Doctoral Dissertation

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Towards enhanced predictability of enzyme reactions for energy reduction and recycling in lignocellulose processing

submitted by

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I

Preamble

This doctoral thesis covers the challenges in enzyme usage for recycling and energy reduction in pulp refining and investigates the mechanisms of the responsible enzymes as well as possible prediction methods. The first section (introduction) explains the necessary steps for paper production with special focus on pulp refining (including key data about energy consumption), followed by the explanation of structure, composition, and biosynthesis of cellulose. Furthermore, a detailed overview about enzymes in pulp refining and recycling of cellulosic waste and their characterization methods is given.

The first publication investigates enzyme formulations that are used in the pulp and paper industry. Enzymes in these formulations are identified and characterized regarding their enzyme activity on classic model substrates and on pulp. Endoglucanase enzymes believed to be responsible for energy reduction were purified and their effect evaluated in laboratory refining trials using a new activity assay with high specificity for these enzymes along with their respective enzyme formulations.

The second publication introduces the use of carbohydrate binding modules as a new prediction method for determination of the most efficient enzymes in pulp refining. The use of different carbohydrate binding modules with varying affinity for the amorphous or crystalline cellulose regions enables the visualization of the enzyme effect on pulp. Analytical methods for determination of the release of sugars or the elucidation of changes to cellulose composition are applied and the results compared with enzyme performance in laboratory refining experiments.

The third paper evaluates the synergistic effect of enzymes in pulp refining with the aim to provide information about the necessity of additional enzymes in complex

enzyme formulations such as hemicellulases or β-glucosidases. Changes to molecular weight and size profiles were evaluated using size exclusion chromatography on model substrates and pulp. Finally, enzyme binding to amorphous cellulose was studied and correlated to refining performance.

The final section of the thesis concludes the findings of the publications and highlights future perspectives. Furthermore, a list of publications, oral presentations, posters, and grants is given as appendix.

II

Abstract

Paper products are omnipresent in our lives, but their production is energy demanding, which impacts the environment. Enzymes came into focus as an environmentally friendly alternative to reduce energy consumption or degrade waste materials. Pulp refining is one step of paper production, which improves paper properties such as tensile strength. Although up to 40% of energy can be saved with enzymes in the process, the high variety of commercial enzyme products makes prediction of enzyme behaviour challenging. More information about enzyme mechanisms for selection of the best enzymes is required.

Commercial enzyme formulations were characterized regarding activity on model substrates as well as on hardwood or softwood pulp. Endoglucanase enzymes responsible for energy reduction were purified. Dosing based on the traditional reducing sugar assays resulted in mixed refining performance. Therefore, the new CellG5 substrate (derivatized cellopentaose) was tested, resulting in improved dosing, however, some variation remained, emphasizing the need for an in-depth mechanistic study. Investigation of sugar release exhibited the highest concentrations for most efficient enzymes. Size analysis showed the highest decrease in molecular weight on carboxymethylcellulose, however, the effect of formulations was a higher, suggesting additional degradation through synergistic action. On hardwood pulp low molecular weight fractions were formed and the total molecular weight was unsuitable for prediction. Changes in amorphous and crystalline cellulose were visualized by carbohydrate binding modules, which assist enzymes in substrate binding, with the most efficient enzymes having the highest decrease in the amorphous region. A correlation between binding of endoglucanases to amorphous cellulose and refining performance was found, as the most efficient enzymes resulted in high binding.

III

Kurzfassung

Papierprodukte sind allgegenwertig in unserem Leben, aber deren Produktion ist sehr energieaufwendig. Enzyme kamen als umweltfreundliche Alternative in den Fokus, um den Energieverbrauch zu senken oder Abfall abzubauen. Die Mahlung von Zellstoffen ist ein Schritt der Papierherstellung, um Papiereigenschaften wie die Zugfestigkeit zu erhöhen. Obwohl dabei mit Enzymen bis zu 40% an Energie eingespart werden kann, macht die hohe Vielfalt an kommerziellen Enzymformulierungen die Vorhersage des Enzymverhaltens schwierig. Mehr Wissen über Enzymmechanismen ist notwendig zur Auswahl der besten Enzyme. Kommerzielle Enzymformulierungen wurden charakterisiert bezüglich Aktivitäten auf Modellsubstraten sowie auf Laubholz und Nadelholz Zellstoffen. Endoglucanase Enzyme, die für die Energieeinsparung verantwortlich sind, wurden aufgereinigt. Dosierung basiered auf traditionelle reduzierende Zuckerassays führte zu gemischtem Verhalten in Mahlungsexperimenten. Daher wurde das neue CellG5 Substrat (derivatisierte Cellopentaose) getestet. Das führte zu verbesserter Dosierung, aber verbleibenden Unterschieden, was die Notwendigkeit einer mechanistischen Studie hervorhebt. Die Untersuchung der Zuckerfreisetzung zeigte die höchsten Konzentrationen bei den effektivsten Enzymen. Die Größenanalyse hat zur höchsten Abnahme des Molekulargewichtes von Carboxymethylcellulose geführt, jedoch war der Effekt bei den Formulierungen durch synergistische Zusammenarbeit höher. Beim Laubholz Zellstoff wurden niedrigere Molekulargewichtsfraktionen gebildet und das gesamte Molekulargewicht war nicht geeignet für die Vorhersage. Änderungen von amorpher und kristalliner Cellulose wurden mit Kohlenstoffbindungsmodulen visualisiert, die Enzyme in deren Substratbindung helfen. Die effektivsten Enzyme zeigten die stärkste Abnahme der amorphen Region. Eine Korrelation zwischen Endoglucanase Bindung zu amorpher Cellulose und Mahlungsleistung wurde gefunden, da die effektivsten Enzyme zu hoher Bindung führten.

1

Aim of the thesis

The aim of this thesis was to establish a better knowledge about the enzyme mechanisms during the refining of cellulose fibers for energy reduction or for the degradation of cellulosic waste for recycling purposes. Thereby, means for prediction of enzyme behaviour of the responsible enzymes should be provided that facilitate their usage for paper manufacturers. Commercial enzyme formulations are commonly used in the pulp and paper industry, as they have a whole set of different enzymes for various processes like pulp refining, bleaching, or deinking. However, the number of commercially available enzyme formulations is huge, each containing different individual enzymes originating from various organisms and in varying concentrations. The selection of the appropriate enzyme formulation is challenging, considering that, also enzyme formulations are available containing only one enzyme like endoglucanases in purified form. In addition, the pulp and paper industry use several pulp types, differing in their origin (hardwood or softwood) or pulping method (e.g., sulfite or sulfate), which have their impact on enzyme accessibility.

Elucidation of the mechanisms of the enzymes responsible for energy reduction during pulp refining or for recycling is therefore essential for identifying beneficial enzyme characteristics of the most efficient enzymes, which enables prediction of enzyme behaviour and reference points for enzyme selection for paper manufacturers. Considering that the addition of enzymes can save significant amounts of energy, the facilitation of enzyme usage in the pulp and paper industry has a huge impact on the environment and can save energy costs in the same process.

2

Introduction

Paper is a remarkable and versatile material that can be used for the acquisition of information, for communication, packaging or as tissue material (e.g., toilet paper) among many other use cases. Hence, we encounter paper already very early in our lives, however, as remarkable as the use cases of paper are, the production of paper also brings environmental concerns through high energy and chemical consumption [1]. In 2020, a total amount of 412.5 million tons of paper and board products was produced around the world, which required 89 777 GWh of electricity. In Austria, the pulp and paper industry is one of the most important industrial sectors with a production of 4.7 million tons of paper in 2020 (5.5 % of the total production within the European Union) [2], [3]. However, the pulp and paper industry in Austria is also among the sectors with the highest energy requirements and needed 28% of the total industrial energy consumption in 2017. Moreover, this industry branch released 7 % of the carbon emissions in the same year. Nonetheless, a lot of effort was already invested into the improvement of energy efficiency of production processes, which resulted in the reduction of carbon emissions by 20 % in the last decades, but still a lot of potential for energy saving exists [4].

2.1 History of Papermaking

Papermaking is a very old craft and has its origins in China around 200 BC, from which it first spread to East and Central Asia by Buddhist monks, further on to Islamic countries and later to the Mediterranean region including Europe. The knowledge reached European Christians in the twelfth century; however, the Christians did not believe that this technology could originate from China; instead, they presumed that the Chinese learned papermaking from Egypt. The proliferation of this knowledge was also strongly depended on available raw materials among factors such as trade, religion, and emigration. For example, in China, paper was made of bast fibers of semi tropical plants, while in the drier regions of Central Asia, the process was adapted to include linen, cotton rags or even old ropes. The most pronounced proliferation of

papermaking technology was achieved by Muslims, which used paper for record keeping and replaced previously used papyrus rolls. However, when the Europeans learned papermaking, their paper makers produced paper so efficient due to their ability to use the power of European water streams in paper mills, that they soon dominated the market [5].

2.2 Steps in Papermaking

The raw material of paper is wood; however, several different steps need to be considered for the transformation of raw wood material to high quality paper sheets that we all know of. The required steps are comprised of:

1.) Pulping
2.) Refining
3.) Dilution (formation of thin pulp slurry)
4.) Suspension in solution
5.) Forming of a fiber web using a thin screen
6.) Pressing (increase of fiber density)
7.) Drying
8.) Finishing (creation of surface for intended use case)

Wood is comprised of cellulose, the most abundant material in the world, which also forms the basis of plant cell walls. Paper consists of cellulose fibers that are formed into sheets, while the weight of the resulting paper sheet defines the final classification into paper (up to 220 g/cm^2) or board (heavier than 220 g/cm^2) [6].

2.3 Composition of wood

Cellulose consists of D-glucopyranose units also named anhydroglucose that are linked together through a β-(1 → 4)-glycosidic bond that enables turning of the chain axis by 180°, thus resulting in a repeating pattern of two anhydroglucose units that form the disaccharide cellobiose (**Figure *1***). These cellulose chains have a varying length depending on their origin and processing method with wood pulps having a length of 300-1700 glucose units. The length of the cellulose chain is called the degree of polymerization. The resulting chains self-assemble themselves into fibrils [7], [8]. The first level of organization is consisting of elementary fibrils with a size of 3-35 nm, which

themselves are arranged into microfibrils (10-35 nm) and finally into macrofibrils that are surrounded by hemicelluloses and lignin. Hemicelluloses consist of xylan (rich in hardwoods like birch), mannan (rich in softwoods like spruce) and xyloglucan (in angiosperms). In contrast to cellulose, hemicelluloses are branched to a varying extent, depending on the respective organism. For example, hardwood xylan is composed of a β-(1 → 4) linked xylose backbone, that is highly substituted with acetylesterifications and to a lesser extent with α-1,2 linked glucuronic acid/4-O-Methylglucuronic acid. Hemicelluloses are regarded to be degradable using enzymes, although complex branching may interfere. However, enzymatic degradation of hemicelluloses requires enzymes such as xylanases that are specialized for this kind of substrate and enhance accessibility for other enzymes such as cellulases, as these enzymes are inhibited by the presence of hemicelluloses [9], [10]. Lignin is the third major component of wood with a share of 20-35 % of the biomass. Lignin is an aromatic compound and composed of three different components: conferyl alcohol, coumaryl alcohol and sinapyl alcohol. The main purpose of lignin is to act as a glue for cellulose and hemicelluloses, therefore it is improving the overall strength of the plant cell wall [8]. The exact ratio of cellulose, hemicelluloses and lignin is determined by the respective plant species, with softwood exhibiting 33-42 % cellulose, 22-40 % hemicelluloses and 27-32 % lignin, whereas hardwood species consist of 38-51 % cellulose, 17-38% hemicelluloses and 21-31 % lignin [11]. A detailed overview over the main components of wood and their chemical structures is given in **Figure *2***.

