliphatic esters like in triacetin. On the other hand, the cutinase (CUT) showed similar activities on both substrates and was previously described to cleave ester bonds in hydrophobic aromatic and aliphatic polyesters [21]. Moreover, different applications of cutinase are known, e.g. a wild type cutinase was reported to esterify the hydroxyl groups of cellulose [22] . This makes cutinase a promising enzyme in cellulose acetate treatment.

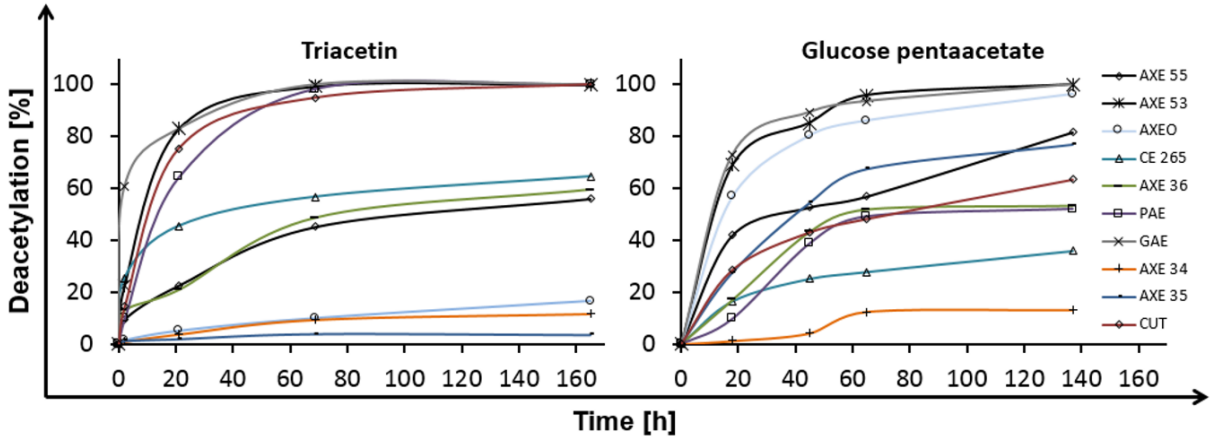

Figure 2. Deacetylation of glucose pentaacetate and triacetin by different esterases. Triacetin is a plasticizer in cigarette filters and glucose pentaacetate serves as small model compound for CA. Axetyl Xylan Estarase (AXE), Carbohydrate Esterase (CE), Cutinase (CUT), Glucomannan Acetyl Esterase (GAE), Pectin Acetyl Esterase (PAE).

AXE 53 and Glucomannan Acetyl Esterase (GAE) showed high capacity to deacetylate both substrates. Especially for GAE, 60% of triacetin were deacetylated in 2 min. For AXEs deacetylation of different model compounds, representing acetylated polysaccharides and non-polysaccharide compounds, was reported before for chitosan, chitin [23] and cephalosporin-C [24].

On the other hand, AXE 34 only weakly deacetylated both substrates. Altaner et al. found evidences for region-selectivity of esterases derived from different families. They claim that enzymes of the carbohydrate esterase (CE) family 1 exclusively deacetylated CA in the C2- and C3-carbon positions, without cleaving C6 in the sugar. Furthermore, they postulate differences in cleavability for different substitution positions on the polymer chain [25]. Complete deacetylation was observed for Pectin Acetyl Esterase PAE, GAE, AXE O and AXE 53. For some enzymes, e.g. AXE 34 only one acetyl group of GPA was attacked. For triacetin, the degrees of deacetylation in Figure 2 are arranged into three groups corresponding to the three acetyl groups of the molecule. This indicates cleavage of only one ester bond for AXE O, AXE 34 and AXE 35. Two bonds were cleaved for CE 265, CUT and AXE 36. All other enzymes deacetylated triacetin completely. An influence of the enzyme family for this behavior is not visible. Apart from GPA, enzymatic hydrolysis of acetylated oligomers, namely cellobiose octaacetate and cellohexose eicosaacetate was investigated. Within 68 h only GAE (45%) AXE 55 (14%) and CUT 1 (13.5%) showed significant deacetylation of cellohexaose eicosaacetate. All other tested enzymes reached deacetylation degrees lower than 4%. Also for GPA an influence of the enzyme family cannot be stated.

In nature, glucomannan acetyl esterase is part of the wood degradation process. O-Acetylgalactoglucomannans (AcGGM) are the principal hemicellulose components in softwoods. They are mainly water insoluble, but their acetylation pattern is influencing their behavior in water [26]. The structure of AcGGM is a linear backbone of (1→4)-linked β-d-mannopyranosyl and (1→4)-linked β-d-glucopyranosyl units, with (1→6)-linked α-d-galactopyranosyl units. Naturally, the mannose subunits can be acetylated at C-2 and C-3, however via chemical acetylation also galactose and glucose subunits were acetylated [27]. It was shown that linear oligosaccharides from AcGGM could be obtained when an acetyl mannan esterases and a α-galactosidases were used in combination [26]. It was also suggested that galactomannan deacetylation is an inherent property of some AXEs [11]. Due to catalytic similarities between acetyl xylan esterases and glucomannan esterases the latter enzyme is of interest for degradation processes of acetylcellulose.

In a next step, enzymatic hydrolysis of CA with different degrees of acetylation was investigated (Figure 3). In general, the activity of the enzymes increased with decreasing degree of acetylation confirming the “protective” function of acetyl groups as reported before [11]. Only for AXE 55, AXE 53 and GAE deacetylation was weaker for CA-DS 0.9 (degree of substitution) than for CA-DS 1.4, which belongs to the same family (Table 3). Poutanen et al. reported the behavior of acetyl xylan esterases on their natural substrates. AXE prefers polymeric substrates, whereas acetyl esterase showed high affinity on acetyl xylobiose. Out of this, they claim a high deacetylation specificity, which depends on the specific position of the acetyl groups and not on the degree of polymerization. Using acetyl xylan esterase in combination with other enzymes they obtained a complete degradation of polymeric substrates to acetic acid and xylose using xylanase and β-xylosidase [28]. Regioselectivity claimed for AXE´s natural model substrates explains the non-complete deacetylation of CA materials. Due to the higher number of acetyl groups per monomer, regioselectivity plays a more important role for CAs with a high degree of substitution. The probability to find a critical position blocked is, therefore, larger for highly acetylated substrates.

Table 3. Esterase screening for several esterases substrates. Very strong deacetylation (++++), strong deacetylation (+++), moderate deacetylation (++), weak deacetylation (+), no deacetylation (-). Axetyl Xylan Estarase (AXE), Carbohydrate Esterase (CE), Cutinase (CUT), Glucomannan Acetyl Esterase (GAE), Pectin Acetyl Esterase (PAE).

Esterase	Triacetin	Glucose pentaacetate	Cellobiose octaacetate	Cellohexaose eicosaacetate	CA-DS 0.9	CA-DS 1.4	CA-DS 1.8	CA-DS 2.3	CA-DS 2.5
AXE O	+++	+++	+	+	+	-	-	-	-
AXE 34	+	+	+	+	++	++	+	-	-
AXE 35	+	++	+	-	++	++	+	-	-
AXE 36	+	++	+	+	++	++	+	-	-
AXE 53	++++	++++	+	+	+++	+++	++	-	-
AXE 55	+++	+++	+	+	+++	++++	+	-	-
CE 265	+	+	-	-	-	-	-	-	-
CUT	++++	++++	+	+	+++	+++	+	-	-
GAE	++++	++++	+++	++	+++	+++	++	-	-
PAE	++++	+++	+++	++	+++	+++	++	-	-

Interestingly, most of the enzymes not belonging to family II, showed almost slightly lower activity on CA-DS 1.4 compared to CA-DS 0.9 while the activity dramatically decreased for CA-DS 1.8. As mentioned before, family II enzymes showed regioselectivity for deacetylation. These three enzymes (AXE 55, AXE 53, GAE) had the highest deacetylation activity. A possible explanation might be given by the distribution of the acetyl groups across the polymer. Hence, for enzymes, cellulose acetates with different degrees of substitutions pose to be substrates with different properties. Based on the conserved motive, AXE 55, AXE 53, and GAE are representatives of the so-called GDSL-family or family II, all esterases within lipolytic enzymes can be classified into the thirteen families [29]. This family shares five highly conserved homology blocks, which are important for their classification. GDSL hydrolases have a flexible active site and they can change conformation in the presence of different substrates [30]. This flexibility might be an explanation for their good deacetylation efficiency over a broad range of substrates. Enzymes of this family were also reported to show broad regioselectivity, which probably makes them suitable for degradation of CAs with a heterogeneous acetylation pattern [31].

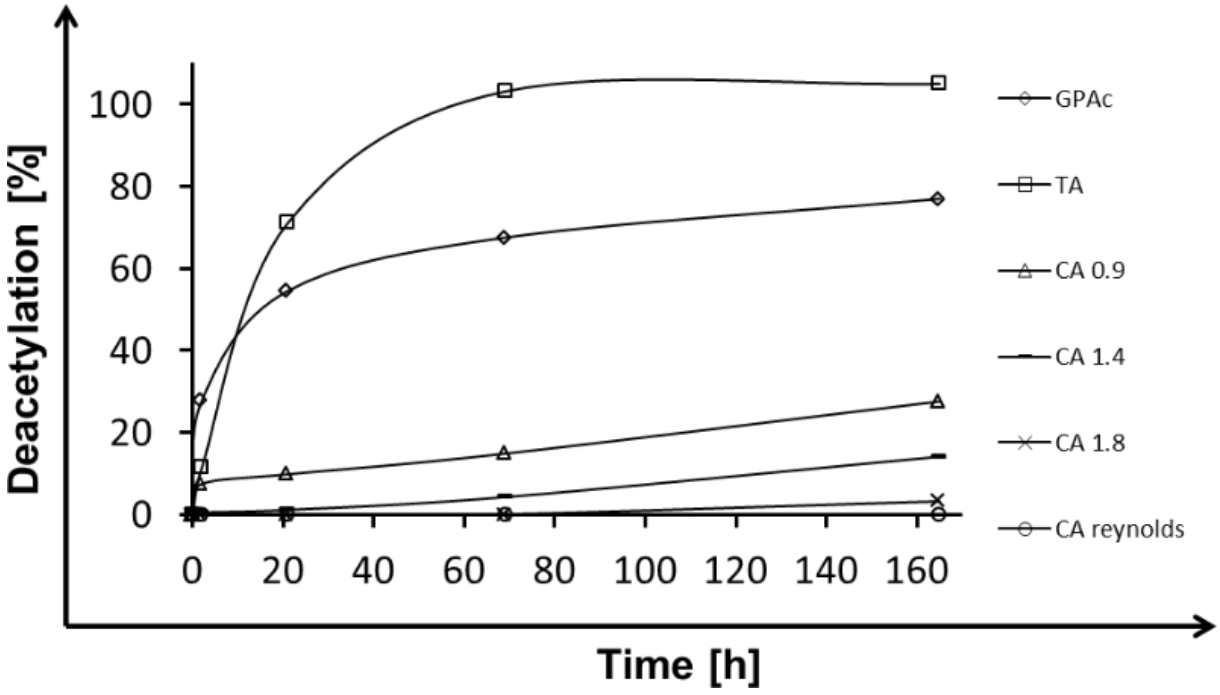

Figure 3. Deacetylation of cellulose acetate with different degree of substitution (DS) and model compounds by Pectin acetyl esterase (PAE) after 168 h of incubation. Glucose pentaacetate (GPA), Triacetin (TA).

Despite investigations for triacetin and glucose pentaacetate (Figure 2), for PAE deacetylation was examined over time by using different cellulose acetate model compounds varying in degree of substitution (CA-DS 0.9, CA-DS 1.4, CA-DS 1.8) and real cigarette filter material (R. J. Reynolds Tobacco Company, United States). Figure 4 compares deacetylation efficiencies for cellulose acetates over time. For the used model compounds, deacetylation was decreasing from 20% for low acetylated substrate (CA 0.9) to 5% for highly acetylated material (CA 1.8) after 165 h of incubation. Real filter material with a degree of substitution of 2.5 revealed high resistance against enzymatic degradation with PAE, resulting in no detectable release of acetic acid. As shown in Figure 2 for glucose pentaacetate and triacetin, PAE exhibited high and medium ability to degraded small acetylated substrates. Whereas larger substrates, like different cellulose acetates (Figure 4), were less deacetylated even when the DS was lower. For the substrates reported in this study, the polymer size was influencing the deacetylation efficiency of PAE on a great extent. Highest deacetylation efficiency was visible for CA-DS 0.9 within the first 2 h. This can be explained by auto-degradation of the polymer in combination with the enzyme action. For all other conditions, deacetylation was linear for the whole reaction time course.

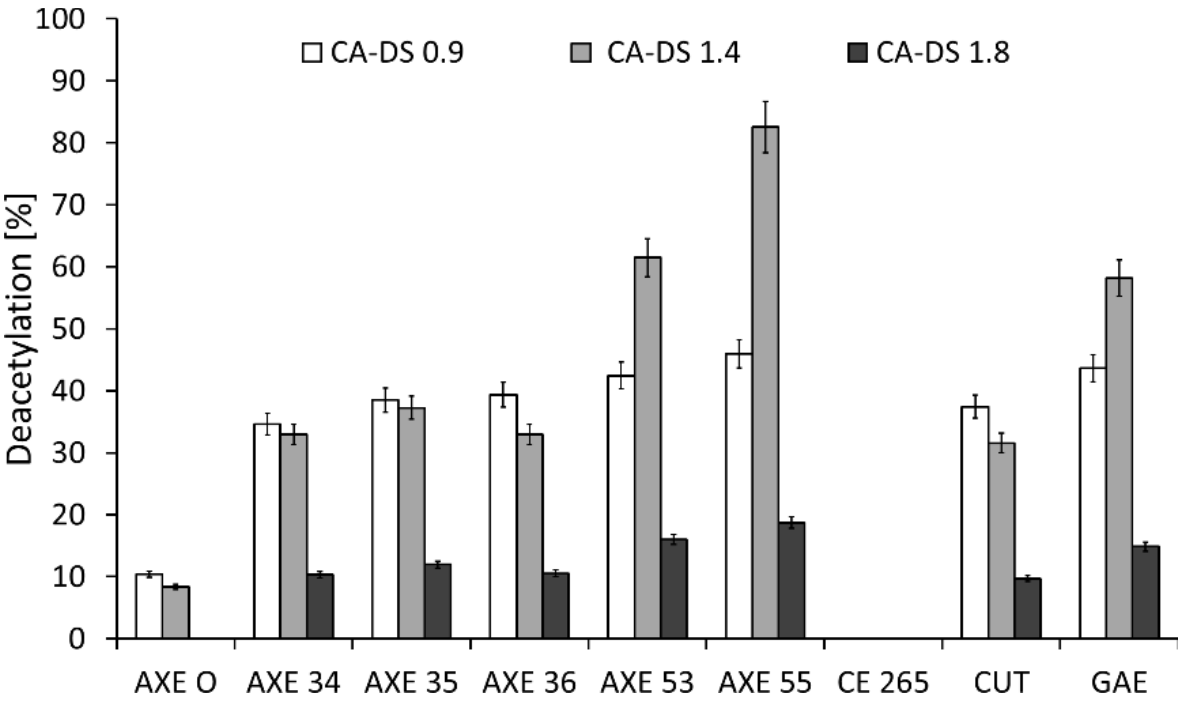

Figure 4. Deacetylation of different cellulose acetates by esterases. Cellulose acetate with 0.9 acetyl groups per monomer (CA-DS 0.9), cellulose acetate with 1.4 acetyl groups per monomer (CA-DS 1.4) and cellulose acetate with 1.8 acetyl groups per monomer (CA-DS 1.8). Axetyl Xylan Estarase (AXE), Carbohydrate Esterase (CE), Cutinase (CUT), Glucomannan Acetyl Esterase (GAE), Pectin Acetyl Esterase (PAE).

The results of enzymatic deacetylation of a variety of substrates with different degrees of acetylation is summarized in Table 4. None of the enzymes was able to deacetylate CA with a degree of substitution higher than 1.8. The most promising enzymes for the degradation of large and highly substituted polymers were of family II (AXE 55, AXE 53, GAE). Small and highly acetylated molecules such as cellobiose octaacetate and cellohexose eicosaacetate were less deacetylated than bigger molecules with less acetyl groups per monomer. An increase in number of glucose subunits from one (glucose pentaacetate) to six (cellohexaose eicosaacetate) was strongly decreasing deacetylation efficiency for all enzymes except for PAE and GAE (compare Figure 4 and Table 4). For example, cellohexaose eicosaacetate was less deacetylated than glucose pentaacetate with a higher degree of substitution. Hence, it seems that for shorter molecules the degree of acetylation has a lower impact on deacetylation efficiency than the chain length.

Table 4. Glucose recovery with Cellic C Tec 3 after esterase pretreatment and without pretreatment of CA with different degrees of substitution. Cellulose acetate with a degree of substitution of 0.9 (CA-DS 0.9). The degree of substitution refers to the number of acetyl groups per glucose unit in the molecule.

Substrate	% Glucose [w/w]	% Acetic acid [w/w]	Glucose recovery pretreated [%]	Glucose recovery not pretreated [%]
CA-DS 0.9	80.6	19.4	53.8	46.5
CA-DS 1.4	72.7	27.3	47.8	34.9
CA-DS 1.8	67.4	32.6	27.6	15.3

5.4.2 Combined CA degradation with esterases and cellulases

To achieve full cellulose acetate degradation, the ability of different cellulases to hydrolyze esterase pretreated CA was investigated. The synergistic mechanism of esterases and cellulases has previously been reported for cellulose acetate degradation in microbial systems [13]. Moriyoshi et al. isolated different enzymes out of *Neisseria sicca* performing a synergistic reaction of deacetylation and degradation [32]. Figure 5 shows the differences in glucose release for a wide spectrum of cellulases preparations applied on pretreated and not-pretreated cellulose acetates with different degrees of substitution (DS). Only, cellulase 8A and E-CELBA did not release glucose. For all cellulases, the glucose release increased upon prior deacetylation with esterases. The best working enzyme approach for pretreated and

non-pretreated samples was Cellic C Tec 3. Multiple enzyme activities like LPMO, endoglucanases, exoglucanases, and cellobiohydrolases are involved in the cellulose degradation in nature and hence also contained in commercial cellulase preparations [33], [34]. This makes a broad screening of enzymes for a possible application necessary. The most pronounced difference between pretreatment and no pretreatment was seen for CA-DS 1.8. Here, deacetylation increased the glucose liberation by almost 50% confirming the "protective" effect of acetyl groups towards enzymatic hydrolysis of cellulosic materials. Irrespective of the pretreatment, the glucose release decreased with increasing acetylation for all enzymes (Table 4).

The glucose recovery halved when the degree of acetylation increases from 0.9 to 1.8. Table 4 indicates that the protective function of acetyl groups is not linear with the degree of substitution. Increasing the acetyl content from 0.9 to 1.4 had fewer effects than an increment from 1.4 to 1.8.

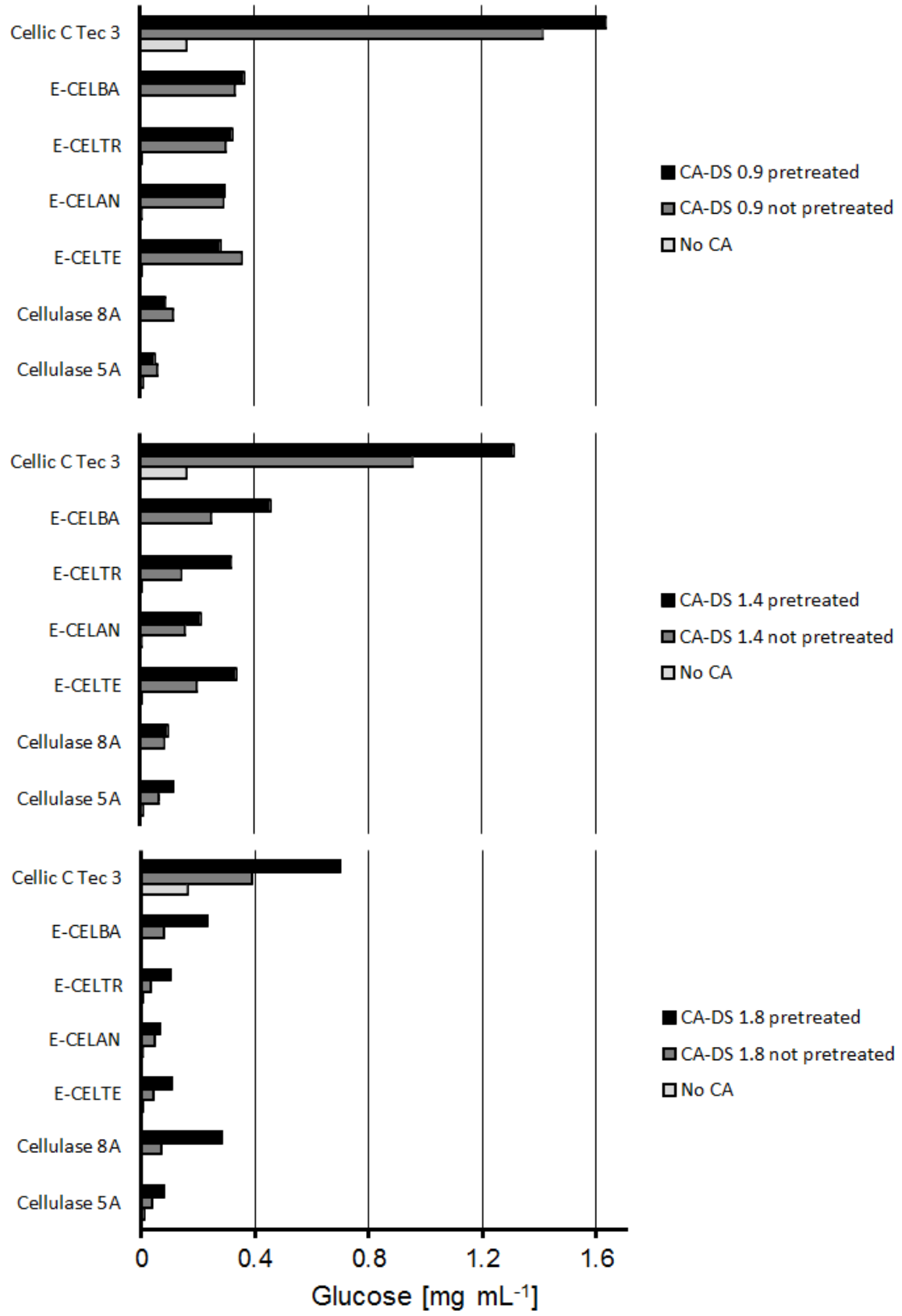

Figure 5. HPLC results of synergistic cellulose acetate degradation by cellulases. Substrates were pretreated with esterase AXE 55. Samples without cellulose acetate were performed to estimate glucose content of the used enzyme formulations.

5.4.3 Lytic polysaccharide monooxygenase (LPMO) hydrolysis of CA

Since the degree of enzymatic deacetylation decreased with increasing polymer chain length, it was speculated that cleavage of the polymer into smaller fragments prior to deactylation increases CA degradation. Lytic Polysaccharide Monooxygenases (LPMOs) are known to attack cellulose by an oxidative mechanism, which cleaves the glycoside bond at either the C1 or the C4 [33]. An interesting feature of LPMO is its flat substrate binding surface, which fits onto the cellulose surfaces and brings the active-site copper with an activated oxygen species into close contact with the species [34]. This enzyme can theoretically bind at any position to cellulose to perform cleavage. Some LPMOs like LPMO-02916 (also known as LPMO 9C) from *Neurospora crassa* can also act on the less structured substrates hemicellulose and cellooligosaccharides [35], [36], which suggests that LPMO-02916 might also be suitable to act on CA. In experiments where LPMO-02916 was incubated with a reductant and cellulose (PASC, Avicel, or steam explodes spruce), fragments between 2–5 monomers were detected [35]. CA with a DS of 0.9 and 1.4 was incubated separately with LPMO-02916 and cleavage fragments were analyzed. For CA with a DS of 0.9 after incubation with LPMO no fragments larger than five monomers and more than one acetyl group were detected. For CA 0.9, the number of different fragments was decreasing for increasing amount of acetyl groups. LPMO liberated fragments for both CA with DS 0.9 and 1.4 (Figure 6.), while no fragments were detected for CA with higher DS. Preferentially fragments with a low DS were released. This indicates that acetyl groups interfere with LPMO's substrate binding site or disturb the catalytic reaction. It also indicates that CA may not be uniformly acetylated, allowing the LPMO to cleave in those regions with a lower DS. In this context synergies of LPMOs in combination with other cellulose degrading enzymes were observed and are worth of further investigations [19]. Cleavage of CA with DS 0.9 and 1.4 by LPMO was investigated by analyzing the released fragments (Figure 6).

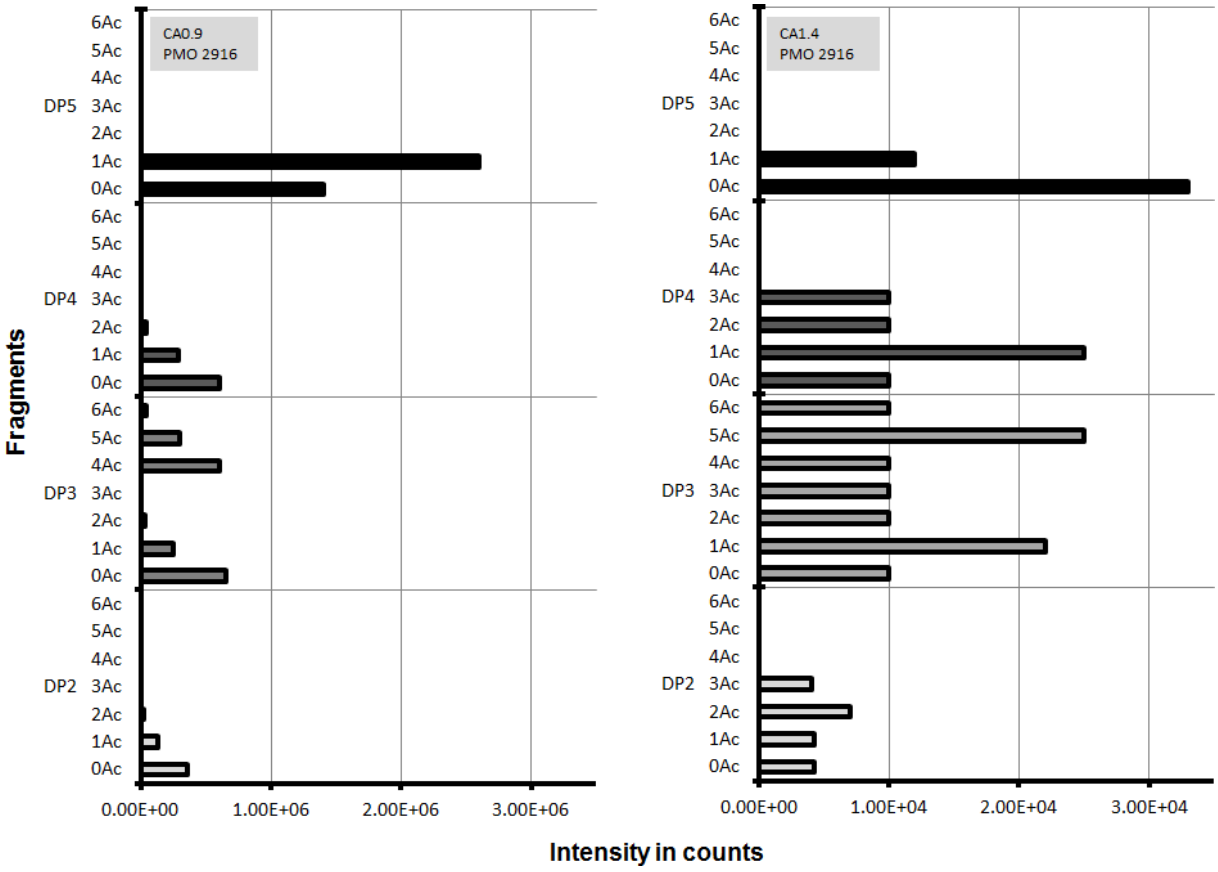

Figure 6. Hydrolysis of cellulose acetates with different degree of acetylation (CA0.9, CA1.4) by lytic polysaccharide monooxygenases (LPMO 2916) monitored using LC-TOF-MS. Degree of polymerization (DP), number of acetyl groups per fragment (Ac).

Figure 7 shows backbone cleavage by cellobiohydrolase I and chitinase after 145 h of reaction. Cleavage activity was monitored photometrically by detection of the reducing sugar ends using the Dinitrosalicylic acid (DNS) method [36]. For cellobiohydrolase I, the amount of reducing ends is increased by factor 3 for low substituted material (CA-DS 0.9). Minor increases are visible for medium substituted substrates (DS 1.4). No cleavage activity was measured for highly substituted cellulose acetate (CA-DS 1.8). As visible for the deacetylation with esterases (Figure 3) decrease in activity, for highly acetylated substrates, is also not linear for glycosidic acting enzymes in backbone cleavage, with the here used substrates. Cellobiohydrolase I is an enzyme with broad product specificity, reported to bind only the hydrophobic parts of the cellulose crystal structure [37]. Ike et al. reported chitinase activity for cellobiohydrolase I [38] and Textor et al. mentioned cleavage of carboxymethyl cellulose by the enzyme. This, presence of a cellulose binding module (CBM) and low end-product inhibition promise applicability of cellobiohydrolase I in industrial degradation processes [39]. Ability of cellobiohydrolases to decrease the degree of

polymerization was reported before by Saake et al. for low and medium acetylated substrates, based on size exclusion chromatography of acetylated cellulose [40].