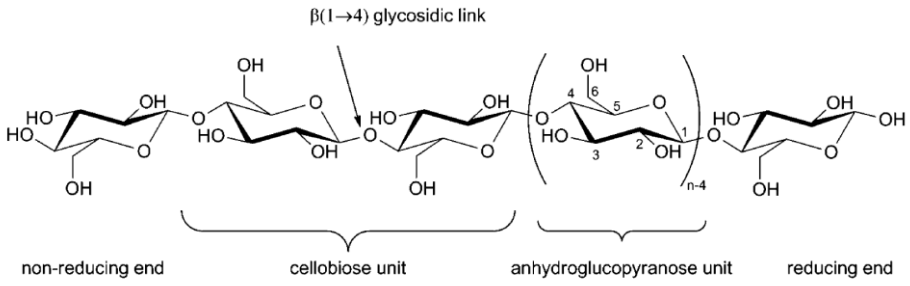

Figure 1 Composition of a single cellulose chain [12]**.**

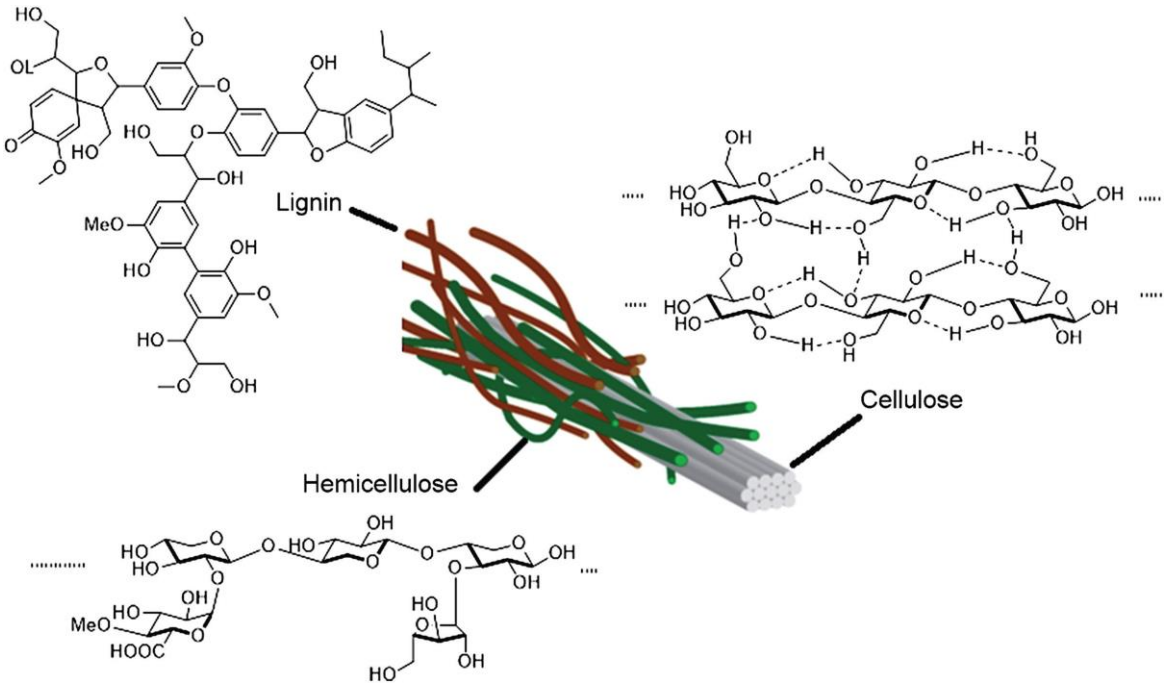

Figure 2 Chemical structures of the main components of wood: cellulose, hemicellulose, and lignin [8]

Cellulose, hemicelluloses, and lignin are connected to each other via hydrogen bonds [13]. This configuration is also known as native cellulose I, one of four polymorphs of cellulose. Cellulose I can be subdivided into the two allomorphs: Iα and Iβ. However, cellulose I can also be transformed into other polymorphs depending on the applied treatment. Cellulose II is obtained through mercerization using sodium hydroxide and exhibits antiparallel packing, while cellulose I is parallel packed. Cellulose III is formed through ammonia treatment of cellulose I and II, while cellulose IV is a modification of cellulose III using glycerol [14]. In plants, microfibrils are embedded in a helically wounded fashion with lignin that acts as protection against biological hazards and provides stiffness to the stem. Each fiber consists of a cell wall, with the primary cell wall being the first layer, followed by the secondary cell wall that is structured in three different layers, while the middle layer defines the final fiber properties. These properties depend on natural factors such as climate, but also on processing methods like mechanical disintegration [15]. A detailed overview over the fiber structure is given in **Figure 3**.

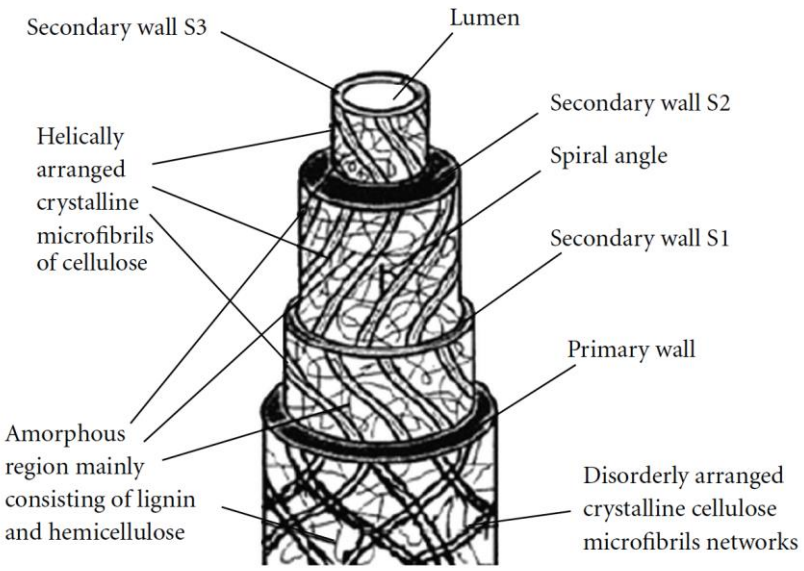

Figure 3 Structure of the cellulose fiber in plants [15]

2.4 Cellulose biosynthesis and role in plants

Cellulose is part of the plant cell wall, where it provides the necessary strength for withstanding the turgor pressure, thereby giving cells their characteristic size and shape. Cellulose producing organisms have large protein complexes, which have a rosette like structure, known as cellulose synthases (CesA). These membrane complexes have a size of approximately 25-30 nm. For the synthesis of cellulose, three different CesA subunits are required, which assemble cellulose using UDP-glucose (**Figure 4**). In detail, the rosette structure enables the formation of cellulose I in two consecutive crystallization steps: in the first step, single molecular glucan sheets are formed, while in the second step six glucan sheets are then guided through the rosette complex aperture, after which the cellulose I microfibril is formed by hydrogen bonds. The specific structure of the rosette complex enables the alignment of the glucan chains into the cellulose I, while it prevents the formation of thermodynamically more stable cellulose II. Crystallization of the chains is occurring quickly, however, not instantaneous and several proteins associated with assembly of the cellulose synthase complexes aid in the crystallization process. Studies suggest that crystallization is achieved during the formation of the hydrogen bond network, which creates the cellulose I microfibril. Depending on primary or secondary cell wall, different cellulose synthase subunits are required, and it was found that many mutations to primary cell wall synthases are lethal to the plants, while mutations to secondary cell wall synthases

lead to collapsed xylem cells that are no longer able to withstand the transpiration pressure [16], [17].

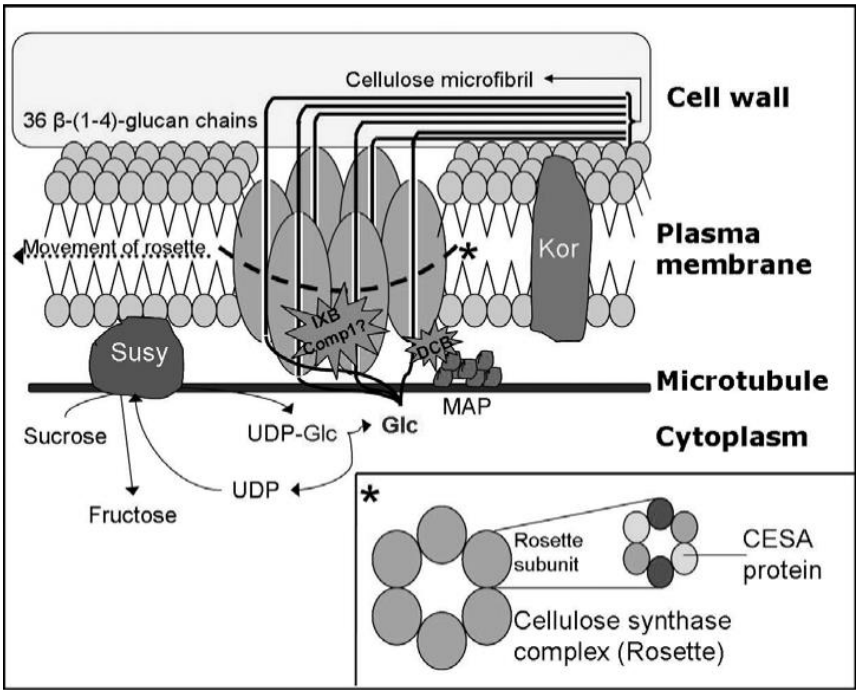

Figure 4 Picture of the rosette cellulase synthase complex embedded into the plasma membrane, releasing cellulose microfibrils [18].

2.5 Cellulose Crystallinity

Cellulose is comprised of crystalline and amorphous regions, originating from the arrangement of the fibrils into different layers of order. Crystalline regions of cellulose are formed by a network of intra-molecular and inter-molecular hydrogen bonds, resulting in an ordered structure, whereas amorphous regions of cellulose are comprised of fewer or broken hydrogen bonds, leading to a less ordered structure [19], [20]. The arrangement of cellulose chains into fibrils is highlighted in **Figure 5**. The crystalline region of Cellulose I consist of the two different allomorphs Iα and Iβ. Cellulose Iα is triclinic, while cellulose Iβ is monoclinic. The exact ratio of these two components depends on the origin of cellulose, with cellulose Iβ being more dominant in plants and cellulose Iα more in algae and bacteria. The crystallites have a size of approximately 5 nm (width), however some of the crystallites are less ordered, also known as amorphous. Therefore, cellulose can be characterized by the ratio of amorphous and crystalline regions, also known as the crystallinity index (CI). This crystallinity index can be determined using various analytical methods like x-ray diffraction, Fourier-transform infrared spectroscopy, Nuclear magnetic resonance

spectroscopy or Raman spectroscopy, however, the resulting values can vary greatly depending on the applied analytical method [21], [22] .

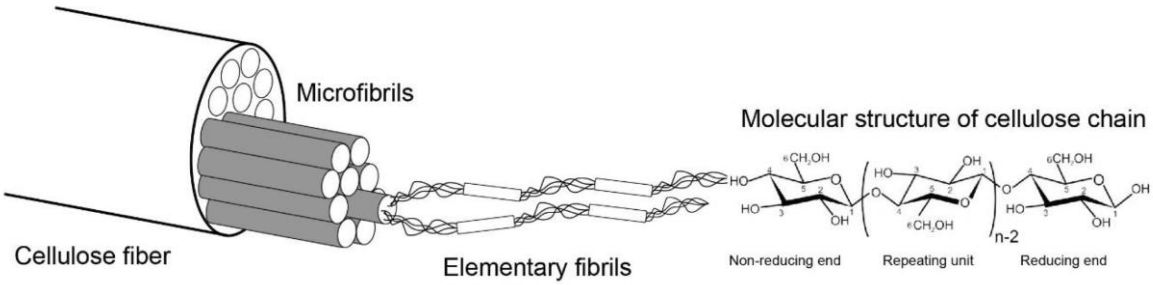

Figure 5 Overview over the arrangement of the cellulose fibers into fibrils [23].

2.6 Wood sources

Paper production begins with the selection of the source of the cellulose fibers. Wood can be divided into softwood and hardwood plants. In addition, also sawdust or sawmill chips can be used, which are by-products from wood processing. The selection of the appropriate wood type is crucial, as the fiber origin also defines paper properties. Softwoods are typically used if papers with higher strength are required. Softwoods can be divided into low- and high-density types, with low density softwoods (e.g., spruce, firs, scots pine) exhibiting high tensile, burst and surface strength, while higher density softwoods (e.g., slash pine, longleaf pine) exhibit higher tear strength and bending stiffness. In Northern Europe and other parts of the norther hemisphere (North America and Russia), softwood plants are dominant.

On the other hand, hardwood plants have shorter fibers, which translates to enhanced uniformity, surface smoothness and opacity, all of which are beneficial properties for printing paper products. Plants like eucalyptus, birch or aspen are part of hardwoods, however, if higher strength sheets are required, birch hardwoods are preferred due to their thinner cell walls. Shorter hardwoods like eucalyptus are the choice for soft tissues that require higher tactile softness properties. To improve the strength of hardwood papers, manufacturers also combine hardwood plants of different origin (e.g., tropical hardwoods), which is achieved through incorporation of longer fibers. Hardwood plants are dominant in Latin America, Africa, and Southeast Asia and generally twice as much hardwood as softwood plants are growing. Other wood source types comprise of

sawmill chips (longer and coarser fibers) and sawdust (lower fiber length through sawblade cutting) [24].

However, due to wood raw material shortages, also sources from non-wood sources came into focus. Hereby, non-wood fibers such as straw, bagasse, reed or bamboo are common, with straw being the most widely used non-wood source material. These plants are very popular in developing countries such as China and India, where they make up 70% of the total raw material for paper production. Non-wood fibers are also essential for regions with poor or no growth of trees as it is the case for Columbia, Pakistan, and Egypt as well as the two previously mentioned countries. Indeed, the use of this kind of raw material has several advantages like being a fast annual growing resource, lower lignin content or the ability to exploit food crops for the paper production. It is expected that the share of non-wood plants will rise as the exploitation of classic wood material meets its limits with increasing paper production volumes [25]. Naturally, wood from different species has a varying composition of cellulose, hemicelluloses, and lignin. A detailed overview of the composition of various plant sources is presented in **Table *1***.

Table 1 Composition of various wood sources [26], [27].

Wood source	Cellulose [%]	Hemicelluloses [%]	Lignin [%]
Eucalpytus	39-46	24-28	29-32
Pine	46	23	28
Miscanthus	45-52	24-33	9-13
Corn stover	37	31	18
Switchgrass	37-42	26-33	17-18
Poplar	42-48	16-22	21-27
Spruce (*Picea abies*)	44.4	22.6	30.6

2.7 The pulping process

Wood is debarked, cleaned from soil as well as other dirt and cut into smaller wood chips, which are then subjected to the pulping process [28]. The pulping process is required to remove lignin from cellulose and can be performed using enzymatic, mechanical, or chemical methods. Mechanical pulping is achieved through application

of mechanical force and thereby involves grinding and refining methods. High pulp yields and beneficial properties such as high opacity and ink absorption are obtained, however, due to high lignin content, yellow discolouring under light exposure and weaker paper strengths are major disadvantages.

2.7.1 Mechanical pulping

Mechanical pulping consists of several different methods: Stone groundwood pulping (SGW), pressure groundwood pulping (PGW), refiner mechanical pulping (RMP), thermo-mechanical pulping (TMP) and chemi-thermo-mechanical pulping (CTMP). During stone groundwood pulping, debarked wood is pressed against a rotating grindstone, which results in the separation of wood in thin fibers. The process generates high pulp yields (around 95 %), but most of the lignin remains, in addition low paper strength and easy discolouring are the main disadvantages. SGW is utilized to produce low-grade products such as newsprints or (low-cost) packaging paper. The refiner mechanical pulping process differs from SGW by the use of rotating metal discs or plates in a refiner, furthermore it can also be combined with thermo- or chemical pulping. Thermo-mechanical pulping (TMP) uses the same refiners, but wood chips are pre-heated under pressure using steam, subsequently refined at elevated temperature and pressure, followed by refining at ambient temperature and normal pressure. The advantage of the TMP pulping is the higher paper strength that is why it is used for furnishes in printed papers, for tissue papers, paperboard, packaging materials and magazines. During chemi-thermo-mechanical pulping (CTMP), steam is combined with chemicals (such as sulfite, peroxides, or carbonates) to remove lignin, resulting in high strength paper, suitable for high-grade products [29], [30].

2.7.2 Chemical pulping

In chemical pulping wood chips are subjected to chemicals at higher temperature and pressure levels, which enables the removal of lignin and hemicelluloses. With their removal, cellulose fibers come in close contact and bond with each other, thereby resulting in highest paper strength. However, the main disadvantage of chemical pulping is the low pulp yield (around 40-55 %). Chemical pulping is divided into the most commonly used kraft (sulfate) as well as the sulfite process [29]:

2.7.2.1 Sulfate (kraft) process

The sulfate process is the dominant chemical pulping method. In the sulfate process cellulose fibers are separated from lignin using sodium hydroxide and sodium sulfide liquor under high pressure (800 kPa) and temperature (155-180°C). The main advantage of the sulfate process is the high strength of the resulting pulp, which earned this process its characteristic “kraft” name [29]. Additional advantages include the ability to recover chemicals and energy as well as the use of a high variety of different hardwood and softwood raw materials. One part of the recovered energy is originating from combustion of the dissolved lignin and hemicelluloses, although recently lignin is also recovered and used to form new products such as animal feed products, binders or lignin-based batteries. For lignin recovery, acid precipitation can be used, as Lignin flocculates if the pH is reduced to values around the pKa of the phenols [31]–[33].

2.7.2.2 The sulfite process

The second major chemical pulping method is the sulfite process. Similarly, to the kraft process, the wood chips are cooked at high temperatures and pressure, however, an aqueous solution of sulfur dioxide and calcium, sodium, magnesium, or ammonium bisulfite is utilized. The sulfite process has several advantages: the ability to use the process over the entire pH range, to handle wood species with low amount of extractives (such as polyphenols, pigments and resins), higher brightness and yield as well as easy refining and bleaching capabilities. The disadvantages are lower pulp strength and longer cooking times as well as the inability to recover chemicals. The sulfite process itself can mainly be divided into acid and alkine sulfite pulping. Acid sulfite pulping is performed under low pH conditions (1.5-2.0) and at temperatures between 125-145°C with cooking times up to 7h, which leads to low hemicellulose composition, but also low pulp strength. During acid sulfite pulping hemicelluloses are in large quantity degraded to mono- or oligomeric compounds and while reprecipitation of xylan on cellulose fibers occurs in kraft pulping, this is not the case for sulfite pulping [29], [34]. For pH values between 3-5, bisulfite pulping can be applied, which uses higher temperatures (160-180°C), and a shorter cooking time (0.25-3h), resulting in higher paper strength suitable for medium grade products such as newspapers. Alkine sulfite pulping consists of equal ratios of sodium hydroxide and sodium sulfite and is utilized to obtain paper strengths comparable to kraft pulping. In addition, the near neutral (slightly alkine) sulfite process can be used on hardwood species for corrugating paper products. Other variations of near neutral sulfite pulping include the

stora or the neutral sulfite anthraquinone process, which are suitable for hardwood and softwood species [29], [35].

2.7.2.3 Dissolving pulp

If wood chips are pre-treated (e.g., with steam) before the kraft or sulfite process, then the obtained pulp is referred to as dissolving pulp. Dissolving pulp exhibits an exceptionally low hemicellulose and lignin content (90-99 % of pure cellulose). Usually, acid sulfite pulping is used to produce dissolving pulp, because of higher hemicellulose degradation at acidic pH, however, acid sulfite pulping cannot remove lipophilic wood extractives. Dissolving pulp is used for the production of cellulose esters and ethers (e.g., cigarette films, binders, pharmaceuticals) or textiles [29], [36], [37].

2.7.3 Composition of cellulose after pulping

The pulping process affects the composition of the cellulose Iα and Iβ allomorphs. It was found that a part of the Iα allomorph fraction is converted into cellulose Iβ during the kraft pulping process, however, this was not the case for acid sulfite treated pulps, but the crystallinity decreased in that case [38]. For example, wood from spruce exhibits an amount of 74% Iα and 26% Iβ. However, after kraft pulping and bleaching of spruce wood, the content of cellulose Iβ increased to 61%, while cellulose Iα decreased to 30%, a small fraction (9%) was even transformed to cellulose II [39]. As expected, kraft pulping also changes the composition of the obtain pulps in regard of hemicellulose and lignin content. For example, woodchips from birch contain 25.1 % hemicelluloses and 26.4 % lignin, which changed to 2.2 % hemicelluloses and 2.9 % lignin content after kraft pulping [40].

2.8 Bleaching

Bleaching is performed after chemical pulping, aiming at higher brightness of pulp, which is necessary for many paper-based products such as sanitary or printed papers. Usually, a sequence of chemicals like chlorine or hypochlorite is applied under cooking, which remove lignin, phenols and resins present in pulp through chemical reaction. However, chlorine based bleaching leads to high levels of water pollution and thereby requires extensive wastewater treatment. Therefore, chlorine free bleaching sequences were developed in recent years: ozone or oxygen bleaching, elemental chlorine free (ECF) or total chlorine free (TCF) bleaching, hydrogen reinforced extraction or the use of peracids. ECF bleaching is the most prominent technique as it

results in bright and strong pulp. Despite the elimination of elemental chlorine, ECF still requires chlorine dioxide and therefore generates halogenated products, although to a much lower extent [41]. Enzymes such as xylanases and laccases came in to focus, as they achieve bleaching of pulp in an environmentally friendly way. Therefore, enzyme formulations used in the pulp and paper industry also contain xylanases, that are also suitable for biobleaching [42], [43]. A comparison of the appearance of bleached and unbleached pulps is shown in **Figure 6**.

Figure 6 Comparison of different pulping methods with bleached pulp. Top: Bleached pulp; Middle: unbleached sulfite pulp; Bottom: unbleached sulfate pulp [44].