Due to the chemical similarities of the polymers chitin and cellulose acetate, chitinase was tested on its ability to cleave glycosidic bonds in different cellulose acetates. Chitinase only showed small changes for CA-DS 0.9 and no shift in absorbance for other substrates. The N-acetamide group seems to be essential for the ability of chitinase to detect glycosidic bonds. Binding ability for a chitinase on chitin and cellulose was determined to be equal for both polymers [41]. Effects of reduced binding affinity to substrates with at least one acetyl group per monomer might be the reason for reduced backbone cleavage of cellobiohydrolase for CA 1.4 and CA 1.8.

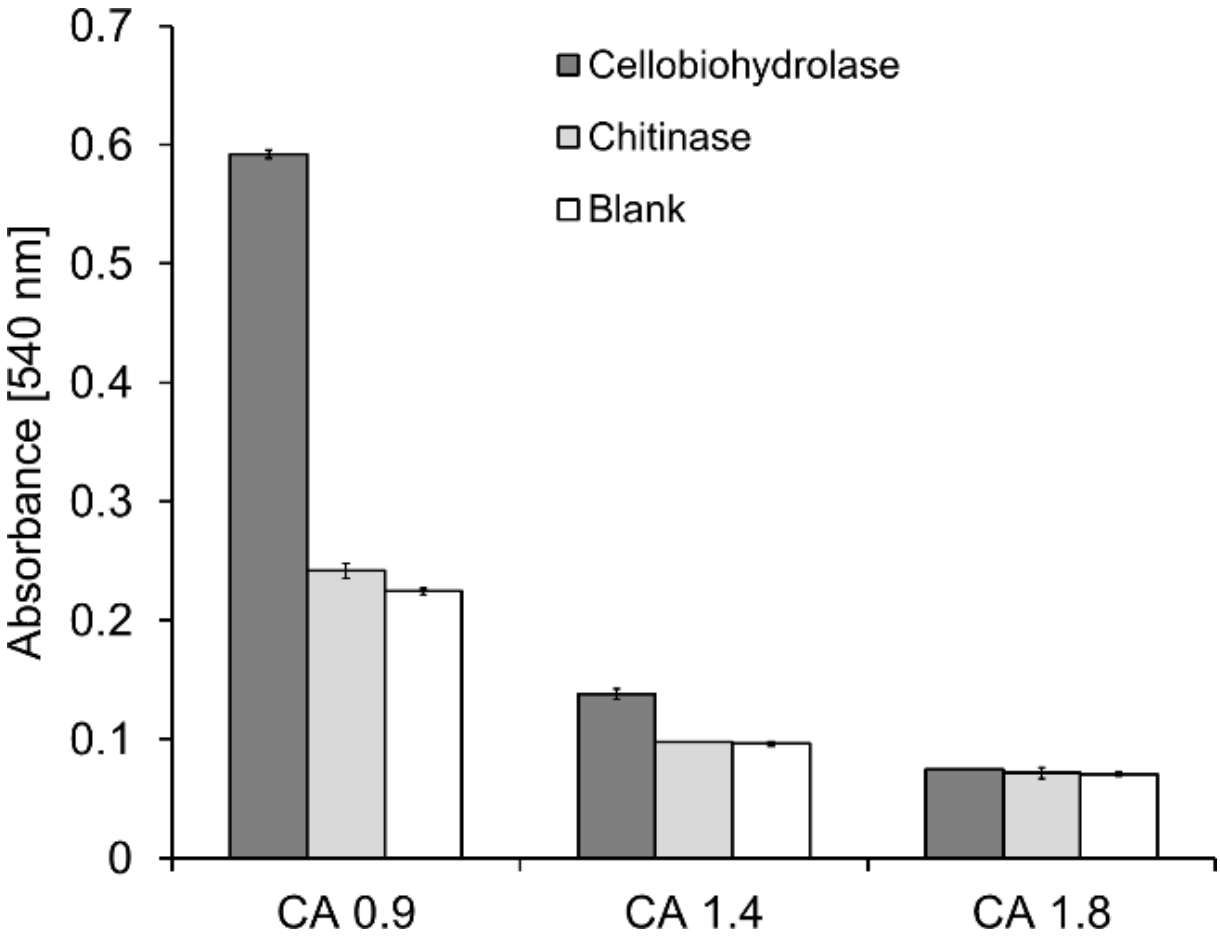

Figure 7. Backbone cleavage of cellulose acetates with chitinase and cellobiohydrolase I after 145 h.

5.5 Conclusions

Here we show an extensive screening of many representatives of different classes of enzymes, which are all involved in the cellulase acetate degradation, either on the acetyl group of the polymer or on the backbone of the chain. Their behavior of different substrates was investigated. To our knowledge the synergistic performance of esterases and cellulases in combination, outside of a microbial system was described for the first time. Additionally we introduced a new enzyme which is called Lytic Polysscharide Monooxygenases to the collection of promising biocatalists for CA degradation.

Most of the investigated esterases were able to completely deacetylate fully acetylated glucose pentacetate and triacetin. However, the ability of the deacetylation by esterases decreased with increasing DS. CAs with a DS up to 1.8 were deacetylated to various extents. Esterases were not able to deacytelate cellulose acetates with higher DS than 1.8. Experiments with small model compounds showed that increasing chain length and degree of acetylation negatively affected the ability of esterases to deacetylate. A combination of esterases with cellulases increased the glucose recovery from cellulose acetate. Furthermore, a hydrolytic enzymes (LPMO) randomly cleaved low acetylated substrates into short fragments with at least one non-acetylated monomer. In summary, combinations of backbone cleaving and deacetylating enzymes can enhance CA degradation up to a maximum DS of 1.8.

5.6 Author contributions

“B.S. and A.S. conceived and designed the experiments; O.H.C. and S.W. performed the experiments; O.H.C., A.P. and G.T. analyzed the data; R. L. expressed and purified the CBH enzyme; O.H.C. and A.P. prepared and formatted the graphs and the manuscript; O.H.C., G.S.N. and G.M.G. wrote the paper.

5.7 Conflict of Interest

The authors declare no conflict of interest.

5.8 Acknowledgments

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6

Cultivation of Heterotrophic Algae on Paper Waste Material and Digestate

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Keywords: Heterotrophic algae cultivation, enzymatic hydrolosis, cellulases, newspaper, paper residue materials

6.1 Abstract

This study investigates the cellulases-catalyzed hydrolysis of newspaper waste and paper slurries as carbon source in combination with digestate from anaerobic digestion as nitrogen source for the heterotrophic cultivation of Chlorella sorokiniana. The enzymatic hydrolysis of paper slurries resulted in a hydrolysate containing 27.2 g l^{-1} glucose reflecting a 78% cellulose conversion. A maximum Chlorella sorokiniana biomass yield of 3.64 g l^{-1} was achieved within 91 h of cultivation under consumption of 9.7 g l^{-1} glucose. The most efficient cultivation converted 1 g l^{-1} glucose into 0.42 g l^{-1} biomass. A strong decrease in pH of the batch cultures due to ammonium consumption and CO_2 production by algae cells inhibited full glucose consumption for high concentrations of 17 g l^{-1} glucose. Characterizing the amino acid content and pattern of the biomass revealed an amino acid fraction of 32.9% with Alanine as the most frequent amino acid.

6.2 Introduction

Paper is a largely consumed commodity with an annual production of 92.18 mio. tons worldwide. Its recycling rate, 71.6% in Europe, is already substantial but there is still room for improvements [1]. Paper consist mainly of cellulose with varying amounts of hemicellulose, lignin and other inorganic constituents Figure 1 [2].

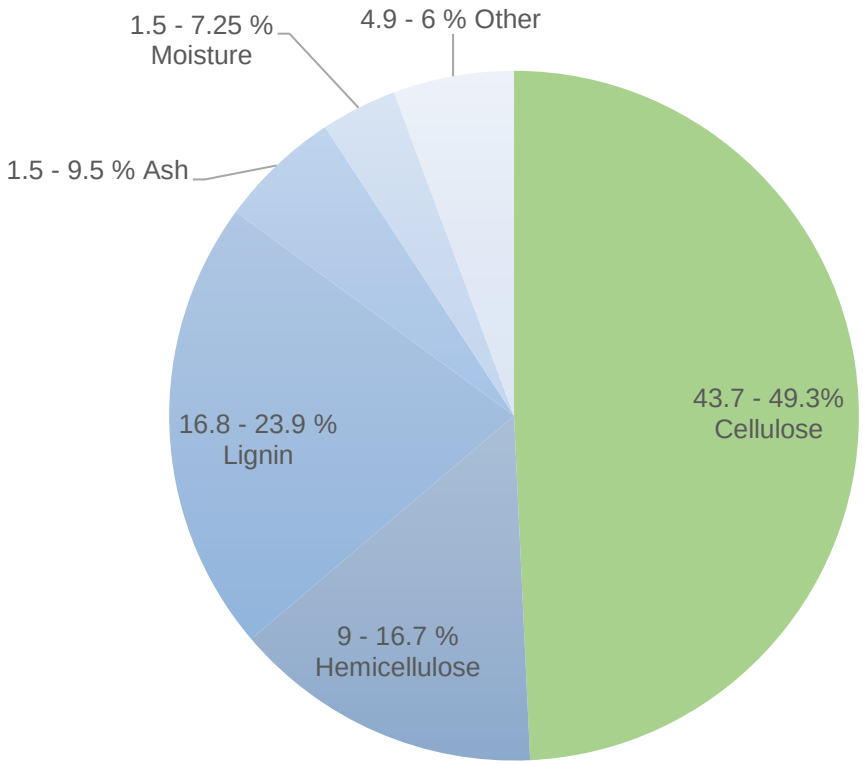

Figure 1. Composition of newspaper according to the work of Guerfali et.al. [2].

Obviously, during recycling contaminants have to be removed in the deinking process while several chemicals like mineral oils, phthalates, phenols and parabens were identified as potentially critical contaminants for paper recycling. These substances can remain in the paper product and accumulate form recycling cycle to recycling cycle. Migration of these substances into foodstuff is potentially possible when recycled paper is used as packaging material [3]. Yet, (hemi)cellulose could provide an attractive substrate of bioproduction after hydrolysis to the respective monosaccharides. Such 2nd generation processes are not in competition to food production and indeed paper waste has already been assessed for bioethanol production [4]. However, enzymes used for the hydrolysis of (hemi)cellulose prior to ethanol production by e.g. Saccharomyces cerevisiae contribute with about 20% to the overall cost for bioethanol

production [5]. In a study from 2019, the actual market price for bioethanol was stated with 0.47$ kg^{-1} [6]. Second generation bioethanol is still not competitive vs first generation or crude oil derived fuels, until its price can be halved [7]. Nevertheless, second generation carbon sources like paper waste and slurries may still be an attractive substrate for production of higher value goods like algae biomass.

Since some of the components from waste paper material might be harmful to microorganisms, the suitability of reside paper materials for microbiological processes is not deemed to be given, and is worth a more in depth investigation.

Chlorella sorokiniana is a unicellular micro algae capable of growing autotrophically with light and carbon dioxide or heterotrophically without light while using organic carbon as energy and carbon source [8]. The bulk selling price of heterotrophically cultivated Chlorella ranges between 10 to 30 $ kg^{-1} [9]. Compounds derived from Chlorella biomass are of interest in several sectors. For example, a high protein content with a similar amino acid pattern makes it a suitable fish feed. Additionally, high concentrations in polyunsaturated fatty acids (PUFAs) are beneficial. A study from 2017 stated similar results in fish production for Chlorella sorokiniana meal compared to traditional fish feed from fishmeal or soybean [10]. Many different alternative utilizations of Chlorella biomass were reported in literature e.g. such as health supplement for humans [11] or coloring ingredient in butter cookies [12].

Next to carbon source, a suitable nitrogen source is essential for economical algae cultivation since nitrogen is contributing to the dry matter of microalgae biomass from 1 to 10% [13]. Digestate is a cheap byproduct of anaerobic digestion, rich in ammonium (NH_4^+). Nowadays digestate is often spread directly on agricultural land with increasing concerns about environmental impacts [14]. Additionally, processing of digestate causes costs of 4 to 9 € m^{-3} for biogas plants depending on the location and their size [15]. The capability of Chlorella to take up ammonium under most conditions is known from literature [16]. In fact, ammonium is the most preferred nitrogen source with the order of preferences is ammonium>nitrate>nitrite>urea [13]. Algae cultivation on different nitrogen rich residue streams as piggery digestate was investigated before, phototrophically [14] as well as heterotrophically on food waste [17]. This makes digestate a potential nitrogen source for heterotrophic algae cultivation with a potential to solve some of the problems mentioned above.

In this work we developed a heterotrophic process cultivating Chlorella sorokiniana on glucose from enzymatically hydrolyzed newspaper waste and two paper slurries of industrial origin. The hydrolysate was then mixed with purified digestate (also of industrial origin) as nitrogen source. To our knowledge, this is the first time that such a feedstock was described for heterotrophic algae cultivation. A visualization and description of the performed process steps is given in Figure 2.

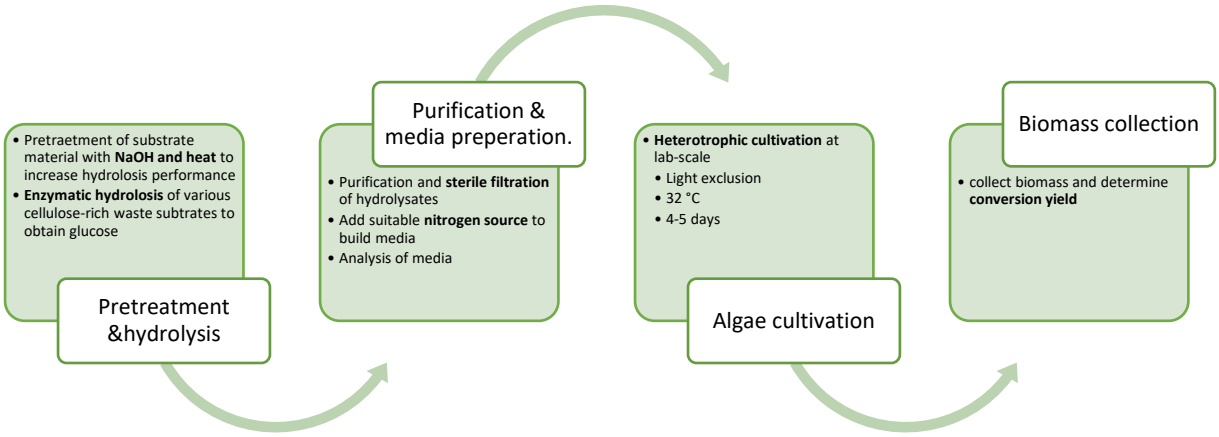

Figure 2. Scheme of the enzymatic substrate hydrolysis and digestate processing for heterotrophic algae cultivation.