2.9 Pulp Refining

The pulp refining process is an integral part of the paper production, as it gives paper sheets its defined properties such as increased tensile strength and smoothness. During refining cellulose fibers are subjected to mechanical treatment involving shear forces and friction among the fibers, leading to internal and external fibrillation, which affect the properties of the final paper product [45]. Internal fibrillation is leading to increased fiber swelling, through creation of voids by mechanical force of the refiner. The voids allow water to enter, which enhances flexibility and plasticity of the fibers, thus increasing tensile strength. On the other hand, fibrils are pilled off from surface of the cellulose fibers during external fibrillation, causing increased bonding between

cellulose fibers [46], [47]. The refining process can be basically divided into three stages, depending on the action between two grooved parallel plates inside of the refiner, of which one is stationary (stator) und the other one a moving plate (rotor). The first stage is comprised of gathering and entrapment of fibers between bars, which are subsequently compressed by the rotor and stator during the second stage, while the third stage applies shear stress on the cellulose fibers (forces between bars and fibers as well as fiber-fiber forces). In the industry large refining devices such as disc, conical or cylindrical refiner types are utilized, which not only differ in the geometry, but also in the used grooves and bars. However, also laboratory refiners were developed to simulate larger processes, but the mechanisms may differ from those in industrial refiners. The most common laboratory refiner is the PFI mill, which is comprised of a rotating stainless-steel roll equipped with bars and a rotating disc containing a smooth bed. Another form of a laboratory refiner is the Valley beater. This device is made of roll bars as well as a bed plate. Valley beaters generate more fines and fiber-cutting, when compared to PFI refining. However, the PFI refiner enhances internal fibrillation and swelling, while it needs a lower sample amount and a decreased refining time. There are also additional laboratory refiners such as the Jokro mill (similarly to the PFI mill, but with smaller groove dimensions), but they are not that widespread. Examples of a laboratory PFI refiner as well as of a conical refiner are highlighted in **Figure 7**. For the optimization of pulp refining, characteristic properties can be measured and compared. One of these values is the Schopper Riegler (SR), which characterizes the drainability of the refined pulp on a scale between 0 and 100, with higher numbers indicating a higher water retention ability [48].

Figure 7 Example of a PFI mill that is used for laboratory refining (left) and an example of a conical refiner used in industry. Image modified from [48].

However, the pulp refining process is very energy intense and requires 30 % of the total energy consumption of a paper mill [49]. Therefore, there is a strong need for optimizations to reduce energy consumption during paper production.

One approach is the pre-treatment of pulp with enzymes such as cellulases, which managed to lower the energy consumption of the refining process between 10 and 40%, depending on which enzyme is utilized [50]. Enzymes are usually available in complex enzyme formulations that contain a set of different enzymes, which complicates predictability, as each individual enzyme contributes to the overall effect.

2.10 The paper machine

After refining, forming, pressing, and drying steps are conducted, which are performed on a paper machine in consecutive order. The classical paper machine is the Fourdrinier machine (**Figure 8**). During forming, the fiber slurry is subjected to the headbox, diluted, and sprayed through a slice lip onto a moving screen called wire on a table. Thereby, the water is drained (from about 0.15 % to about 20 % consistency), resulting in a continuous fiber web. Afterwards the wet fiber sheets are compressed in the pressing section and dried by passing through heated metal rollers. The final paper sheet is obtained after a smoothing procedure, which is called calendaring. Calendaring is an essential process resulting in higher smoothness and gloss with the aim to improve printability. Usually, paper is compressed using two rolls, thereby applying a pressure of 10-50 MPa, which leads to previously mentioned surface modifications, while the thickness is decreasing at the same time. With different calendaring settings, paper products of varying paper grades and properties are produced, adjusted to the versatile needs of the customers [51]–[53].

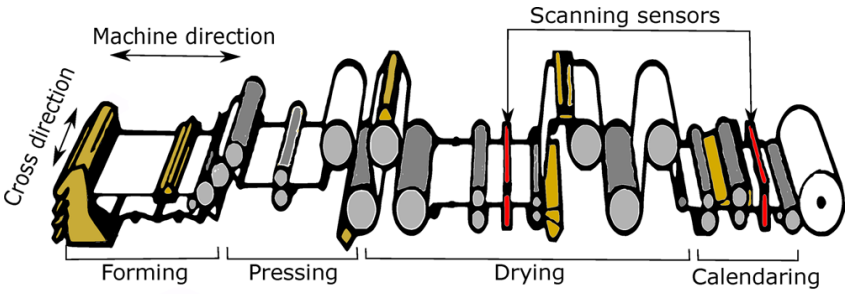

Figure 8 Set-up of a Fourdrinier paper machine with its characteristic steps [51].

2.11 Enzymes in pulp refining

2.11.1.1 Cellulases

Cellulase is an umbrella term for a class of enzymes, comprising of endoglucanases, cellobiohydrolases, β-glucosidases as well as oxidative enzymes such as lytic polysaccharide monooxygenases (LPMOs) or cellobiose dehydrogenases. Endoglucanases randomly cleave β-1,4-glycosidic bonds in the amorphous region of cellulose and are therefore known as β-1,4-D-glucanases. Endoglucanases are considered to be the primary drivers for energy reduction in pulp refining [54]–[56]. Cellobiohydrolases (CBH I and CBH II) are exoglucanases and attack the reducing and non-reducing ends of chains in the crystalline region of cellulose [57], [58]. β-glucosidases finally turn released cellobiose units into glucose, the main building block of cellulose. The combination of endoglucanases, cellobiohydrolases and β-glucosidases enables the degradation of cellulose through synergistic action, which is compelling for recycling purposes [59] . Additional enzymes such as LPMOs degrade cellulose through oxidative depolymerization, while cellobiose dehydrogenase oxidizes cellobiose into cellobionolactone (**Figure 9**) [60], [61] . The action of cellulase enzymes also depends on the crystallinity of cellulose and the type of the dominating cellulose allomorph (Iα or Iβ). Generally, cellulose Iβ is thermodynamically more stable and denser than Iα, but more difficult to access by enzymes, thereby resulting primarily in the enzymatic degradation of cellulose Iα [62]. However, for endoglucanases only the less ordered amorphous region of cellulose is of interest, as these enzymes can only degrade amorphous cellulose, which is primarily located on the surface of cellulose and between microfibrils [63].

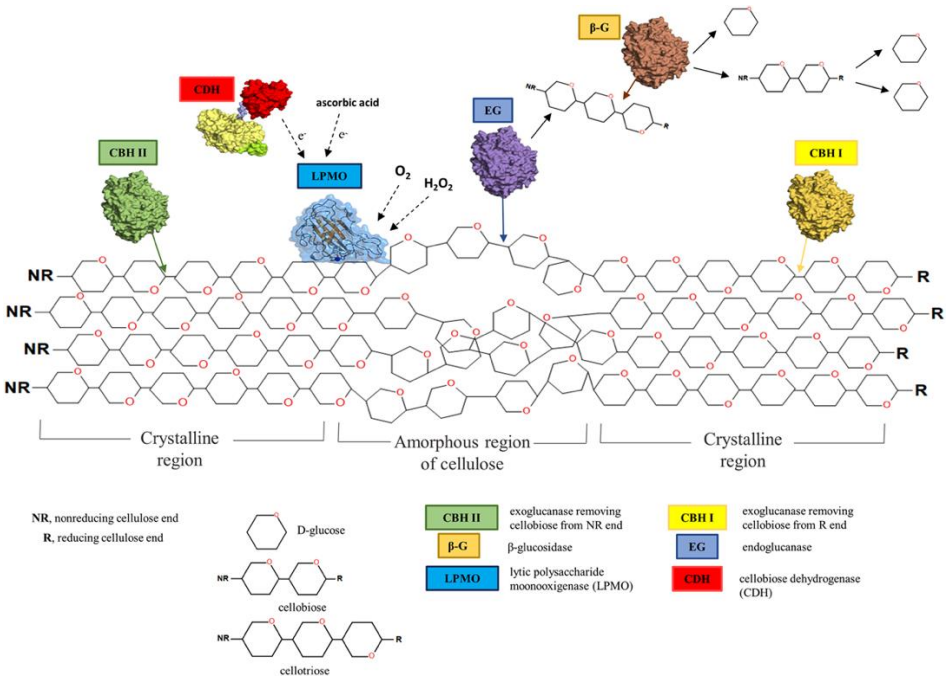

Figure 9 Enzymes of the cellulase family and their preferred location of attack on the cellulose fiber. Cellulose is divided into crystalline and amorphous regions, with endoglucanases having the ability to attack the amorphous region, whereas cellobiohydrolases have a high affinity for crystalline entities. Products of cellobiohydrolases and endoglucanases are turned over to glucose by β-glucosidases, while enzymes like lytic polysaccharide monooxygenases or cellobiose dehydrogenases attack cellulose oxidatively [64].

2.11.1.2 Origin of cellulases

Enzymes such as cellulases are usually isolated from organisms in nature, which require them to take up the essential components they need for living. The most characterized cellulase enzymes were discovered in the filamentous mesophilic fungus *Trichoderma reesei* over 70 years ago on rotting equipment of second world war soldiers. The most prominent *Trichoderma reesei* strain is called QM6a, which is the last remaining original strain from that time and also the strain, from which all mutants that are used today are derived from. Cellulases from *Trichoderma reesei* are secreted extracellularly and the ability of these enzymes to degrade cellulosic materials to glucose, was of special interest for the exploitation of these enzymes to produce fuel. The first isolated enzyme from this fungus was cellobiohydrolase I, which was also the first enzyme, of which a structure was obtained. For the production of fuels, the expression of high concentrations of these enzymes was a major goal, which also required further characterization of the cellulose system [65].

The *Trichoderma reesei* enzyme system consists of β-1,4-D-glucanases from families GH7 (Endoglucanase I/Cel7B), GH5 (Endoglucanase II/Cel5A), GH12

(Endoglucanase III/Cel12A) and GH45 (Endoglucanase V/Cel45A). Another enzyme was previously characterized as GH61 endoglucanase IV, due to its low endoglucanase activity, however, recent studies suggest that this enzyme rather belongs to the lytic polysaccharide monooxygenase family AA9. This enzyme aids in the oxidative degradation of crystalline cellulose, which also explains the rather low endoglucanase activity in comparison with other cellulase members of *Trichoderma reesei* [66], [67]. The reaction mechanisms of the endoglucanases are versatile, with Cel7B producing cellobiose and glucose, Cel5A and Cel12A in addition cellotriose, while the main product of Cel45A is mainly cellotetraose with almost no generation of glucose or cellobiose [68]. Examples of the structures of a cellobiohydrolase (Cel7A) and an endoglucanase (Cel7B) are shown in **Figure 10**.

Furthermore, two cellobiohydrolases from family GH7 (Cellobiohydrolase I/Cel7A) and GH6 (Cellobiohydrolase II/Cel6A) are part of the cellulose system, which can also attack crystalline regions of cellulose. Additionally, at least ten β-glucosidases are expressed by *Trichoderma reesei,* which convert products of cellobiohydrolases or endoglucanases into glucose. These β-glucosidase can be divided into GH1 (two enzymes) and GH3 families (8 enzymes). GH1 β-glucosidases (Cel1A and Cel1B) are intracellular enzymes, while most of GH3 family enzymes are extracellular [67], [69]. Evaluation of the ratios of the secreted enzymes of *Trichoderma reesei* concluded that 60% of the total protein content is originating from CBH I, while CBH II (20%) and endoglucanase enzymes (10%) only contribute to a small fraction. The low β-glucosidase production of this fungus was the main reason for genetic engineering efforts, as this imposes a serious limitation for industrial applications, which require glucose as the final product [70].

Although the cellulose system of the fungus *Trichoderma reesei* is well characterized and used for various industrial processes, it also exhibits limitations such as the maximal temperature stability of around 65 °C beside low enzyme titers. Although considerable improvements were implemented such as the development of recombinant strains, optimization of cultivation conditions or co-cultivation of different strains of *Trichoderma reesei*, also other fungal organisms came in to focus like *Aspergillus* or *Penicillium* strains, with cellulases of some *Penicillium* strains even exhibiting higher activity and thermal stability [71]. Moreover, bacterial production

systems are now available, which produce cellulases with high specific activities and thermostability. Aerobic, anaerobic, mesophilic, and thermophilic organisms can be chosen depending on the desired use case, with common microorganisms being *Chlostridium* spp., *Cellumonas* spp. or *Bacillus* spp. as well as thermophilic bacteria such as *Thermonospora* spp., *Thermobifida fusca* and *Ruminococus albus* [72], [73]. Finally, also plants are considered for cellulose production as they are inexpensive expression systems e.g., to produce the GH5 family endoglucanase E1 in *Zea mays* [74].

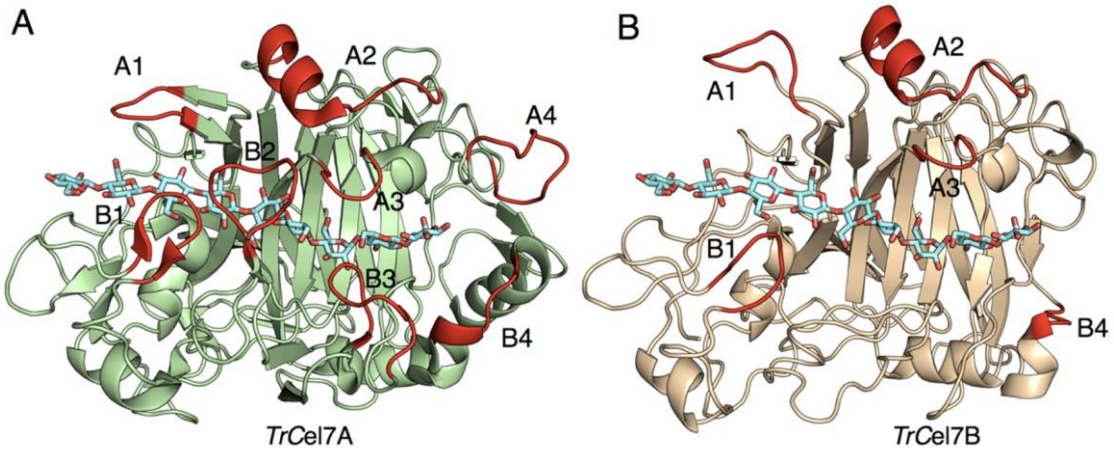

Figure 10 Enzyme structures of cellobiohydrolase Cel7A (A) and endoglucanase Cel7B (B) of *Trichoderma reesei.* Important loops of the enzymes are labelled and the binding of the cellulose like nonamer substrate cellononaose to the enzyme is indicated [75].

2.11.2 Hemicellulases

Hemicellulases are enzymes that attack and degrade hemicelluloses. Similar to cellulases, several different hemicellulases degrade hemicelluloses in a synergistic manner. Required enzymes consist of endo-xylanases (endo-1,4-ß-xylanase), ß-xylosidase, ß-mannosidase, α-glucoronidase, α-arabinofuranosidase, arabinase (endo α-L-arabinase), acetyl xylan esterase and ferulolyl xylan esterase. Because xylan is the dominant component of hemicelluloses, endo-xylanases (endo-1,4-ß-xylanase) and ß-xylosidases are the most characterized enzymes. ß-mannosidases are responsible for the degradation of linkages between mannan chains, α-arabinofuranosidases and arabinases hydrolyze arabinose sidechains, while α-glucoronidases remove α-(4-O-methyl)-D-glucoronic acid from the xylan backbone. Similarly, acetyl xylan esterase hydrolyzes acetyl groups from xylan sidechains and ferulolyl xylan esterase remove ferulate ester groups from arabinose sidechains connected to the xylan backbone [76]. The hemicellulases that are of interest for pulp

refining are endo-xylanases. Endo-xylanases randomly cleave the β -1,4-linked xylan backbone, the products of which are degraded to xylan by ß-xylosidases. Analogous to endoglucanases the presence of ß-xylosidases may be required in industrial formulations to avoid product inhibition [77], [78]. Endo-xylanases showed positive effects in pulp refining in a previous study through degradation of xylan, which increased susceptibility of pulp to refining [79]. In addition, hemicelluloses are mainly on the outer surface of cellulose and can limit the accessibility of pulp to cellulase enzymes, thereby addition of xylanases can aid synergistically to the action of endoglucanases [80]. Furthermore, other enzymes such as arabinosidases assist xylanases in the degradation of xylan as they remove arabinose from its sidechains [81].

2.11.3 Carbohydrate binding modules (CBMs)

Another factor for the success of enzymes is the presence or absence of carbohydrate binding modules (CBMs). Carbohydrate binding modules are small sequences of amino acids (30 to 200 units) that are associated with enzymes and have the ability to bind to certain carbohydrates. The main purpose of these CBMs is to bring the catalytic center of the enzymes they are associated to into proximity of their substrates (**Figure 11**). Therefore, a high number of different CBM classes have been identified, which can bind to amorphous or crystalline cellulose to a varying extent. For example, CBM 1 from the cellobiohydrolase Cel7A of *Trichiderma reesei* exhibits a high affinity for the crystalline region of cellulose, while CBM 28 of the endoglucanase Cel5a of Bacillus sp. 1139 binds strongly to amorphous cellulose. However, the substrate specificity of CBMs is not restricted to cellulose only. CBMs were identified from xylanases (with binding capabilities to hemicelluloses), esterases, mannanases, chitinases among many more. Hence, the binding properties of carbohydrate binding modules were exploited for various applications ranging from use as purification tags, design of recombinant enzymes with adapted targeting capabilities, enhancement of enzyme activity, immobilization aids or as probes and sensors (e.g., for characterization of the pulp surface). Furthermore, it was shown that some carbohydrate binding modules express the ability to modify fibers, for example, the surface of cotton fibers was roughened using isolated CBMs [82]–[84].

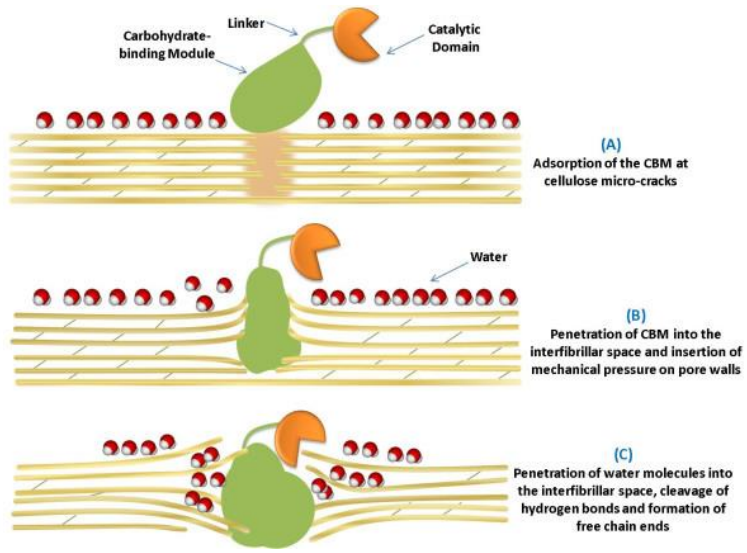

Figure 11 Carbohydrate binding modules acting on cellulose fibers. Carbohydrate binding modules bring enzymes in proximity to their substrates, thus allowing the catalytic center to reach them and fulfil its purpose [85].

2.12 Enzyme synergisms for saccharification of cellulose

Enzymes such as the cellulase endoglucanase act individually in pulp refining and a complete or enhanced degradation of cellulose into its building blocks is undesired, as it would negatively affect paper properties. Additional enzymes such as ß-glucosidases are often added by enzyme suppliers in complex enzyme formulations to avoid product inhibition of endoglucanases [86]–[88]. However, for recycling of waste such as newspapers or textiles, the complete conversion into the main building block glucose is needed to be able to produce new products such as biofuels. For total hydrolysis of cellulosic materials, several types of enzymes are required to act in concert. Endoglucanases attack the cellulose randomly within the cellulose chain, whereas cellobiohydrolases provide new points for attacks to endoglucanases by degradation of reducing and non-reducing ends of the cellulose chain, while ß-glucosidases turn the released soluble sugar fragments into glucose [59]. For the complete degradation of waste materials, also oxidative enzymes such as lytic polysaccharide monooxygenases (LPMOs) or cellobiose dehydrogenases came into focus. A study showed that the incubation of cellulose with LPMOs enabled cellulose enzymes to degrade crystalline cellulose areas, which are usually not accessible to endoglucanases [89]. Furthermore, waste materials also contain hemicelluloses or old ink, which need to be degraded as well. Xylanases and cellulases act in concert to remove hemicelluloses and ink, by removal of surface fibrils, which leads to detachment of ink [90]. However, the action of enzymes such as endoglucanases

during total hydrolysis of cellulosic materials depends on their efficiency to bind to their substrates and their catalytic action. Industrial enzymes are derived from organisms occurring in nature; hence, a high versatility of different enzymes is available, with each enzyme exhibiting different binding and catalytic properties.

2.13 Characterization of enzymes using activity assays

The action of enzymes and specifically endoglucanases can be analyzed using a wide range of analytical methods. One pillar for enzyme characterizations are activity assays that measure enzyme activity on either model substrates such as soluble carboxymethylcellulose (**Figure 12**) or filter paper in the case of cellulases or directly on the material that they are intended to be used on like pulp [91]. The activity assays comprise the incubation of these substrates with enzyme solution for one or more defined timepoints and the calculation of enzyme activity from a previously measured standard curve. In case of cellulases, the classical activity assay for activity determination is the reducing sugar assay on carboxymethylcellulose, which measures the release of reducing sugars by cellulases through colorization using the DNS (dinitrosalicylic acid) reagent [92]. The assay uses the ability of reducing sugars that are released by enzymatic hydrolysis of the cellulose chain to reduce dinitrosalicylic acid to 3-amino-5-nitosalicylic acid, which has a high absorbance at 540 nm [93]. The detection of reducing sugars can be utilized for all kinds of enzymes that release reducing sugars such as carbohydrases including cellulases, xylanases, pectinases, ß-mannanases and many more [94]. Therefore, this reagent lacks specificity, especially when complex enzyme formulations are used that contain a whole set of different enzymes. Hence, new substrates were developed recently e.g., derivatized cellooligosacherides, which achieve specificity for endoglucanase enzymes through blocking of cellulose ends through introduction of ketone groups [95].