6.3 Material and methods

Paper substrates from before of the deinking process were provided from Laakirchen Papier AG (Laakirchen, Austria). Slurries came from the stacking tower (Paper Slurry A) with a consistency of 7% (w/w) and from the dissolution process (Paper Slurry B) with a consistency of 4% (w/w). Cellic CTec3, developed by Novozymes (Bagsværd, Denmark) and purchased from Pointner-Rothschädl (Salzburg, Austria) was used for enzymatic hydrolysis of cellulose constituents in waste paper / slurries. Ammonium rich digestate was provided from a biogas plant in Austria utilizing slaughterhouse waste as substrate (Sankt Martin, Austria). Ultra-pure water was produced via a Sartorius Arium pro water purification system (Göttingen, Germany). The standards and solvents for HPLC analysis were from VWR (Vienna, Austria) in HPLC/GC grade. All other chemicals used were purchased from Sigma Aldrich (Vienna, Austria) in HPLC grade.

6.3.1 Microorganisms and synthetic medium

Chlorella sorokiniana (SAG strain number: 211-8k) was obtained from the Culture Collection of Algae at Goettingen University (Goettingen, Germany). Before cultivation on glucose from paper slurries, algae were cultivated in liquid medium (synthetic medium) containing yeast extract 10 g l^{-1} and glucose 10 g l^{-1} with artificial sea salt (YEG-ASS). For cultivation on agar plates, the same medium was prepared containing additionally 2% agar. The final concentrations of the artificial sea salts (ASS) in the medium were 9.7 mM sodium chloride, 5 mM magnesium sulfate and 5 µM manganese(II)chloride. The medium was sterile filtered (0.02 µm) into sterile baffled flasks. All experiments, whether liquid medium (hydrolysate or artificial medium) or plates were performed in biological duplicates.

6.3.2 Hydrolysis

For evaluating hydrolysis, 15 g dry newspaper waste or corresponding amount of Paper Slurry A and B were weight into 1000 ml bottles. 30 ml 1 M NaOH was added and samples were filled up with ultra-pure water to a final volume of 300 ml. Samples were incubated for 15 h at 50 °C and 150 rpm. Thereafter, the pH of all samples was adjusted to pH 5.0 using 5 M HCl. The enzyme cocktail Cellic CTec 3 was added (3% w/w), the volume was filled up to 600 ml and the samples were incubated at 50 °C for 168 h at 150 rpm. Hydrolysis was monitored via reducing sugar determination over time.

For large scale hydrolysis, samples (approx. 115 g dry weight) were weight in 2000 ml bottles and incubated in 0.1 M NaOH for 15 h at 50 °C stirring at 150 rpm. The material was then washed with 50 ml ultra-pure water per gram of dry substrate using a sieve with 500 µm mesh width. A sample of approximately 5 g was withdrawn for dry weight determination at 100 °C. According to the dry weight determination after washing, 75 g l^{-1} dry weight of the substrates in ultra-pure water were used for hydrolysis. The pH was adjusted to 5.0 and cellulase (Cellic CTec3) was dosed to a concentration of 5% (w/w). Hydrolysis of 1.2 l was performed in a 1.5 l glass reactor (double jacket) equipped with an AwiFlex Series 7 process control system from AWITE Bioenergie GmbH (Langenbach, Germany). The reactor was constantly stirred with 150 rpm at 50 °C for 24 h and the pH was monitored and adjusted. After hydrolysis, samples were centrifuged at 9,000 rpm and 4°C for 20 min. The supernatant was characterized regarding its glucose and xylose content with HPLC and stored at -20 °C until media preparation. Large scale hydrolysis was performed one time with each substrate to obtain hydrolysate for algae cultivations.

6.3.3 Media preparation and algae cultivation

Digestate was centrifuged at 4 °C for 9,000 rpm for 20 min and the supernatant was collected and adjusted to pH 7 with 5 M HCl. A Microjet ML2 microwave autoclave (Basildon, United Kingdom) was used for sterilization of 100 ml centrifuged digestate. An aliquot of 10 ml was sampled and adjusted to pH 7 using 5 M HCl, the amount of added HCl was recorded. The corresponding volume of sterile 5 M HCl was added to the remaining 90 ml of digestate to bring it to the desired pH of 7. Afterwards, a sample was taken to determine ammonium concentration and pH. Digestate was stored at 4 °C when not used the same day. Hydrolysate was adjusted to pH 7 (1 M NaOH) and sterile filtered (0.2 µm). Cultivations of 100 ml volume were performed in 500 ml baffled flasks The medium for the cultivation contained cellulose hydrolysate and sterile digestate in different ratios and dilutions (ultrapure water) as indicated in Table 1, to reach the target concentrations of glucose and ammonium. Furthermore, an antifoam agent from Bussetti Glanopon DG160 (Vienna, Austria) was added to a final concentration of 0.01% (v/v). Medium in baffled flasks was inoculated with 10 % of an algae preculture cultivated on synthetic medium for 4 days at 32°C with 100 rpm in the dark. All cultivations were performed in a New Brunswick Innova44 incubator as biological duplicates while error bars indicate standard deviation.

Table 1. Glucose and ammonium concentrations from the substrate materials for media preparation and the glucose and ammonium levels performed in algae cultivation.

Substrate	Glucose in hydrolysate for media preparation [g l^{-1}]	Ammonium in digestate for media preparation [g N l^{-1}]	Glucose levels [g l^{-1}]	Ammonium levels [g l^{-1}]
Newspaper waste	15.19	6.24±0.02	5.8, 11.7	0.0, 0.5, 0.7, 1.0
Paper Slurry A	27.23		10, 15, 20	0.7
Paper Slurry B	20.94		7, 12, 17	0.6

Digestate with a dry substance of 2.7% showed low concentrations in heavy metals, lead < 5.00 mg kg^{-1} dry weight (DIN IN ISO 11885), cadmium < 0.500 mg kg^{-1} dry weight (DIN IN ISO 11885) and mercury < 0.500 mg kg^{-1} dry weight (DIN IN 1483 (E 12-4)). Results of standard analysis characterizing the digestate are summarized in Table 2. HPLC performed in an Agilent 1100 series (Santa Clara, USA) system with an RI detector (Agilent 1100 series) and MW detector (Agilent 1100 series) to analyze potential carbon sources in the digestate found 23 mg l^{-1} acetic acid as highest concentrated carbon compound (mobile phase: 0.01 N H_2SO_4, runtime: 60 min, flow rate: 0.325 ml min^{-1}, temp: 65 °C, column: Transgenomic IC SEP ION300, injection volume: 40 µl). To 960 µl of diluted sample, 20 µl of a 250 mM $K_4[Fe(CN)_6] \cdot 3\ H_2O$ solution were added and samples were vortexed. Thereafter, 20 µl of 1 M $ZnSO_4 \cdot 7\ H_2O$ solution were added and samples were vortexed again. Samples were centrifuged at 12,700 rpm for 30 minutes; the supernatant was filtered through a 0.2 µm polyamide (PA) syringe filter into glass vials and stored at 4 °C. Calibration was performed in a range from 10-1000 mg l^{-1}.

Table 2. Characterization of digestate for algae cultivation according to DIN ISO 11466 (extraction) and DIN ISO 11885 (analysis).

Analyte	Concentration in original digestate [mg kg^{-1}]
Al (Aluminum)	513
B (Boron)	0.14
Ca (Calcium)	598
Co (Cobalt)	0.38
Cr (Chromium)	0.28
Cu (Copper)	2.05
Fe (iron)	109
K (Potassium)	708
Mg (Magnesium)	72.2
Mn (Manganese)	5.9
Mo (molybdenum)	0.21
Na (sodium)	741
Ni (nickel)	0.27
P (Phosphor)	675
S (Sulfur)	225
Se (Selene)	0.015
Zn (Zink)	9.96

On a daily bases, 5 ml samples were collected from algae cultivations and characterized according to its optical density at 750 nm in plastic cuvettes (single use) with 1 cm path length using a Hach-Lange DR3900 photometer (Manchester, United Kingdom). Samples were diluted in ultra-pure water to an OD below 1, against ultra-pure water. Remaining samples were stored at -20 °C until HPLC and ammonium determination. In the end of each cultivation, the volume of the remaining medium was measured. Biomass was recovered by centrifugation of the culture at 4 °C for 5 min with 5,000 rpm. The pellet was washed two times with ultra-pure water and centrifuged again. After washing, the pellet was dried in a freeze dryer, type Christ Alpha 2-4 LSCplus (Osterode, Germany). The dried biomass was weighted and stored in the dark at 4 °C.

6.3.4 Determination of reducing sugars, glucose, xylose and ammonium

Cleavage of cellulose and hemicellulose, resulting in the formation of reducing sugars, was monitored using the DNS method as previously reported by Ghose [18] with slight modifications. To 500 µl of a properly diluted sample (diluted in ultra-pure water) 500 µl DNS solution was added. Samples were vortexed and placed into a boiling water bath for 5 min. After cooling down to room temperature, 200 µl of the colored solution were transferred into a 96 well plate and absorbance was measured at 540 nm in a Tecan Infinite M200 plate reader (Männedorf, Switzerland). A calibration with glucose standards in the concentration range from 0 to 10 mM was prepared and treated the same way as the samples. The DNS reagent contained 10 g l^{-1} dinitrosalicylic acid, 16 g l^{-1} NaOH and 300 g l^{-1} sodium potassium L(+)-tartrate tetrahydrate. Experiments were performed in duplicates and error bars indicate standard deviation.

Products from large scale enzymatic hydrolysis of cellulosic substrates and the cultivation medium for C. sorokiniana was analyzed according to the content of glucose and xylose. For precipitation of impurities, to 960 µl of diluted sample, 20 µl of a 250 mM $K_4[Fe(CN)_6] \cdot 3\ H_2O$ solution were added and samples were vortexed. Thereafter, 20 µl of 1 M $ZnSO_4 \cdot 7\ H_2O$ solution were added and samples were vortexed again. Samples were centrifuged at 12,700 rpm for 30 minutes; the supernatant was filtered through a 0.2 µm polyamide (PA) syringe filter into glass vials and stored at 4 °C. The HPLC system, a 1260 series system from Agilent Technologies (Santa Clara, USA) was equipped with a Transgenomic ION-300 column (New Haven, United States). As mobile phase, tempered at 45 °C, 0.01 N H_2SO_4 was used with an isocratic flow rate of 0.325 ml min^{-1}. For analysis, 40 µl of sample were injected at a runtime of 45 min. The method was calibrated using glucose and xylose standards over a 25-1000 mg l^{-1} range.

For ammonium determination, 1 ml of undiluted sample was analyzed in an AutoKjeldahl Unit K-370 (Büchi, Switzerland). NH3 was extracted from the samples using 50% NaOH and transferred into 20 g l^{-1} boric acid. Ammonium was titrated using 0.05 M HCl. Ammonium content was calculated in mg ammonium ml^{-1}. Distillation was performed under the following parameters: water dosage of 40 ml, NaOH dosage of 40 ml, reaction time of 10 s and distillation time of 180 s.

6.3.5 Protein determination and amino acid pattern

To 30 mg of lyophilized algae powder in glass tubes, 2 ml of 6 M hydrochloric acid were added and incubated under regular mixing at 105 °C for 24 h. Samples were centrifuged at 1000 rpm for 5 min and neutralized with 0.5 M NaOH. For precipitation, to 960 µl of sample, 20 µl of a 250 mM $K_4[Fe(CN)_6] \cdot 3H_2O$ solution were added and samples were vortexed. Thereafter, 20 µl of 1 M $ZnSO_4 \cdot 7H_2O$ solution were added and vortexed again. Sample tubes were centrifuged at 12,700 rpm for 30 min. The supernatant was filtered through a 0.2 µm PA syringe filter into glass vials and stored at 4 °C until analysis. HPLC analysis was performed on a 1260 series HPLC from Agilent Technologies (Santa Clara, USA) specially equipped with a 1290 series ELSD detector from Agilent Technologies (Santa Clara USA). For analysis, 5 µl sample at a flow rate of 0.2 ml min^{-1} were injected. The evaporation and nebulizer temperature of the ELSD was set to 60 °C and the gas flow rate of N_2 was set to 1.6 standard liters per minute (SLM). The gradient of the mobile phase consisted of three solutions, A, B and C. Solution A contained 20 mM perfluoropentanoic acid (NFPA) + 0.6% trifluoroacetic acid (TFA), Solution B consisted of 20 mM NFPA +0.8% TFA and Solution C was pure acetonitrile (ACN). An overview of the gradient program is given in Table 3. For calibration standards in a range of 100-1250 mM were used. The method was evaluated according to DIN 32645. The amino acid profile from biomass cultivated on hydrolysate/digestate mixtures was measured in duplicates and error bars indicate the standard deviation.

Table 3. Gradient of mobile phase for amino acid determination via HPLC. A: 20 mM perfluoropentanoic acid (NFPA) + 0.6% trifluoroacetic acid (TFA), B: 20 mM NFPA + 0.8% TFA, C: acetonitrile (ACN).

Time [min]	**0**	**10**	**11,5**	**20**	**30**	**40**	**45**	**50**	**51**
A [%]	0	0	83,6	26	50	50	100	100	0
B [%]	100	85	0	0	0	0	0	0	0
C [%]	0	15	16,4	74	50	50	0	0	100

6.4 Results and discussion

6.4.1 Enzymatic hydrolysis

Enzymatic hydrolysis of three different cellulosic substrates (newspaper waste, paper slurry A from the stacking tower & paper slurry B from dissolution) was investigated to compare the effects of NaOH pretreatment at a moderate temperature of 50 °C. For all three substrates, the pretreatment resulted in a stronger increase of the reducing sugar concentration after 168 h (Figure 3A). Strongest effects of the pretreatment were seen for Paper Slurry A. For this sample, the pretreatment led to an increase of the reducing sugar concentration from 8.2 g l^{-1} to 10. 2 g l^{-1}. Considering the values for cellulose and hemicellulose from Figure 1, a hydrolysis efficiency for non-pretreated paper slurry A from 49.7-62.2% and for pretreated from 61.8 to 77.4% was reached. For all materials and treatments, the minimum efficiency was above 44.2%. Considering the fact that all materials contained the same starting concentration of paper material (25g l^{-1}), differences in hydrolysis reflects differences in accessibility of the substrate by the enzyme. In the paper mill process, paper slurry A is longer dispersed in liquid than paper slurry B, meaning that fibers in paper slurry A had more time to swell, being a potential explanation for more efficient hydrolysis. Further, a longer and stronger mechanical treatment of the material during the passage (from dissolution to stacking tower) might increase accessibility additionally, due to friction and tumble in the facility. Moreover, some cellulose fibers may be generally inaccessible to the enzymes since entrapped e.g. in adhesives, inks or other impurities and additives. The maximum hydrolysis level for Paper Slurry A was reached after only 24 h of reaction while for a longer time no significant increases in reducing sugar concentration could be detected (paper slurry A, Figure 3B). For this sample, the hydrolysis pattern for pretreated and non-pretreated materials was equal until 6 h. Afterwards, pretreated samples showed a further increase in reducing sugar than non-pretreated samples did. Obviously, in the first phase, material components which are better accessible by the enzymes are hydrolyzed. In a second phase, those areas where the pretreatment increased accessibility of the fiber are hydrolyzed. For single cellulose fibers, Novy et.at. showed the presence of zones of lower structural organization on the surface of a fiber. During the initial phase of the hydrolysis enzymatic degradation predominates at these zones. This effect is that strong, that an increase in less accessible areas was observed during monitoring the hydrolysis

process. So, degradation of highly ordered structures is delayed [19]. For corn stover different pretreatments were investigated according to their potential to increase digestibility. As a result of air drying, digestibility of the samples substantially decreased. Dilute acid pretreatment and xylan removal increased cellulose degradation. The same study found that most of a Cellulase from *T. resii* was bound in the beginning of an hydrolysis and amount decreased constantly over a time course of 120 h [20]. A study from 2019 mentioned the hydrolysis of lignin and hemicellulose using NaOH in a pretreatment and stated its profitability for sugarcane bagasse and trash lignocelluloses in a biorefinery [21].

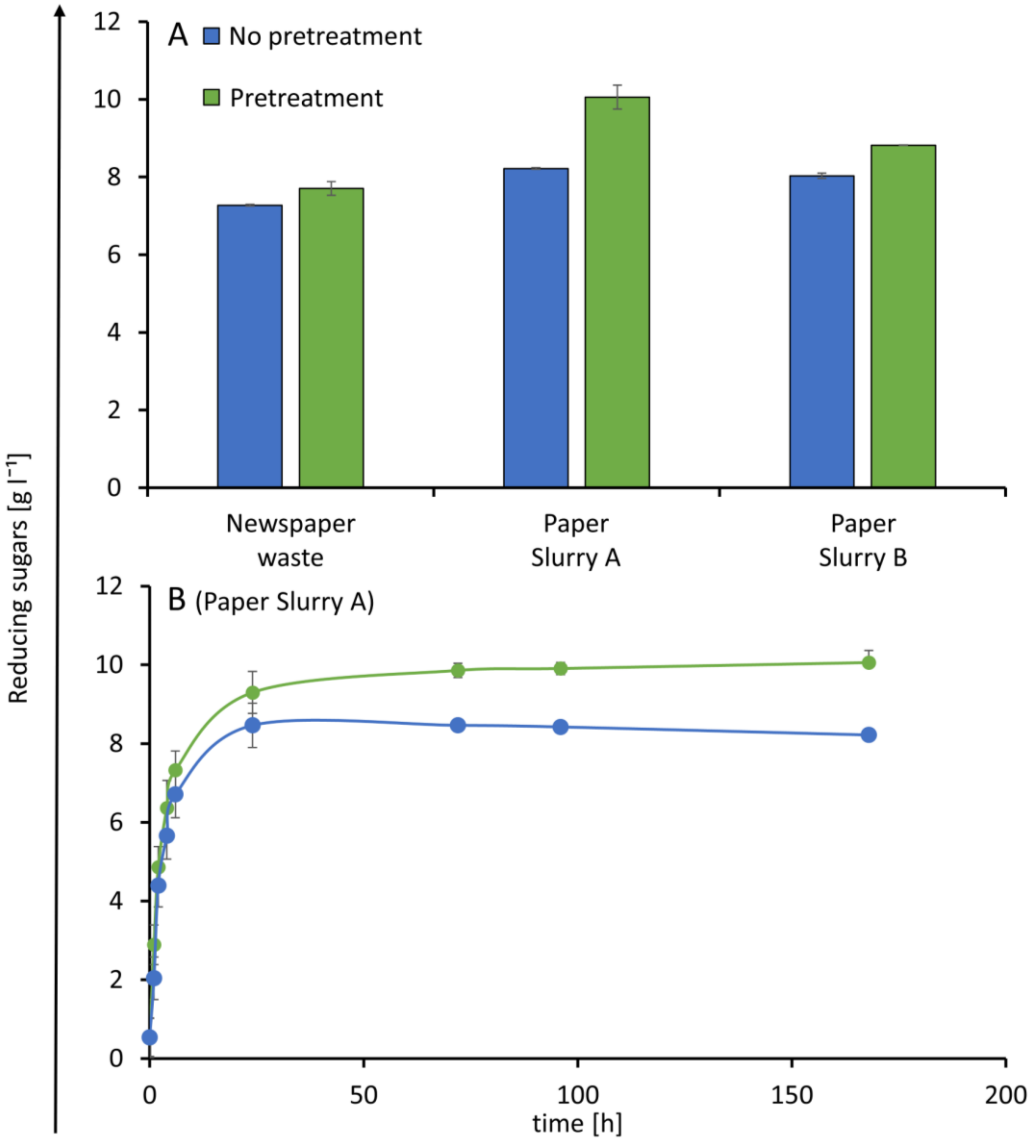

Figure 3. Enzymatic hydrolysis of pretreated and non pretreated newspaper waste and paper slurries. For all substrates, an incresed reducing sugar release due to the pretretment was recorded which was lowest for newspaper waste and highest for paper slurry A. Also, highest total glucose concentration was obtained from paper slurry A while all substrates dosed to the identical starting concentration (25 g l⁻¹ substrate). Error bars indicate standard deviation of duplicates measured.

From the results presented in Figure 3 it can be concluded that at the used enzyme concentration the maximum hydrolysis yield is reached after 24 to 50 hours. However, regardless the incubation time a maximum yield based on theoretical cellulose content of 77% was reached. Therefore, more severe treatments opening the lignin/cellulose structures, reducing the cellulose crystallinity, increasing the surface area, and altering the degree of cellulose and hemicellulose polymerization may contribute to higher overall yields. A study from 2017, showed best effects of a heat treatment at 80 °C for 30 min in a hot air oven and 110 °C for 40 min in an autoclave [22]. Heat production is energy intensive, but residual heat from different heat streams in the facility could be used. Therefore, a heat treatment at lower temperatures might slightly limit the pretreatment effects but could be more reasonable, especially when processing huge amounts in short periods. This is why a possible heat treatment prior to hydrolysis must be evaluated in individual cases [23]. A positive effect of a chemical pretreatment with sodium hydroxide even at low temperatures (50 °C) was shown for different sodium hydroxide levels (0.5, 1.0 and 2.0%) close to the level used in our study (0.4%). A similar hydrolysis pattern was obtained, and hydrolysis was completed within the first 24 h. An increase in the sodium hydroxide concentration from 0.5% to 1.0% increased the efficiency, but a further increase to 2.0% did not lead to a significant improvement. The highest glucose yield was reported for lignocellulosic substrates (Swithchgrass) with 275 mg g^{-1}, reflecting a yield of 27.5% [24]. In general, product inhibition is another important issue in glucose recovery from lignocellulosic material and is worth to be considered in continuous removal of glucose [25].

In this study, for larger scale hydrolysis experiments using a stirred tank reactor several adaptions were applied. Pretreatment time and hydrolysis time were set to 24 h each, the enzyme load was increased to 5% and the substrate load was increased to 75 g l^{-1} for enzymatic hydrolysis. Using this system higher hydrolysis efficiencies were obtained (Figure 3&4) with a maximum glucose recovery of 73.6-83.1% from paper slurry A. In addition to the higher enzyme load, better stirring in the reactor might be beneficial. The reactor was stirred mechanically with a propeller, enabling better distribution than in bottles shacked in an orbital shaker. This is highly relevant, especially in the beginning of a hydrolysis when the solution is highly viscous due to a high substrate concentration. From newspaper waste only approximately half of the calculated amount of glucose could be obtained.

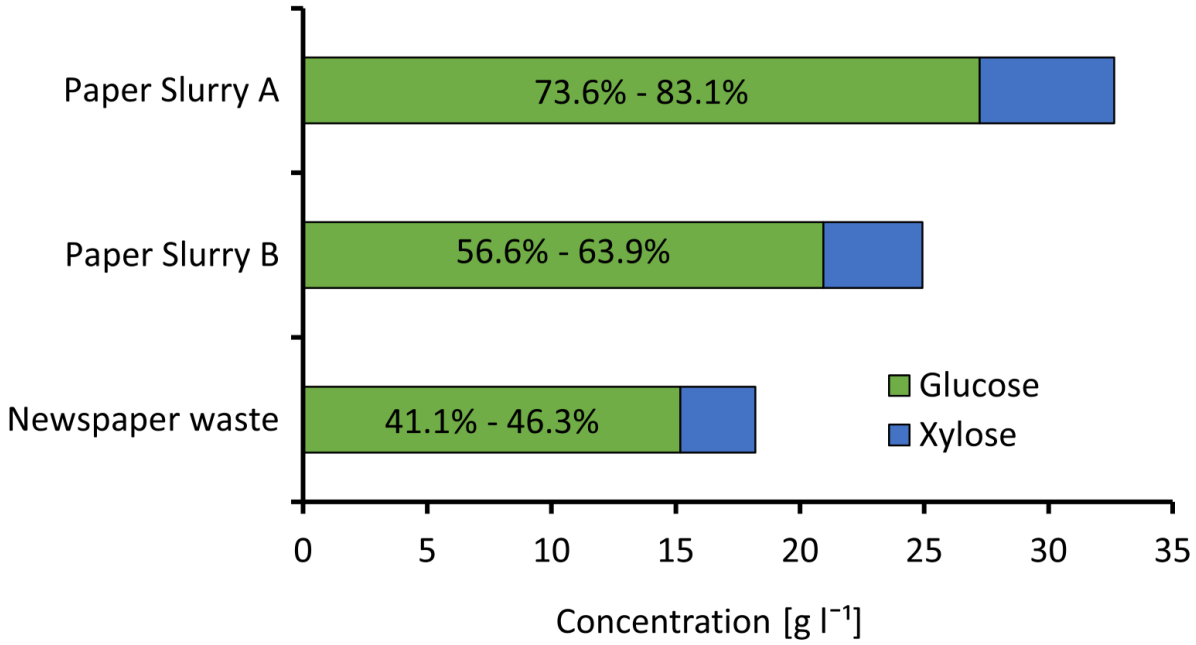

Figure 4. Glucose and xylose concentration of larger scale hydrolosis (1.5 l stirred reactor) of newspaper waste, Paper Slurry A and Paper Slurry B (75 g l⁻¹) with CellicCtec3 (5%) at 50 °C and pH5. Numbers in the green bars indicate glucose recovery based on the cellulose content given in Figure 1.

6.4.2 Algae cultivation

Based on the cellulose's hydrolysate and digestate as carbon and nitrogen sources, respectively, defined media for the cultivation of C. sorokiniana were prepared. Carbon derived from the hydrolysate and nitrogen from the digestate. Further essential elements as sulfur, phosphor and metals also derived from the digestate (Material and methods, Table 2). Phosphorus and sulfur as essentials for algae cultivation were reported in literature before, but also growth under P and S limitation was reported [26]. Successful heterotrophic algae cultivation with Chlorella vulgaris was reported on a medium congaing the following trace metals H_3Bo_3, $MnCl_2 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ [27]. Therefore, digestate can provide essentials elements for algae cultivation.

For newspaper waste, two glucose levels (5.8 g l^{-1} and 11.7 g l^{-1}) and four digestate levels (0.0, 0.5, 0.7 and 1.0 g l^{-1}) were investigated. For samples containing 5.8 g l^{-1} glucose the optical density increased when the ammonium level was 0.5 g l^{-1} and 0.7 g l^{-1} while no growth was visible without ammonium and at the highest ammonium concentration (Figure 5A). Therefore, quite expectedly paper hydrolysate alone does not provide enough nitrogen for growth of C. sorokiniana. On the other hand, an ammonium concentration of 1 g l^{-1} seems to be too high and inhibited growth of the

organism. The toxicity of high ammonium concentrations on different Chlorella strains with half maximal effective concentration values (EC50) between 0.4 and 1.6 g l^{-1} was shown in literature [28]. Higher amounts of digestate lead to an extended lag phase. At 0.5 g l^{-1} ammonium, accelerated growth started after 8 h while for 0.7 g l^{-1} there was a further delay of 16 hours but a higher final OD value (8.0) after four days was reached. For the last 24 h OD remained stable (0.5 g l^{-1} ammonium) or slightly decreased (0.7 g l^{-1} ammonium). At a glucose level of 5.8 g l^{-1} optimal cultivation time is four days at an ammonium concentration of around 0.7 g l^{-1}. Glucose concentrations measured in the growth medium fully supported the findings from OD measurements. In fact, glucose decreased only for ammonium levels of 0.5 g l^{-1} and 0.7 g l^{-1}. As for OD data, a delay was visible when higher amounts of digestate was present. For both ammonium levels the glucose concentrations decreased close to zero, indicating that C. sorokiniana consumed all available glucose at 5.8 g l^{-1}. For OD and glucose both concentration levels show the same pattern (Figure 5A, C & Figure 5B & D). Interestingly, the glucose concentration did not drop to zero for the higher level concentration level (11.7 g l^{-1} glucose) as shown in Figure 5D. The utilization of higher concentrations of glucose did not increase algae growth, indicating that the maximum growth rate was already reached at the 5.8 g l^{-1} glucose concentration level. Further, for higher glucose concentration the growth decelerated after five days without consuming all the starting feedstock. It must be considered that metabolites such as CO_2 produced by C. sorokiniana reduced algae growth under heterotrophic conditions. In particular, CO_2 has the effect of lowering the pH of the medium and therefore deteriorates growth conditions. Results for Paper Slurries A & B clearly show a negative effect of decreasing pH on the algae cultivation (Figure 6 & 7). A similar behavior can be assumed for newspaper waste, since cultivation conditions were similar. Limiting effect caused by other elements present in the digestate cannot be excluded completely, for such a complex natural compound as digestate and high ammonium concentrations. A nitrogen rich digestate with low concentrations of other components is favorable. But it must be considered, that digestate got diluted by mixing it with hydrolysates. In our study dilutions were between 1:6.2 for 1.0 g l^{-1} ammonium and 1:12.4 for 0.5 g l^{-1} ammonium (Table1).