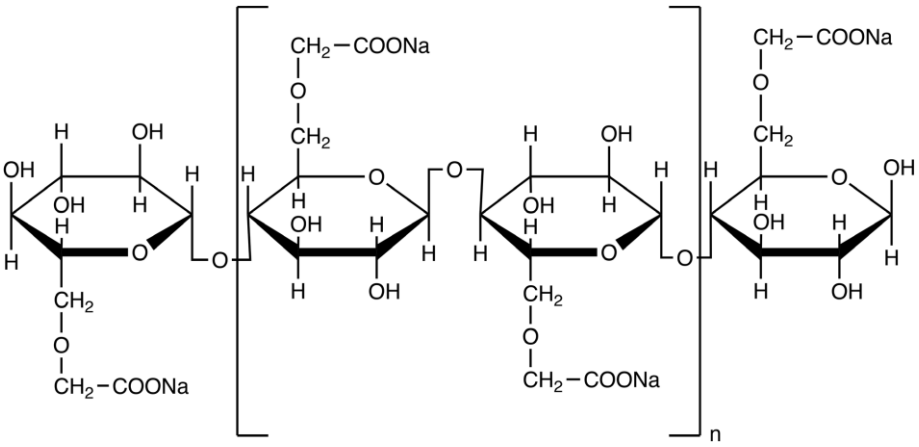

Figure 12 Structure of the soluble carboxymethylcellulose substrate [96]

2.14 Analytical methods for enzyme characterization

Analytical methods like HPLC (High-performance liquid chromatography) enable the identification and quantification of soluble products released from enzymes such as endoglucanases or xylanases [97]. However, changes to insoluble substrates are difficult to track using this method, therefore alterations to crystallinity or functional groups of cellulose can be assessed by FTIR (Fourier-transform infrared spectroscopy) or NMR (Nuclear magnetic resonance spectroscopy) [21], [98]. Enzymes attack cellulose in different ways, depending on their catalytic center, hence different sizes of fragments are released by the enzymes, which can be used to monitor the mechanisms of hydrolysis [99]. Size exclusion chromatography with a coupled multi-angle laser light scattering detector (SEC-MALLS) offers not only the analysis of the size of released fragments from soluble model substrates such as carboxymethylcellulose, but also of insoluble cellulose after dissolution in DMAc/LiCl, which enables the study of enzyme mechanisms directly on pulp [100], [101]. The action of enzymes often also depends on the number of bound enzymes, which can be elucidated by SPR (surface plasmon resonance). However, only regenerated cellulose films can be applied, which requires dissolution in DMAc/LiCl as well [102]. The binding of the catalytic center to the substrate can be enhanced by the presence of carbohydrate binding modules that act as an anchor for carbohydrate substrates, thus bringing enzyme and its substrate in vicinity [103]. This property of carbohydrate binding modules can also be used to monitor changes in crystallinity or the morphology of cellulose fibers if they are coupled with fluorescent dye and visualized with imaging techniques such as the confocal laser scanning microscope (CLSM) [104]. Analytical methods enable the identification of beneficial enzyme properties for most efficient energy saving in pulp refining or for complete degradation of cellulose for recycling purposes. Therefore, these methods

are essential to offer new ways of prediction of enzyme behaviour and ultimately the selection of the most suitable enzymes.

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3

Biorefining: The role of endoglucanases in refining of cellulose fibers

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3.1 Abstract

With an annual production of more than 400 million tons, paper is the main product of the largest biorefinery process industrially implemented. Enzymes have been used for pulp refining to dramatically reduce energy consumption. However, exact mechanisms related to the individual enzymes are hardly understood. Yet, this knowledge would be important to predict enzyme performance in industrial processes.

Three commercial refining enzyme formulations showed different endoglucanase (1.25 nkat mg^{-1} to 13.7 nkat mg^{-1}), β-glucosidase (0.57 nkat mg^{-1} to 1.34 nkat mg^{-1}) and xylanase activities (1.78 nkat ml^{-1} to 62.1 nkat mg^{-1}) on model substrates. Additionally, distinct amounts of reducing sugars from hardwood sulfate pulp were released. Endoglucases were purified from each formulation by using hydrophobic interaction and anion exchange chromatography and showed molecular weights from 20 kDa to 55 kDa and specific activities ranging between 3.11 nkat mg^{-1} and 26.3 nkat mg^{-1} according to endoglucanase specific derivatized cellopentaose (CellG5). Refining trials of hardwood sulfate pulp were conducted using a PFI laboratory mill and fiber properties such as degree of refining or fiber length and properties of formed sheets like tensile index were monitored. Thereby, enzymes were dosed based on identical

endoglucanase activity on CellG5. Enzyme formulations and purified endoglucanases led to an increase of the degree of refining of up to 47.9 [°SR] at 6000 PFI revolutions while the tensile index was improved by up to 76.0 Nm g^{-1}.
In summary, refining effects can be primarily attributed to endoglucanases indicating activity on CellG5 being a suitable parameter for enzyme dosing.

Keywords: Cellulase, Endoglucanase, Refining, Pulp, Cellulose fiber, CellG5

3.2 Introduction

Annually, more than 400 million tons of pulp are converted to paper and boards representing one or the largest biorefinery processes. During the paper making process, refining of pulps is a key process step to increase fiber-fiber bonding, tensile strength properties, homogeneity, air resistance and many other quality parameters important for the resulting paper. Cellulose is built of chains of unbranched β(1 → 4) D-glucopyranosyl units that form highly ordered crystalline structures, which are appearing in the two different forms I_α and I_β [1]. In the pulping process of wood chips, cellulose is (partially) separated from lignin and hemicelluloses. Traditionally, refining of paper pulps involves mechanical action loosening the structure of cellulose fibers. Refining leads to internal and external fibrillation of cellulose fibers as hydrogen bonds are broken causing increased flexibility and fibrils are peeled off, respectively. The principal drawback of mechanical refining technologies is the high energy consumption, usually ranging from 150 to 500 kWh/ton paper and accounting for up to 30 to 50% of the total energy used for paper making [2], [3]. Enzymes are emerging as ideal tools for cellulose fiber refining leading to a reduction of energy consumption of up to 40% [4], [5]. However, many studies have shown that different commercial formulations perform differently and effects are difficult to predict [6].

Cellulases (EC number 3.2.1.4) is an umbrella term for enzymes involved in cellulose degradation in nature, including endoglucanases, cellobiohydrolases, lytic polysaccharide monooxygenases and β- glucosidases [7]. Endoglucanases attack the amorphous regions of cellulose by cleaving β-1,4-glycosidic bonds, which leads to the formation of new chain ends that can be attacked by cellobiohydrolases, exo-acting enzymes that can degrade cellulose chains either from their reducing or non-reducing ends, releasing cellobiose units. Cellobiose is a disaccharide that can be cleaved by

β-glucosidases yielding glucose units, the elemental building blocks of cellulose. However, it is believed that in enzymatic refining cellobiohydrolases and β-glucosidases are not beneficial as they lead to excessive degradation of the fibres [8], [9]. On the other hand, endoglucanases are believed to play an important role as they fibrillate the fibres causing higher freeness without negatively affecting fibre properties [10]. Moreover, endoglucanases lead to the reduction of cellulose chain length, but the lateral hierarchical structure of the wood fibers is unaffected [11].

The use of cellulase enzymes during the refining process also leads to a reduction of chemical additives in paper production, as a lower amount of fines is generated, thus improving the drainage rate and eliminating the need for drainage aid chemicals [12], [13]. Today, a variety of enzymes preparations have been assessed for refining containing (besides cellulolytic enzymes) biocatalysts having other activities such as xylanase or laccases [14], [15]. Xylanases are also able to reduce the refining energy to reach a certain degree of refining. However, some xylanases also negatively affected paper properties depending on the rate of degradation of hemicelluloses [16]. Laccases aid the refining process though degradation of lignin and increasing the amount of carboxyl groups, thus leading to increased bonding of fibres [17]. Commercial refining enzyme preparations contain a variety of (hemi-)cellulolytic enzymes in different ratios. It is still not fully clear which individual enzyme characteristics are essential for refining and based on which activity enzyme formulations should be dosed by paper manufacturers. Considering a total amount of 419.7 million tons of paper and board was produced in 2018[18], exact and targeted dosing of enzymes is highly important for both technological and economic reasons to achieve beneficial effects while avoiding excessive fibre damage. This lack of ability to control enzyme activity is thus hampering the wider adaption of these environmentally friendly catalysts in the pulp and paper industry.

In this study, different refining enzyme formulations were characterized using activity assays and rheological studies. The contained endoglucanases were isolated to elucidate the mechanisms occurring in the modification of pulps and their effect in refining was compared based on activity on the endoglucanase-specific cellopentaose substrate (CellG5).

3.3 Materials and methods

3.3.1 Enzyme formulations, pulps and chemicals

Enzyme formulations from three different commercial enzyme suppliers were provided by paper manufacturers and termed EnzA, EnzB and EnzC. The commercial FiberCare® R endoglucanase formulation was kindly provided by Novozymes A/S (Bagsværd, Denmark). A commercial hardwood short fibre sulfate (kraft) pulp from eucalyptus (SF Sulfate) was provided by paper manufacturers. The CellG5 Cellulase assay kit and the XylX6 Xylanase assay kit as well as Cellobiohydrolase I were purchased from Megazyme (Bray, Ireland). All other chemicals were purchased from Sigma-Aldrich (Austria), in HPLC grade and used without further purification if not stated elsewhere.

3.3.2 Enzyme activity assays

3.3.2.1 Endoglucanase activity on derivatized cellopentaose (CellG5 assay)

Endoglucanase activity on the specific CellG5 substrate was determined using the Megazyme CellG5 Cellulase kit (Bray, Ireland) according to the principle described by Mangan [19]. Dilutions were performed in 50 mM citrate buffer (pH 4.8) or 50% tap water + 50% ddH_2O (pH 7). A volume of 100 µl of CellG5 substrate (blocked 4-nitrophenyl-β-D-cellopentaoside) solution was added to 100 µL diluted enzyme solution and incubated for 10 minutes at 45°C. The reaction was stopped by adding 3 ml of 2% (w/v) Tris solution (pH 10). Afterwards, the reaction volume was transferred into 3 ml cuvettes and the absorbance was measured at 400 nm using a Hitachi U2900 Photometer (Chiyoda, Japan). Endoglucanase activity was calculated in nkat ml^{-1}. Measurements were performed in duplicates and error bars indicate the standard deviation. The resulting activities on derivatized cellopentaose were then used for determining enzyme dosage in laboratory refining trials.

3.3.2.2 Endoglucanase activity on carboxymethylcellulose

Endoglucanase activity was also measured on carboxymethylcellulose (CMC) similar to measurements performed by Liu [20]. Therefore, a 2% carboxymethylcellulose substrate solution was prepared with an average molecular weight of 90.000 g/mol and a degree of substitution of 0.7 (Sigma-Aldrich, Austria). CMC was either dissolved in 50 mM citrate buffer (pH 4.8) or 50% tap water + 50% ddH_2O (pH 7). The assay is based on the reducing sugar determination with colour formation by dinitrosalicylic acid

[21]. A volume of 200 µl substrate solution was placed in 15 ml glass tubes and equilibrated at 50°C for two minutes. To start the reaction, 50 µl of properly diluted enzyme solution was added. A volume of 250 µl of 1 M NaOH was added after 0. 20, 40 and 60 minutes to stop the enzyme reaction. For blank reactions (timepoint 0) 1 M NaOH was added before addition of the diluted enzyme solution. For colour development, 250 µl of DNS solution was added to each sample, the tubes were sealed and incubated for 5 minutes in a boiling water bath. A volume of 200 µl of each sample was transferred to a 96 well plate and the absorbance was measured at 540 nm using an Infinite 200 Pro Tecan Reader (Zurich, Switzerland). A standard curve using glucose standards ranging from 0-20 mM was measured. Activities were measured in duplicates and expressed as nkat ml^{-1}.

3.3.2.3 β-Glucosidase activity

β-Glucosidase activity was measured according to Parry [22] with the following modifications: 2 mM 4-Nitrophenyl β-D-glucopyranoside was dissolved in 50 mM citrate buffer pH 4.8 or 50% tap water + 50% ddH_2O (pH 7) and used as substrate solution. A volume of 200 µL substrate solution was placed in 15 ml glass tubes and the solution was equilibrated at 45°C for two minutes. A volume of 50 µl diluted enzyme solution was added and the reaction was stopped after 0, 20, 40, 60 minutes by addition of 500 µl methanol. For the blank reaction, methanol was added before enzyme addition. After stopping the reaction, 750 µl of 500 mM $NaPO_4$ buffer (pH 7) were added and 200 µL of each sample were transferred into 96-well plates. Absorbance was measured at 410 nm using an Infinite 200 Pro Tecan Reader (Zurich, Switzerland). A standard curve was created using 4-Nitrophenol (Sigma-Aldrich, Austria) as product ranging from 0- 0.2 mM. The β-glucosidase activity was calculated in nkat ml^{-1}. Measurements were performed in duplicates and error bars indicate standard deviation.