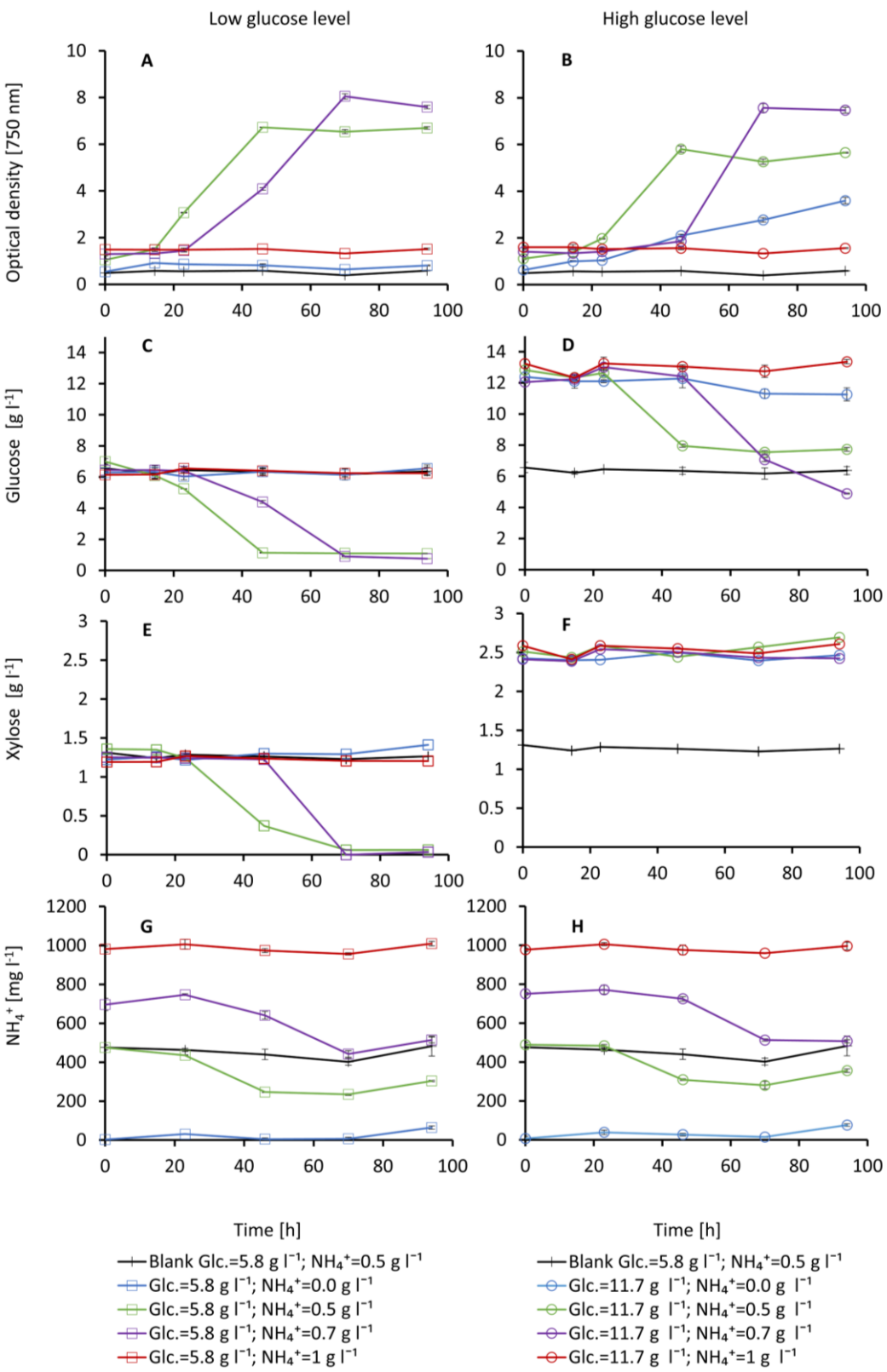

Figure 5. Cultivation of C. sorokiniana on newspaper waste hydrolysate and digestate at different glucose and ammonium levels. Xylose was only consumed at low glucose concentration. Optical density measured at 750 nm at low glucose level (5.8 g l^{-1})(A) and high glucose level (11.7 g l^{-1})(B). Glucose consumption at low glucose level (C) and high glucose level (D). Xylose consumption at low glucose level (E) and high glucose level (F). Ammonium consuption (NH_4^+) at low glucose level (G) and high glucose level (H). Error bars indicate the standard deviation of biological duplicates performed. Error bars indicate standard deviation of biological duplicates.

When using low glucose concentrations, a diauxic consumption of xylose was detected. In Figure 5E it is possible to observe that the xylose level is slightly reduced after the glucose level started to decrease. For higher concentrations of glucose (11.7 g l^{-1}) a decrease in glucose is visible Figure 5D. But the xylose level remained stable for five days at 2.5 g l^{-1} for higher glucose concentrations. Turon et.al. reported diauxic growth for C. sorokiniana on acetate and butyrate at a concentration level of 0.3 g l^{-1} for heterotrophic and mixotrophic conditions [29]. Also on waste streams under heterotrophic conditions diauxic growth was reported for glucose and acetate at 20 g l^{-1} and 2 g l^{-1}, respectively [30] In 2014, a study investigated the glucose and xylose consumption mechanism: xylose is transported across the cell membrane by an inducible hexose symporter, when cells grew on glucose before (glucose induced algae cells). Further, the xylose uptake increased under presence of light while concurrent presence of glucose inhibited xylose consumption [31]. Algae cultivation on newspaper waste/digestate medium showed a similar behavior for high glucose concentrations but parallel consumption of both sugars at lower glucose concentrations. In literature, various studies report on the ratio of carbon to nitrogen (C:N ratio) required for optimal growth of C. sorokiniana. Silaban et. al. investigated growth on glucose and using sodium nitrate as nitrogen source and found best growth at a C:N ratio of 15:1 under mixotrophic conditions [32]. Another study used a 29:1 ratio with glucose and KNO_3 under heterotrophic conditions, and obtained 0.5 g biomass from approx. one gram glucose [33]. For the medium used in Figure 5, the C:N ratio determination has to take into account whether xylose was consumed. For example, for the experiment with 5.8 g l^{-1} glucose and 0.7 g l^{-1} ammonium the C:N ratio would be 5:1 (glucose only) but 6:1 (glucose + xylose). Low carbon concentrations compared to the amount of ammonium present might explain that not more nitrogen was used from the medium (Figure 5 G&H). Although, for higher glucose concentrations (11.7 g l^{-1}) with a higher C:N (9.9:1 for 0.7 g l^{-1} ammonium & 13.9:1 for 0.5 g l^{-1} ammonium) also no increase in ammonium consumption was visible for the investigated time frame of 96 h (Figure 5H). C:N ratios of 3-20 were reported to have similar biomass productivities from 0.928±0.042 g d^{-1} l^{-1} (C:N 5) to 0.957±0.043 g d^{-1} l^{-1} (C:N 20) [34]. It seem, that also for a paper hydrolysate/digestate medium C:N is not significantly influencing the biomass productivity.

Another interesting fact is the comparison of growth at different carbon levels in comparison with different ammonium levels. For a glucose concentration of 5.8 g l^{-1} and no additional nitrogen source, no growth was detected. Increasing the glucose concentration to 11.7 g l^{-1} showed a limited growth of C. sorokiniana from an OD of 0.63 (0h) to 3.6 (96 h) without ammonium from digestate. The only nitrogen source was the very small amount from the hydrolysate (Figure 5G & H). It seems, that algae were able to adopt their metabolism to a nitrogen limited environment at high glucose concentrations but not at low glucose concentrations. Growth of Chlorella vulgaris in a nitrogen limited environment was shown, under phototrophic conditions, before [35]. For heterotrophic conditions growth was obtained under a significant change of the cell composition. Nitrogen limitation caused an increase in lipid content of the algae cells [36]. In conclusion, it seems that there are no contaminants present in the hydrolysates potentially inhibiting growth at higher concentrations. The hydrolyzed newspaper waste used in our study seems to fulfill the requirements of a substrate for algae.

Due to more complete enzymatic conversion of the cellulose content in paper slurries A and B compared to newspaper waste, hydrolysates with higher glucose concentration were obtained (Figure 4). Consequently, growth media with higher glucose concentrations when compared to newspaper waste hydrolysates were investigated. For all hydrolysate/digestate combinations a lag phase after inoculation with C. sorokiniana grown on synthetic medium was observed (Figure 6). For higher glucose concentrations (15 & 20 g l^{-1}) the lag phase was shorter (14.5 h) than for 10 g l^{-1} (38.5 h). The highest OD (11.5) was reached for a glucose concentration of 15 g l^{-1} within 38.5 h (Figure 6A). Afterwards OD remained stable without showing any significant variation. The next higher glucose level (20 g l^{-1}) showed a similar course but stopped at a slightly lower OD of 8.2. The lowest glucose concentration (10 g l^{-1}) led to the same OD within 96 h. Measured biomass amounts reflect the OD values (Figure 6B). An OD of 11.5 yields 3.6 g l^{-1} algae biomass.

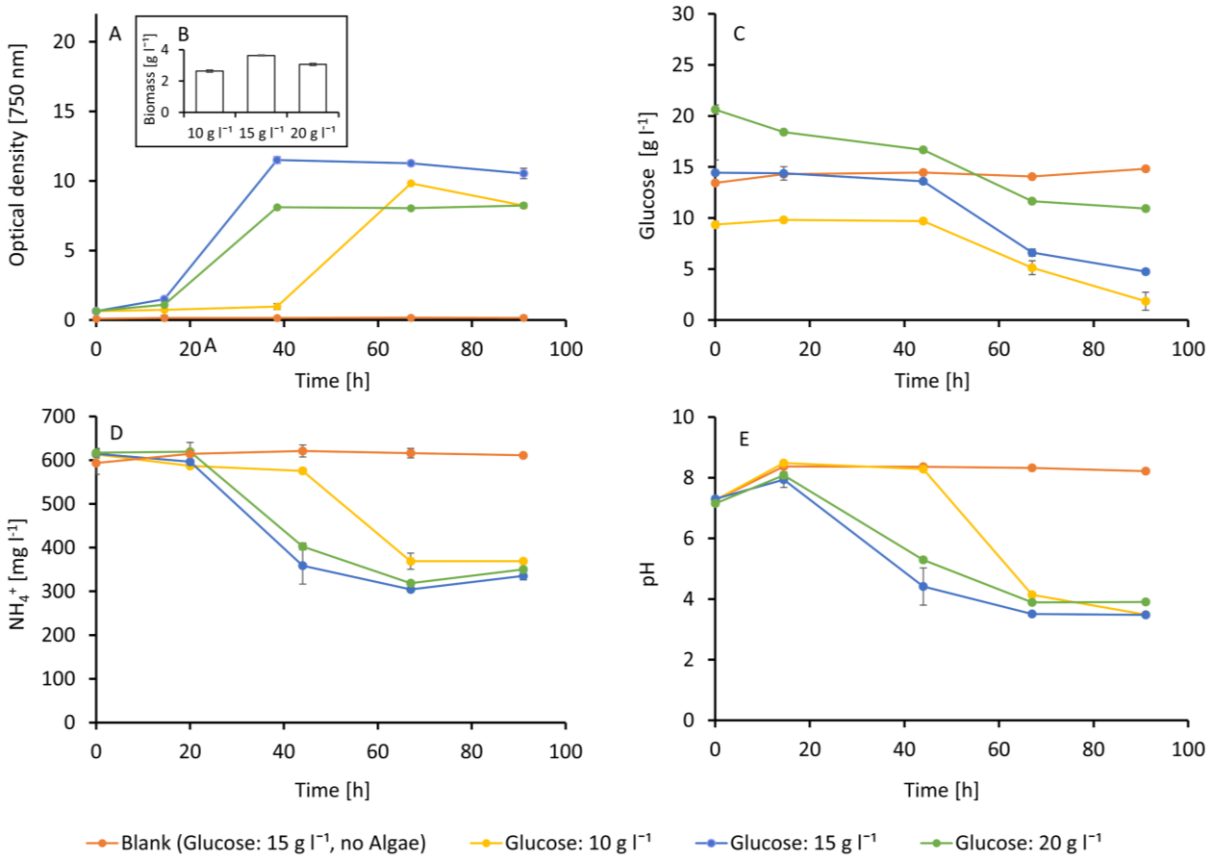

Figure 6. Cultivation of C. sorokiniana on Paper Slurry A at glucose concentrations of 10 g l⁻¹(yellow), 15 g l⁻¹(blue), 20 g l⁻¹(green) and a blank measurment without algae at 15 g l⁻¹ (orange). Optical density at 750 nm (A). Biomass yield after five days (B). Glucose consumption (C), ammonium (NH_4^+) utilisation (D) and pH course (E). The Blank shows no grwoth. Glucose and ammonium consumption was visible for all media. The pH increased in the first 24 h for all sampes (including the blank) and was around 4 after five days of cultivation. Algae growth was slower for 10 g l⁻¹ glucose for the first 48 h but increasds afterwards to similar values compared to the other glucose levels. Error bars indicate standard deviation of biological duplicates.

Glucose was incompletely consumed for all concentration levels except for the lowest concentration of 10 g l^{-1} (Figure 6C). A higher initial glucose concentration did not cause a faster glucose consumption or growth rate. The fastest growing glucose concentration was 15 g l^{-1}, obtaining 0.42 g biomass per gram glucose, whereas 10 g l^{-1} produced 0.35 g g^{-1} biomass and 20 g l^{-1} resulted in 0.32 g g^{-1}. Biomass yields reported in literature vary for heterotrophic Chlorella cultivation on glucose. A study from 2019 by Kim et.al. reported a productivity of 0.58 g g^{-1} on an optimized medium under pH controlled conditions at a glucose concentration of 72 g l^{-1} [37]. Another study using glucose from whey as substrate reported of 0.5 g g^{-1} with 10.5 g l^{-1} glucose over a total period of 216 h [38]. Results presented here for slurries A&B (Figure 6 & Figure 7) let conclude, that higher glucose concentrations fasten the adaption to a new environment. Precultures grew on the synthetic medium for four days at a glucose concentration of 10 g l^{-1}. It can be assumed, that almost all the

glucose was consumed when hydrolysate/digestate flasks were inoculated. The adaption to the new environment was faster for higher glucose concentrations, at least in a range from 7-20 g l^{-1} of glucose used in our study.

The ammonium course (Figure 6D) was in agreement with the values obtained for OD and glucose. Ammonium decreased, when the OD increased and the glucose decreased. After 96 h, ammonium levels between 330 and 350 mg l^{-1} were achieved, meaning a total consumption of approximately 300 mg l^{-1}. An explanation for the incomplete glucose consumption and lower glucose conversion efficiency can be seen from the pH course (Figure 6E). During cultivation the pH in the beginning increased from 7 to 8 and afterwards decreasing to pH 4 since ammonium consumption and CO2 production cause a decrease in pH. It was previously reported that in mixotrophic conditions an excess in CO2 had blocked the metabolization of organic substrates but stimulated photosynthesis [39]. These results indicate that an active pH regulation or a medium with a higher buffer capacity might allow to use media with higher glucose contents and total glucose consumption by the algae cells. Trends visible for OD, glucose and ammonium are conclusive with pH values. Accelerated algae growth caused an accelerated decrease of the pH.