3.3.2.4 Xylanase activity

Xylanase activity was measured using the Megazyme XylX6 Xylanase assay kit (Bray, Ireland). The assay is based on the procedure published by Mangan [23]. A volume of 50 µl XylX6 substrate was placed in 15 ml glass tubes and 50 µl of diluted enzyme solution was added. The reaction was performed at 45°C and stopped after 10 minutes by addition of 2% Tris (w/v) pH 10 solution. The reaction volume was transferred into 3 ml cuvettes and the absorbance was measured at 400 nm against distilled water

using a Hitachi U2900 Photometer (Chiyoda, Japan). Dilutions were performed either in citrate buffer (50 mM, pH 4.8) or 50% tap water + 50% ddH_2O (pH 7). Xylanase activity was calculated in nkat ml^{-1}. Measurements were performed in duplicates and error bars indicate standard deviation.

3.3.2.5 Filter paper assay

Enzyme activity on filter paper was performed according to the guidelines published by the International Union of Pure and Applied Chemistry (IUPAC) in 1987 with some modifications [24]. Stripes of 0.75 x 7.5 cm Whatman grade No.1 filter paper pieces were prepared and rolled. These stripes were added to 800 µl 50 mM citrate buffer (pH 4.8) 50% tap water + 50% ddH_2O (pH 7) in 15 ml glass tubes. A volume of 200 µl properly diluted enzyme solution was added (=start of reaction) and the reaction was stopped at timepoints of 0, 5, 10, 20, 40 and 60 minutes with 1000 µl 1 M NaOH. Afterwards, 1000 µl DNS solution was added for colour development and the samples were incubated in a boiling water bath for 5 minutes. A volume of 200 µl of each sample was transferred into a 96 well plate and the absorbance was measured at 540 nm using an Infinite 200 Pro Tecan Reader (Zurich, Switzerland). A glucose standard curve ranging from 0-20 mM was measured along with every newly prepared DNS solution. Duplicate measurements were performed, and error bars indicate the standard deviation. The activity on filter paper was calculated in nkat ml^{-1}.

3.3.2.6 Cellobiohydrolase I activity

Cellobiohydrolase I cleaves cellulose from reducing ends of the cellulose chain and its activity was determined using 4-nitrophenyl cellobioside as substrate. The measurement principle was escribed by Percival Zhang [25]. A solution containing 2 mM 4-nitrophenyl cellobioside in 50 mM citrate buffer (pH 4.8) was prepared. Additionally, 100 mM D-glucono-1,5-lactone to supress any β- glucosidase activity was added. A volume of 200 µl of substrate solution was placed in 15 ml glass tubes and equilibrated at 50°C and 50 µl of appropriately diluted enzyme solution was added. The reaction was stopped after 0, 10, 20, 40 and 60 minutes using 500 µl methanol. For the colour reaction, 750 µl of 500 mM $NaPO_4$ buffer pH 7 were added. From each sample, a volume of 200 µl was transferred into a 96 well plate and the absorbance was measured at 410 nm using an Infinite 200 Pro Tecan Reader (Zurich, Switzerland). A 4-nitrophenol standard curve ranging from 0- 0.2 mM was measured along the

samples for activity calculation. For method validation a Megazyme Cellobiohydrolase I (E-CBHI) was used. Measurements were performed in duplicates.

3.3.2.7 Activity assays on pulps

Enzyme activity on pulp was determined using a short fiber sulfate eucalyptus pulp in 50% tap water + 50% deionized water (pH 7) as dilution buffer to mimic the conditions used in the refining trials. The reactions were prepared in 15 ml glass tubes, by adding 10 mg of each pulp and suspending them in 200 µl dilution buffer. Diluted enzymes were added at timepoints 0, 10, 20, 40 and 60 minutes. Reactions were stopped by adding 250 µl of 1 M NaOH. Colours were developed by addition of 250 µl of DNS solution, followed by incubation in a boiling water bath for 5 minutes. Absorbance was measured at 540 nm using an Infinite 200 Pro Tecan Reader (Zurich, Switzerland). A glucose standard curve ranging from 0-20 mM was measured along with every newly prepared DNS solution. Duplicate measurements were performed, and error bars indicate the standard deviation. The activity on pulps was calculated in nkat ml^{-1}.

3.3.3 Endoglucanase purification

Endoglucanases from enzyme formulations (EnzA, EnzB, EnzC) were purified using a two-step process involving hydrophobic interaction chromatography (HIC) as first step and anion exchange chromatography (AEC) as second. The final purified endoglucanases were then named EndoA, EndoB and EndoC, according to their corresponding enzyme formulations. All purification steps were performed using an Äkta pure 25 purification system (Cytiva, Vienna). For HIC, three 5 mL HiTrap Phenyl HP columns (Cytiva, Vienna) were connected in series. Columns were equilibrated in HIC binding buffer A (10 mM acetate buffer + 1.5 M $(NH_4)_2SO_4$, pH 4.8) and HIC elution buffer B (10 mM acetate buffer pH 4.8) before sample application. Enzyme formulations were diluted in binding buffer A to enhance binding to the columns to a total volume of 70 ml. Precipitates were removed by centrifugation with 3700 rpm, at 4°C for 10 minutes. After loading the proteins (approx. 70 ml) onto the HIC columns, the columns were washed with binding buffer A until the UV 280 nm sensor displayed values under 50 mAU. Elution was performed depending on the respective endoglucanase. For EndoA, the elution was performed by applying a 30%B step for 5 column volumes, followed by a linear gradient of 30-80%B for 16 column volumes, yielding the desired enzyme fraction. EndoB was purified by applying a 50% B step (5 column volumes), followed by a 50-100% B linear gradient for 10 column volumes, whereas EndoC was purified by applying a 40 %B step (5 column volumes), followed

a linear gradient of 40-90%B for 10 column volumes. A flow rate of 5 ml/min was used except during loading of the columns with enzymes (1 ml min^{-1}) and elution (2.5 ml min^{-1}). Fractions were collected in a 96-well plate by the fraction collector and screened for the desired enzyme fractions by running an SDS-PAGE.

3.3.3.1 Anion Exchange Chromatography (AEC)

For AEC, a 5 ml HiTrap DEAE Sepharose FF column was used (Cytiva, Vienna). Prior to AEC, pooled fractions from HIC were concentrated using a 5 kDa Vivaflow 50 membrane (Sartorius, Göttingen, Germany). Thereafter, additional 10 ml anion exchange buffer A (10 mM Tris buffer pH 7.5) were added for further desalting, after reaching an end-volume of 5 ml, concentration was stopped, and the buffer exchanged using PD-10 columns (Cytiva, Vienna, Austria). 1 PD-10 column was loaded with 1 mL concentrate and exchanged to 50 mM citrate buffer pH 4.8 for activity assays after HIC. Further PD-10 columns were used to the change the buffer of the residual concentrate to anion exchange buffer A (10 mM Tris, pH 7.5). After elution using 10.5 ml of buffer A, additional 14.5 ml of buffer A was added to keep salt concentration low. The DEAE column was equilibrated in binding buffer A (10 mM Tris, pH 7.5) and elution buffer B (10 mM Tris + 1 M NaCl, pH 7.5) before sample application. A total volume of 25 ml sample was loaded onto the DEAE column. Elution was performed using a linear gradient of elution buffer B. EndoA was purified by applying a linear gradient of 0-7%B for 15 column volumes, EndoB through a linear gradient of 0-10%B for 15 column volumes, EndoC by applying a 1.5%B step for 5 column volumes. A flow rate of 5 ml/min was used except during loading of the columns with enzymes (1 ml min^{-1}) and elution (1 ml min^{-1}). Fractions were collected in 96-well plates by the fraction collector and desired enzyme fractions were identified by running an SDS-PAGE. Fractions containing the purified enzymes were pooled and concentrated using Vivaspin 20, 3000 Da centrifugal concentrators (Sartorius, Göttingen, Germany). Buffer was exchanged to 50 mM citrate buffer pH 4.8 using PD-10 columns. Purified enzymes were aliquoted and stored at -20°C until analysis.

3.3.4 Determination of protein concentration

The concentration of the protein content of the enzyme formulations and purifications was determined by the Implen NanoPhotometer NP80 (Munich, Germany). A volume of 2 µl of was used for each measurement. 50 mM citrate buffer (pH 4.8) was used as buffer for sample dilutions and blank measurements. Measurements were performed

in duplicates. For calculation, the extinction coefficient of BSA was utilized (Molar Extinction Coefficient: 44.289 [M^{-1}*cm^{-1}]).

3.3.5 SDS-PAGE

The size of the enzymes in the enzyme formulations and the purified endoglucanases was determined using SDS-PAGE. Therefore, samples were diluted accordingly in 50 mM citrate buffer (pH 4.8). Laemmli buffer was prepared according to Laemmli [26]. A volume of 5 µl of 5x Laemmli buffer was then added to 20 µl of sample. Samples were incubated at 99°C and 300 rpm on an Eppendorf thermomixer comfort (Hamburg, Germany) for 10 minutes for denaturation of the proteins. A volume of 5 µl of peqGOLD Marker IV (VWR, Austria) and 10 µL of each sample was applied to a 4-15% StainFree TGX gel from Biorad (Hercules, California). The SDS-PAGE gel was run at 150 V for 45 minutes. Afterwards the gel was stained in Coomassie staining solution (ddH_2O, acetic acid, ethanol in the ratio 6:3:1 with 0.125 % Coomassie G-250) for 1 hour and 3 times in destaining solution (ddH_2O, acetic acid, ethanol in the ratio 6:3:1) for 1 hour. Image acquisition of the stained gel was performed using the Biorad ChemiDoc (Hercules, California).

3.3.6 Laboratory refining trials, sheet forming and paper testing

Refining trials were conducted using 50% tap water + 50% deionized water at pH 7. Commercial SF eucalyptus sulfate pulp (130 g dry) was adjusted to a consistency of 4%, soaked overnight, and desintegrated for 10 minutes before enzyme addition. Enzyme formulations as well as purified endoglucanases were added at a dosage of 5.1 nkat based on the results of the endoglucanase specific CellG5 activity assay to 50% tap water + 50% deionized water (pH 7) and the temperature was set to 45 °C and kept constant over the whole experiment. After addition of the appropriate enzyme amount, the pulp solution was incubated under agitation for 30 minutes and inactivated using 30% H_2O_2. The enzyme treated pulp solution was subsequently thickened to 10% using filters. Refining was performed using a PFI laboratory mill type Mark IV (Hamjern Maskin AS, Norway) at 0, 1500, 4000 and 6000 PFI revolutions according to ISO 5264-2:2011 and the achieved degree of refining was determined using the Schopper-Riegler method (ISO 5267−1:1999). For quantitative characterization of weighted fiber length according to ISO 16065-2 and fibril area a L&W Fiber Tester Plus from ABB (Kista, Sweden) with an optical resolution of 3,3 µm/pixel was applied. Laboratory handsheets were formed according to the Rapid–Köthen procedure

(ISO 5269−2:2004) using a RK4-KWT sheet former from Frank PTI (Austria). Tensile properties of the formed handsheets (ISO 1924-2:2008) and air permeance Gurley (ISO 5636-5:2013) were determined after conditioning the samples for at least 24 hours in the climate room at 23°C and 50% relative humidity. For the determination of the tensile properties 13-15 paper strips were analyzed, for the determination of the fibre length 10500 to 15000 fibers were analyzed. Error bars indicate the standard deviation.