The second hydrolysate (Paper Slurry B, Figure 7) had a lower sugar concentration than paper slurry A (32.6 vs 24.9 g l^{-1}). Therefore, media with a slightly lower glucose concentration were prepared (Figure 4). Behavior of paper slurry A and B were relatively similar, both were generally suitable for heterotrophic algae cultivation. The lag phase in the beginning seems to be slightly more distinct for paper slurry B than for Paper Slurry A. this might be due to the slightly higher ammonium concentration in the media from paper slurry B. The algae derive from a synthetic medium with yeast extract as nitrogen source. Adaption to a different medium (from synthetic medium to hydrolysate/digestate medium) with a different nitrogen source as ammonium requires an adjustment time. Precultivation on hydrolysate/digestate medium might reduce the lag phase significantly. Significant differences can be seen for the glucose to biomass conversions. For 7 g l^{-1} glucose and 12 g l^{-1} glucose complete consumption was seen within 91 h (Figure 7A). Biomass production is equal for all glucose levels, resulting in different conversion efficiencies. Highest yield was reached for 7 g l^{-1} with 0.48 g g^{-1}, compared to 0.31 g g^{-1} for 12 g l^{-1}. A contribution of xylose to biomass production can

be excluded for both slurries, as proven by equal xylose levels for both slurries and all sugar levels at 0 h and 91 h (Figure S1).

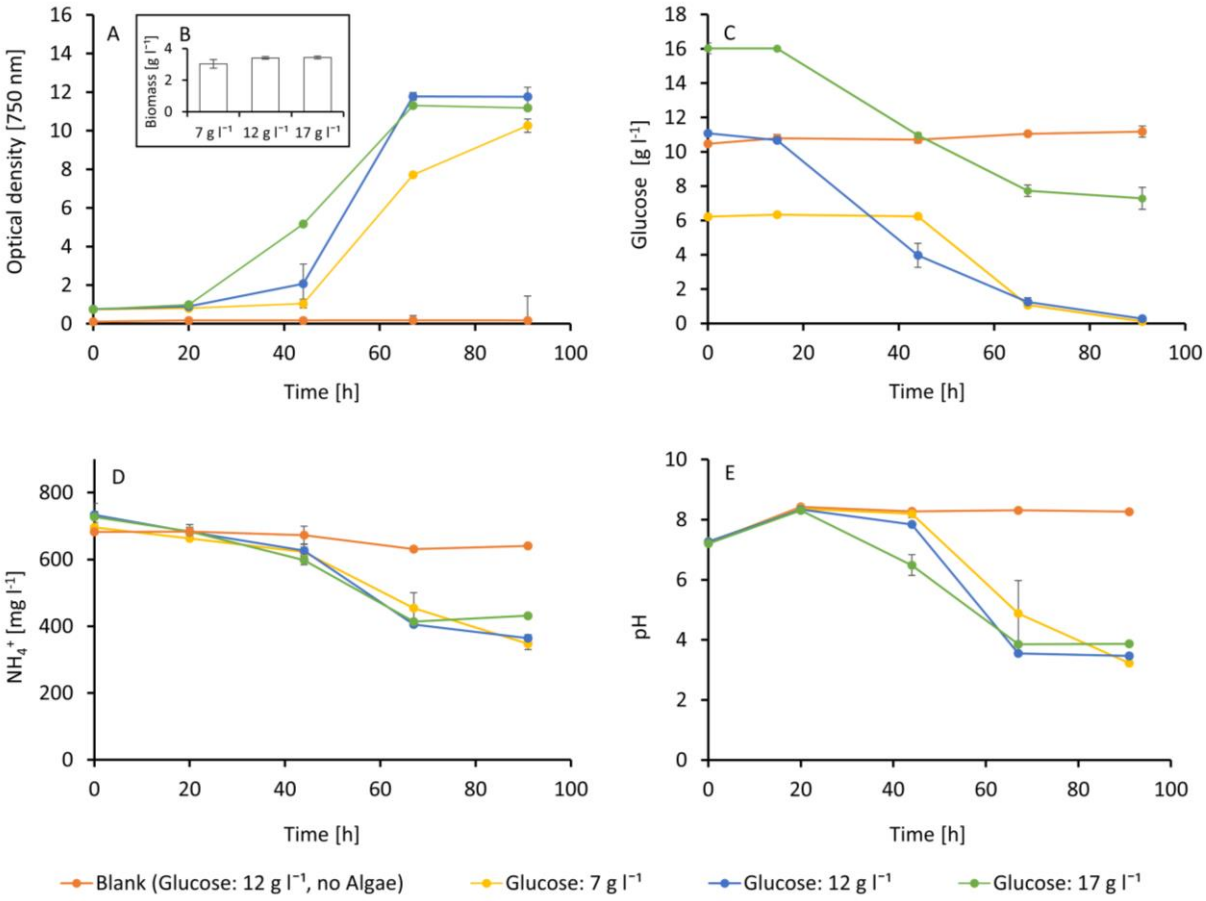

Figure 7. Cultivation of C. sorokiniana on Paper Slurry B at glucose concentrations of 7 g l⁻¹(yellow), 12 g l⁻¹(blue), 17 g l⁻¹(green) and a blank measurment without algae at 12 g l⁻¹ (orange). Optical density at 750 nm (A). Biomass yield after five days (B). Glucose consumption (C), ammonium (NH_4^+) utilisation (D) and pH course (E). The blank shows no alage grwoth. Glucose and ammonium consumption were visible for all samples containng algae. The pH increased in the first 24 h for all sampes (including the blank) from 7 to 8 and with a value of 4 after five days. Error bars indicate standard deviation of biological duplicates.

As for paper slurry A, also for paper slurry B a distinct decrease in pH was reported (Figure 7E), probably causing an incomplete consumption of glucose (Figure 7C). The pH dropped fastest for 17 g l^{-1} glucose, followed by 12 g l^{-1} and 7 g l^{-1}. For higher glucose levels a higher conversion rate to CO_2 can be assumed, since OD and biomass yields did not significantly increase with higher glucose concentration (Figure 7 A&B). The ammonium consumption was uniform for all cultivations (Figure 7D) as was biomass formation, independent from the glucose level. Therefore, the nitrogen uptake correlates with the produced biomass, assuming a uniform protein content of the algae biomass produced from paper slurry B medium.

6.4.3 Protein content and amino acid spectrum of algae biomass

The amino acid concentration was determined via HPLC. The sum of the found amino acids relates to the protein content, not considering the water molecule that is added during hydrolysis of a peptide bond. However, protein content can be indicated from the amino acids found. The amino acid content fluctuated between 31.1 ± 4.0% (slurry B, Glc.=12 g l^{-1}; NH_4^+=0.6 g l^{-1}) and 33.5 ± 4.2% (slurry A, Glc.= 15 g l^{-1}; NH_4^+=0.7 g l^{-1}). The amino acid pattern for newspaper (Glc.= 11.7 g l^{-1}; NH_4^+=0.7 g l^{-1}) is given in Figure 8. With 48.6 ± 2.9 mg g^{-1}, alanine was the most frequent amino acid, whereas leucine was determined the rarest with 4.6 ± 0.1 mg g^{-1}. The amino acid concentration for all samples were summarized in Table S1.

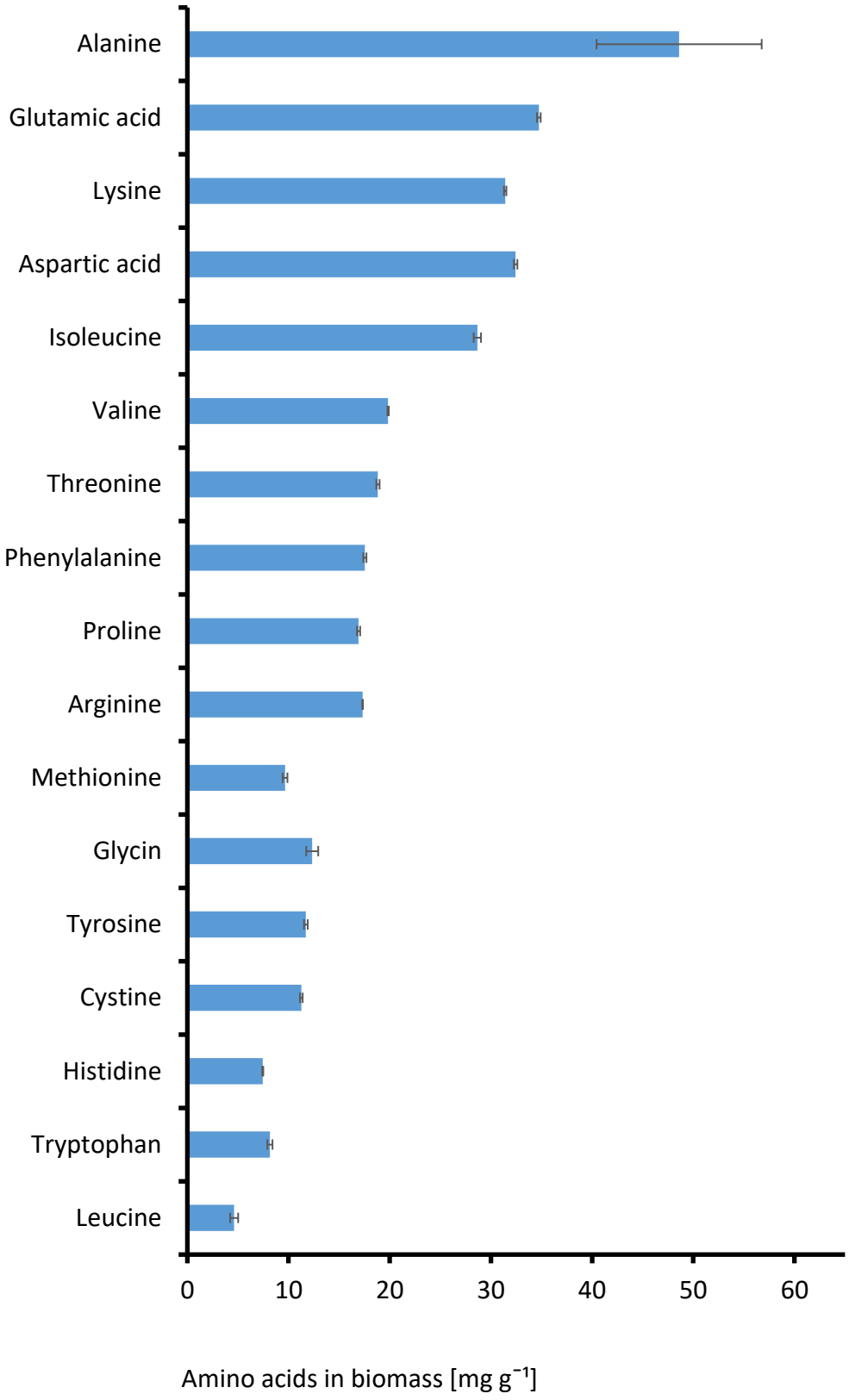

Figure 8. Amino acid concentration and pattern of lyophilized algae biomass cultivated on newspaper with glucose= 11.7 g l^{-1} and NH_4^+=0.7 g l^{-1}). Alanine is the most frequent amino acid, whereas leucine is the rarest amino acid.

A study cultivating Chlorella under heterotrophic conditions reached protein levels from 25 to 40% when nitrate was used as nitrogen source at a biomass productivity comparable to the results found in the present study (3 g l^{-1}) [40]. A different study performed by the authors used the same algae strain and method for amino acid determination found a similar but not identical amino acid pattern. For paper derived material with digestate, as for food waste, leucine was the rarest amino acid. In contrast, for food waste aspartic and glutamic acid were most common. Here aspartic acid and glutamic acid also belong the most frequent amino acids but rank only place 2 and 4 on the paper material medium [30]. Considering the low fluctuations of the amino acid content (33.1%-33.5%) for glucose concentrations ranging from 5.8 g l^{-1} to 15 g l^{-1} the protein production is stable over a wide range of carbon source concentrations. It is important to mention, that the biomass productivity was equal for all samples measured (Figure 6 & 7). Therefore, for a later pH controlled system with higher biomass productivity, amino acid concentrations might change. Literate shows, that also at higher biomass concentrations comparable protein levels were achievable [30].

6.5 Conclusion

The general suitability of newspaper waste and paper slurries as C-source in combination with digestate from anaerobic digestion as N-source for heterotrophic cultivation of Chlorella sorokiniana was demonstrated. Enzymatic hydrolysis of the substrates resulted in reducing sugar concentrations between 18.2 g l^{-1} and 32.6 g l^{-1}, namely glucose and xylose. An amount of 3.6 g l^{-1} algae biomass was produced within 3 days, with an amino acid content between 31.1% and 33.5%. This approach contributes to the creation of a biorefinery concept addressing different industrial sectors as paper and pulp manufactures and biogas producers as well as different socioeconomic factors as waste utilization and nitrogen usage. Different waste streams also from other sectors, such as from textile products or the agriculture industry are worth a deeper investigation. Further improvements of the process should aim on pH control to increase sugar to biomass conversion efficiencies and the characterization of the lipid content. Additionally, efforts to maintain xylose utilization by algae at high glucose concentrations might increase sustainability and the economic value of the approach.

6.6 Author contributions

O. Haske-Cornelius: investigation, methodology, formal analysis, project administration, writing - original draft. S. Gierlinger: investigation. R. Vielnascher: investigation, methodology. W. Gabauer: investigation, methodology. A. Pellis: supervision, project administration, writing - review & editing. G.M. Guebitz: supervision, writing - review & editing.

6.7 Conflict of interest

The authors declare no conflict of interest.

6.8 Acknowledgments

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6.10 Supplementary Information

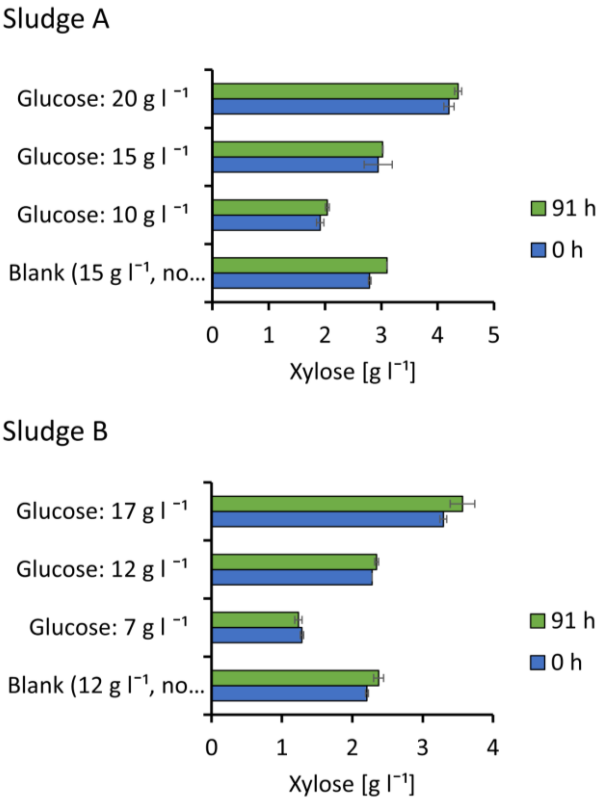

Figure S1. Xylose concentration in the beginning and after 91 h of heterotrophic algae cultivation on Paper Slurry A and Paper Slurry B. No significant consumption was visible for both slurries at all measure glucose levels. Error bars indicate standard deviation of biological duplicates.

Table S 2. Amino acid concentration of lyophilized algae biomass cultivated on Newspaper with digestate and paper slurries with digestate at different concentrations of glucose and ammonium.

Description	Newspaper, Glc.=5.8 g l^{-1}; NH_4^+=0.7 g l^{-1}	Newspaper, Glc.=11.7 g l^{-1}; NH_4^+=0.7 g l^{-1}	Slurry B, Glc.=7 g l^{-1}; NH_4^+=0.6 g l^{-1}	Slurry B, Glc.=12 g l^{-1}; NH_4^+=0.6 g l^{-1}	Slurry A, Glc.=15 g l^{-1}; NH_4^+=0.7 g l^{-1}
Alanine [mg g^{-1}]	55.7 ± 8.2	48.6 ± 1.3	46.5 ± 2.9	42.6 ± 4.8	43.5 ± 5.5
Glutamic acid [mg g^{-1}]	33.9 ± 0.2	34.7 ± 0.5	36.7 ± 0.9	32.6 ± 0.4	34.6 ± 0.6
Lysine [mg g^{-1}]	31.9 ± 0.1	31.4 ± 0.5	33.1 ± 0.9	30.4 ± 0.5	31.2 ± 0.8
Aspartic acid [mg g^{-1}]	31.4 ± 0.2	32.4 ± 0.6	29.4 ± 4.7	23.8 ± 4.6	28.3 ± 4.1
Isoleucine [mg g^{-1}]	27.9 ± 0.4	28.7 ± 0.5	29.8 ± 0.8	26.1 ± 0.3	27.9 ± 0.5
Valine [mg g^{-1}]	19.4 ± 0.1	19.8 ± 0.3	20.2 ± 0.6	17.7 ± 0.2	19.1 ± 0.4
Threonine [mg g^{-1}]	18.0 ± 0.2	18.8 ± 0.3	28.9 ± 17.5	41.5 ± 24.9	42.8 ± 25.1
Phenylalanine [mg g^{-1}]	16.7 ± 0.2	17.5 ± 0.2	18.1 ± 0.6	15.3 ± 0.2	16.5 ± 0.5
Proline [mg g^{-1}]	16.2 ± 0.2	16.9 ± 0.3	18.0 ± 0.5	15.6 ± 0.2	17.1 ± 0.5
Arginine [mg g^{-1}]	15.5 ± 0.0	17.3 ± 0.3	18.2 ± 0.5	15.1 ± 0.4	16.5 ± 0.5
Methionine [mg g^{-1}]	12.1 ± 0.2	9.7 ± 0.2	3.0 ± 0.2	2.1 ± 0.4	2.4 ± 0.7
Glycin [mg g^{-1}]	12.2 ± 0.6	12.3 ± 0.5	11.5 ± 0.5	12.4 ± 0.2	12.0 ± 0.1
Tyrosine [mg g^{-1}]	10.9 ± 0.2	11.7 ± 0.1	13.0 ± 0.4	11.0 ± 0.5	12.2 ± 0.4
Cystine [mg g^{-1}]	10.8 ± 0.1	11.3 ± 0.4	10.7 ± 0.5	8.4 ± 0.2	9.4 ± 0.3
Histidine [mg g^{-1}]	7.0 ± 0.1	7.5 ± 0.2	7.7 ± 0.3	6.5 ± 0.1	7.1 ± 0.2
Tryptophan [mg g^{-1}]	6.6 ± 0.3	8.2 ± 0.6	6.5 ± 1.5	6.2 ± 0.7	8.1 ± 1.5
Leucine [mg g^{-1}]	4.4 ± 0.4	4.6 ± 0.1	3.3 ± 0.1	4.0 ± 1.0	6.2 ± 0.3
Total Protein [mg g^{-1}]	**330.9 ± 11.4**	**331.5 ± 6.9**	**334.7 ± 33.5**	**311.3 ± 39.6**	**335.0 ± 41.8**
Total Protein [%]	**33.1 ± 1.1**	**33.2 ± 0.7**	**33.5 ± 3.3**	**31.1 ± 4.0**	**33.5 ± 4.2**

7

General Conclusion

This thesis investigated the production, conversion and degradation processes of cellulose materials and related derivatives with the aid of enzymes. The possibilities to create more energy efficient production methods to obtain new innovative materials and utilize residue streams to protect the environment and produce high value bio products was demonstrated. Cellulose as a rigid but flexible and renewable polymer can be used for multiple applications as newspaper packaging material or cloths. Different derivatives can serve as plastic replacement in consumer products or can be used in industry e.g. as membranes or as building material. Functionalization can be performed, expanding possible product properties and application fields.

Enzyme cocktails showed a positive effect in pulp refining presumed knowledge about composition of enzyme formulations and properties of a specific pulp is available, to allow correct dosing and application. So far, enzyme application is used in pulp refining, but bases on individual experiences and on-site test trials. The behavior of some enzymes on model substrates and real pulps was shown in this work. A correlation between several standard activity tests with pulp and hand sheet properties was obvious. But a general forecast of enzyme performance is not possible yet. Therefore, the detailed interaction of single fibers from different pulps which each component present in a cellulase formulation is important. To improve enzyme performance, after all the mechanism are under control, mixing different activities will lead to efficient individually tailored enzyme solutions for manufacturers.

Paper as a starting material for functionalization showed its ability to alter its properties towards a higher hydrophobicity. The paper sheets could be oxidized chemically and enzymatically without losing its cohesion. The later bound proteins, as hydrophobins, were coupled covalently without losing its functions. Actually most packaging material for food is plastic based and therefore not renewable. Using more paper is an actual trend in the food sector, and packaging material demand is rising with increased online shopping shares over the whole retail sector. More hydrophobic cellulose-based materials can help to replace these fossil based materials in the future, accomplishing more sustainable packaging solutions. Large scale production, implementation to existing production facilities and price competitiveness are key points for a successful market introduction. Further, the approach can be beneficial in another higher value sector of paper production. Paper materials with individualized bound proteins can make paper products, even a single sheet, trackable over time and distance. The suitability for daily use is still necessary to be elucidated, but for e.g. banknotes and passports this approach seems feasible. In addition, the possibility to couple catalytic proteins to impart the paper with a specific function is interesting. Paper-based biosensors or renewable smart surfaces can be provided by proteins coupled to cellulose.

Additionally, the utilization and recycling of cellulose and its derivatives have been one of the researcher's focus for many years. Paper materials can be deinked several times and reprocessed to new products. The number of cycles is limited and with each round of processing, some of the material gets lost. The recycling of composite materials is possible but very intensive in labor and cost, so that incineration is often the preferred utilization. New strategies involving enzymes can help to further increase the reuse of cellulose. One main strategy can be to degrade a potentially harmful cellulose derived polymer as cellulose acetate to a degree where natural processes in the environment can deal with the reaction products. This approach aimed to support natural degradation mechanisms by esterases and cellulases to get rid of cellulose acetate in the environment in a feasible amount and time. This contributes on the path to make cellulose acetate a renewable and biodegradable polymer, whereby the degree of substitution and polymerization are main factors influencing enzyme action. The conditions in the soil have to promote enzyme action to completely degrade cellulose acetate. The degradation in soil and the characterization of involved microorganisms

are important features that need to be further investigated before cellulose acetate can be stated as fully biodegradable.

Another strategy was the utilization of cellulose rich material streams from the paper industry in combination with nitrogen rich waste from anaerobic digestion, to produce algae biomass. The benefit of this approach is the conversion of waste, which causes disposal cost, to a valuable product with demand in the market. Further, a connection is created, where a material not useful for nutrition (paper) was successfully converted into a feedstock feasible for nutrition (algae biomass). Simultaneously, a potential harmful nitrogen rich waste material as digestate, that can contaminate ground water resources was converted to valuable algae protein. Utilization of starch rich materials to glucose and microbial production is well known and performed in large scale for crops as corn. The generated bio-ethanol is used as fuel in large scale, but competes with global food production. Glucose production from cellulose is also applied, but high cost, among others for the needed enzymes, make its production not capable of competing in the global market. A new process as the production of high value algae might be more promising, but requires further investigations according to its potential field of application. In some arears, as the production of bio-diesel from oils form algae via transesterification, knowledge exist. In other fields as the feeding of aqua culture fish with heterotrophic algae more knowledge is required.

Due to the enormous production capacity of the worldwide cellulose industry and the high-energy demand of cellulose processing, environmental concerns and socioeconomically impacts can be strong. A consequent transformation towards a fully integrated biorefinery concept might overcome negative impacts and will open new opportunities. Therefore, all material and energy streams must be considered as resource and the implementation of new processes is essential. Limitations in resources must be overcome by improvement in efficiency and adaptations of material streams. Cooperation with other industrials sectors can help to close production cycles and can enable access to new raw materials, beneficial for all participants.

8

Appendix

8.1 Publications

1. Andreas Ortner, Daniela Huber, **Oskar Haske-Cornelius**, Hedda K. Weber, Karin Hofer, Wolfgang Bauer, Gibson S. Nyanhongo, Georg M. Guebitz - Laccase mediated oxidation of industrial lignins: Is oxygen limiting? *Process Biochemistry, **2015**, Volume: 50, Issue: 8, Pages: 1277-1283, doi: 10.1016/j.procbio.2015.05.003*

2. **Oskar Haske-Cornelius**, Alessandro Pellis, Gregor tegl, Stefan Wurz, Bodo Saake, roland Ludwig, Andries Sebastian, Gibson s. Nyanhongo, Georg M. Guebitz - Enzymatic Systems for Cellulose Acetate Degradation. *Catalysts, **2017**, Volume: 7, Issue: 10, Pages: 1-15, doi: 10.3390/catal7100287*

3. **Oskar Haske-Cornelius**, Simone Weinberger, Felice Quartinello, Claudia Tallian, Florian Brunner, Alessandro Pellis and Georg M. Guebitz - Environmentally friendly covalent coupling of proteins onto oxidized cellulosic materials. *New Journal of Chemistry, **2019**, Volume: 43, Issue: 36, Pages: 14536-14545, doi: 10.1039/c9nj03077h*

4. **Oskar Haske-Cornelius**, Sabrina Bischof, Bianca Beer, Miguel Jimenez Bartolome, Eyitayo Olatunde Olakanmib, M. Mokobac, Georg M. Guebit, Gibson S. Nyanhongo - Enzymatic synthesis of highly flexible lignin cross-linked succinyl-chitosan hydrogels reinforced with reed cellulose fibre. *European Polymer Journal, **2019**, Volume: 120, Issue: August: Pages 1-10, doi: 10.1016/j.eurpolymj.2019.08.028*

5. **Oskar Haske-Cornelius**, Alexandra Hartmann, Florian Brunner, Alessandro Pellis, Wolfgang Bauer, Gibson S. Nyanhongo, Georg M. Guebitz - Effects of enzymes on the refining of different pulps. *Journal of Biotechnology, **2020**, Volume: 320, Issue: December 2019, Pages 1-10, doi: 10.1016/j.jbiotec.2020.06.006*

6. **Oskar Haske-Cornelius**, Thang Vu, Christoph Schmiedhofer, Robert Vielnascher, Moritz Dielacher, Verena Sachs, Markus Grasmug, Stefan Kromus, Georg M. Guebitz - Cultivation of heterotrophic algae on enzymatically hydrolyzed municipal food waste. *Algal Research, **2020**, Volume: 50, Issue: June: Pages 1-10, doi: 10.1016/j.algal.2020.101993*

8.2 Oral presentations as presenting author

1. **Oskar Haske-Cornelius**, Alexandra Hartmann, Florian Brunner, Hedda K. Weber, Gibson S. Nyanhongo, Wolfgang Bauer, Georg M. Guebitz - Enzymes, Specialists in Cellulose Fibrillation – Prediction of Action on Fiber. *6th International IUPAC Conference On Green Chemistry, 4-8 September **2016**, Venice, Italy*

2. **Oskar Haske-Cornelius**, Sara Vecchiato, Renate Weiß, Gibson S. Nyanhongo, Wolfgang Bauer, Georg M. Guebitz - Enzymes in Circular Cellulose Fibre Biorefinery. *Paper & Biorefinery, 5-6 June 2019, Graz, Austria*

3. **Oskar Haske-Cornelius**, Alexandra Hartmann, Hedda K. Weber, Gibson S. Nyanhongo, Wolfgang Bauer, Georg M. Guebitz – Enzymes, Specialists in fibrillation – Prediction of their Refining Performance. *Paper & Biorefinery, 11-12 May 2016, Graz, Austria*

8.3 Poster presentations as presenting author

1. Andreas Ortner, Daniela Huber, **Oskar Haske-Cornelius**, Hedda K. Weber, Karin Hofer, Wolfgang Bauer, Gibson S. Nyanhongo, Georg M. Guebitz – Oxygen can limit enzymatic oxidation of industria ligins. *12th International Conference on Material Chemistry (MC12), 20-23 July **2015**, York, UK*

2. **Oskar Haske-Cornelius,** Wolfgang Bauer, Hedda K. Weber, Gibson S. Nyanhongo, Georg M. Guebitz – Turning the Austrian Pulp and Paper Industry Green. *International Symposium on Wood, Fiber and Pulping Chemistry (18th ISWFPC), 9-11 September **2015**, Vienna, Austria*

3. **Oskar Haske-Cornelius,** Wolfgang Bauer, Hedda K. Weber, Gibson S. Nyanhongo, Georg M. Guebitz – Application of Enzymes in the Austrian Pulp and Paper Industry. *The 6th Nordic Wood Biorefinery Conference (NWBC2015), 20-22 October **2015**, Helsinki, Finnland*

4. Katrin Greimel, **Oskar Haske-Cornelius**, Veronika Perz, Karolina Härnvall, Enrique Herrero Acero, Georg M. Guebitz, - Enzymatic systems for oxidative cross-linking of coating polyesters. *6th International IUPAC Conference On Green Chemistry, 4-8 September **2016**, Venice, Italy*

5. **Oskar Haske-Cornelius**, Stefan Kromus, Georg M. Guebitz – Microbial Media Preparation from Waste – Enzymatic Hydrolysis as Starting Point. *Biogas Science 2018, 17-19 September **2018**, Torino, Italy*

8.4 Fellowships

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Statutory declaration

I declare that I have authored this thesis independently, that I have not used other than the declared sources/resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used source.

X

Oskar Haske-Cornelius

Vienna, 31 August 2020