3.3.7 Scanning electron microscopy (SEM):

For visualisation of the formed handsheets using SEM, small pieces were cut off the handsheets and analysed using the Hitachi TM3030 scanning electron microscope (Chiyoda, Japan). Prior to imaging, samples were coated with 1 nm platinum using the Leica EM Ace200 sputter coater (Wetzlar, Germany) for contrast enhancement. SEM pictures were obtained from refined handsheets at 1000x magnification and are available in **Fig. S21**, **Fig. S22**, **Fig. S23** and **Fig. S24** of the Online Resource.

3.3.8 Viscosity measurements

Differences in the ability of enzyme formulations and purified endoglucanases to hydrolyse the model substrate carboxymethylcellulose (CMC) were measured with the Anton Paar MCR 302 rheometer (Graz, Austria) with a CP50-1 plate cone measurement system attached. Measurements were conducted as described by Lee [27] with some modifications: endoglucanase activities of enzyme formulations and purified endoglucanases were adjusted to 0.83 nkat ml^{-1} using the measured activities on the endoglucanase specific CellG5 substrate. A volume of 1 ml of 1% carboxymethylcellulose with an average molecular weight of 700.000 g mol^{-1} and a degree of substitution of 0.9 was placed on the pre-tempered (45°C) plate and the cone lowered to the measurement gap. After trimming of the sample, the measurement was started while the plate cone system rotated with a shear rate of 100 s^{-1}. After a constant viscosity was reached, a volume of 50 µl of diluted enzyme solution was slowly added with a pipette between the rheometer and the rotating plate cone measurement system. 900 datapoints were recorded which corresponds to a total measurement time of 15 minutes. As blank, 50 µl of 50 mM citrate buffer pH 4.8 was added without any enzymes (= Upper limit). As lower limit, 1% CMC with a degree of substitution of 0.7 and an average molecular weight of 250.000 g mol^{-1} was measured. Measurements

were performed in duplicates and average values of the recorded viscosity were calculated and displayed graphically.

3.4 Results and discussion

3.4.1 Comparison of refining enzyme formulations

In a first step, the commercial enzyme formulations received from the pulp and paper industry were compared. The highest protein concentrations were measured in EnzC (254 mg ml^{-1}) followed by EnzB (176 mg ml^{-1}), while EnzA exhibited the lowest protein concentration of 27.1 mg ml^{-1}. All enzyme preparations showed endoglucanase activity which has been reported to play a major role in refining of pulps [28]. However, activities measured on the endoglucanase specific CellG5 substrate (derivatized cellopentaose) varied over a wide range from 1.3 nkat mg^{-1} (EnzA) to 13.7 nkat mg^{-1} for EnzC (**Fig. 13**). EnzC also showed highest activity on carboxymethylcellulose and the second highest on filter paper, however, the ratio of activities on the three substrates was different. For example, the difference between the activities of the three enzymes was higher on CMC when compared to CellG5. This difference was much less pronounced when filter paper was used as a substrate. CellG5 was reported to be highly specific for endoglucanase activity [29] when compared to CMC which can also be cleaved by cellobiohydrolases or even xylanases [30], [31]. On the other hand, in the filter paper assay released reducing sugars are quantified as a result of the synergistic action of a variety of different enzymes including cellobiohydrolases, β-glucosidases or lytic polysaccharide monooxygenases (LPMOs). Whatman filter paper (as used for the activity assays) is derived from cotton fibers and contains a high percentage of cellulose (96%) and only 4% of hemicelluloses. The use of filter paper is therefore often not an appropriate measure of the predictability of cellulase mixtures, as they do not reflect the composition of other cellulosic materials like pulps [32], [33].

The enzyme formulations did not show any cellobiohydrolase I activity when measured on 4-nitrophenyl cellobioside as substrate. Since β-glucosidases and also some endoglucanases [34] can also hydrolyse this substrate, D-glucono-1,5-lactone was added as inhibitor for these enzymes. Although this inhibitor was used in slightly higher concentration than previously reported [35], this did not have any impact on the measurement of the activity of a commercial cellobiohydrolase I **(**as shown in table 1 of the Online Resource**)**. Nevertheless, cellobiohydrolase activity may be detrimental in refining due to excessive degradation of cellulose in combination with β-

glucosidases [36]. On the other hand, a study indicated that cellobiohydrolases used in refining did not affect overall handsheet properties of the resulting paper, but led to dislocations and disruptions on the surface of cellulose fibers [37]. The activity of β-glucosidase differed greatly between the investigated enzyme formulations (0.57 nkat mg^{-1} to 1.3 nkat mg^{-1}). β-Glucosidase activity may be necessary to avoid product inhibition of endoglucanases by accumulation of cellobiose and cellotriose [38]. There is a sensitive balance point since release of sugars by β-glucosidase does not only reduce pulp yield but can cause considerable cost for waste-water treatment. The enzyme formulations exhibited a wide range of xylanase activities. EnzA only showed an activity of 1.78 nkat mg^{-1}, EnzB had the highest value of 62.1 nkat mg^{-1}, while for EnzC a xylanase activity of 8.21 nkat mg^{-1} was measured. The activity of the commercial endoglucanase FiberCare R was in comparison neglectable (0.024 nkat mg^{-1}). Different amounts of hemicelluloses may also be responsible for the differences in the performance of EnzB as this enzyme formulation also exhibited a high xylanase activity, while filter paper only exhibits low amounts of hemicelluloses. Several studies have reported that xylanases contribute positively to refining of pulps in terms of energy reduction [39]. Despite the successful demonstration of the effectiveness of energy reduction by xylanases, their use can also affect the paper properties like tensile strength negatively by the removal of xylan [2], [40], [41]. Determined volumetric activities in nkat ml^{-1} of the enzyme formulations are listed in **Table S*2*** of the Online Resource.

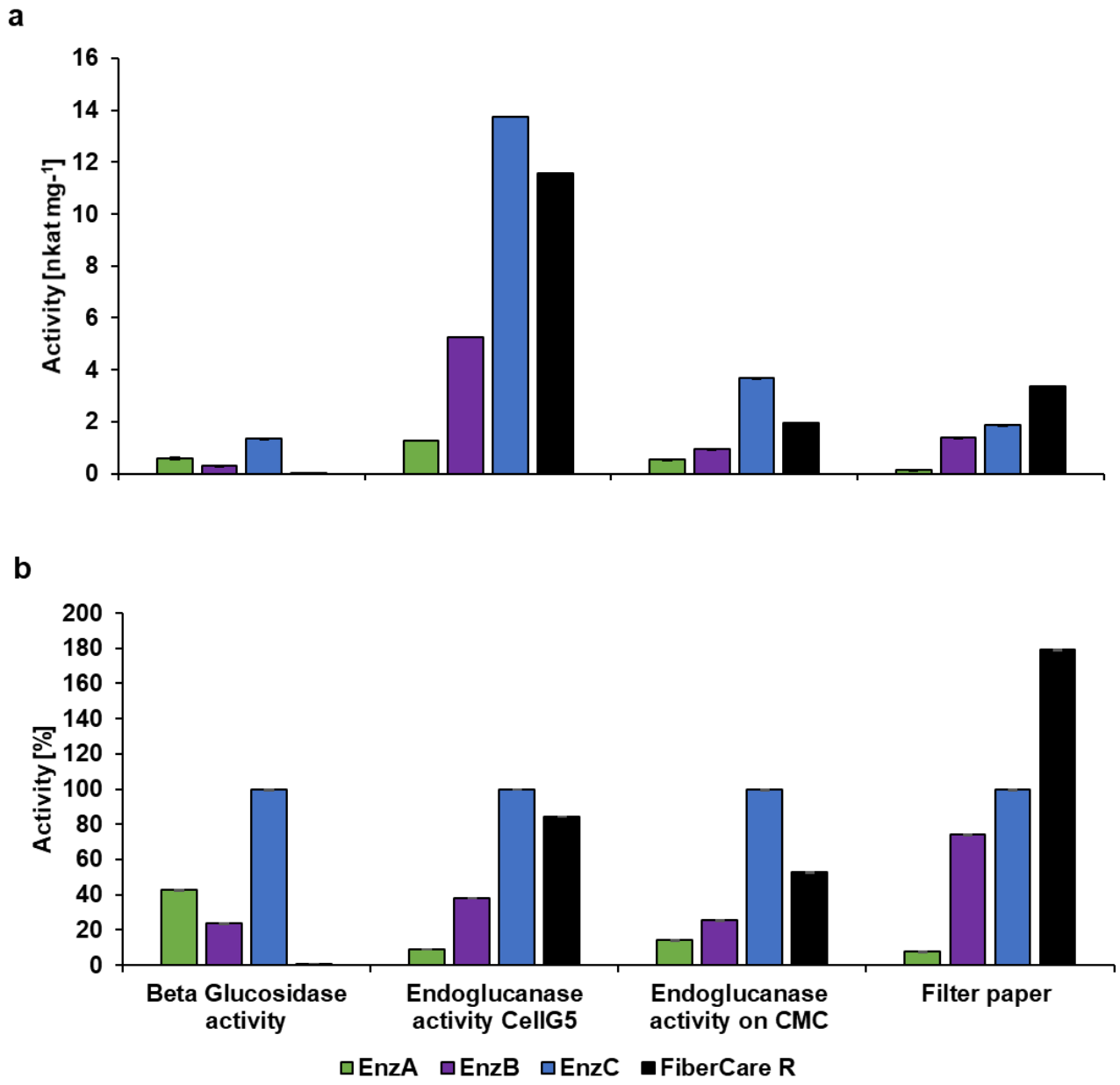

Fig. 13 Relative activities of commercial refining enzyme formulations measured in 50 mM citrate buffer (pH 4.8). (a) shows the activities determined on the endoglucanase specific CellG5 substrate, on carboxymethylcellulose (CMC) and on filter paper as well as the β-Glucosidase activities in nkat mg^{-1}, (b) shows the relative activities in percent related to EnzC for better visualization of the differences between the enzyme formulations.

3.4.2 Endoglucanase purification

In a next step, endoglucanases were purified from the formulations EnzA, EnzB and EnzC that are used in the industry in order to investigate their effect on the refining process. Purification was necessary to eliminate interfering enzymes such as xylanases or β-glucosidases, that may also be active on cellulose fibers or influence their performance. In addition, the purified endoglucanases were also compared with FiberCare R, a commercial endoglucanase formulation, that already contains

endoglucanases in purified form. Endoglucanases are the most promising enzymes in the refining process, due to their ability to cause a more intense fibrillation [3]. Based on several preliminary experiments, all enzymes were successfully purified with a combination of HIC and AEC. Other than in many research studies characterizing and purifying endoglucanases like Karnchanatat et al., HIC was applied prior to AEC [42]. The fact, that HIC was suitable for the purification of all the endoglucanases in the enzyme formulations suggests the presence of hydrophobic regions in all endoglucanases, this is supported by the findings of a study that the divergence in sequence in the hydrophobic core is less pronounced [43]. The disadvantage of this combination is the requirement of an buffer exchange after HIC, as high salt concentrations would interfere with binding to the AEC column [44]. The purified enzymes had a similar protein content ranging between 7.1 and 10.9 mg ml^{-1} (**Fig. *14***)

Endoglucanase EndoA from EnzA had the lowest molecular weight of approximately 20 kDa, endoglucanase EndoB from EnzB has a size of approx. 55 kDa, and endoglucanase EndoC from EnzC 37.5 kDa (**Fig. *14***). This is in the range of published endoglucanases, for comparison, the endoglucanase I from the well characterized fungus *Trichoderma reesei* has a MW of 58 kDa, an endoglucanase of *Thermoascus aurantiacus* a molecular weight of 35 kDa, while the endoglucanase Cel12A from *Gloeophyllum trabeum* show a MW of 26 kDa [45]–[47]. The SDS-PAGE pictures of the enzyme formulations are shown in **Fig. S*19*** of the Online Resource.

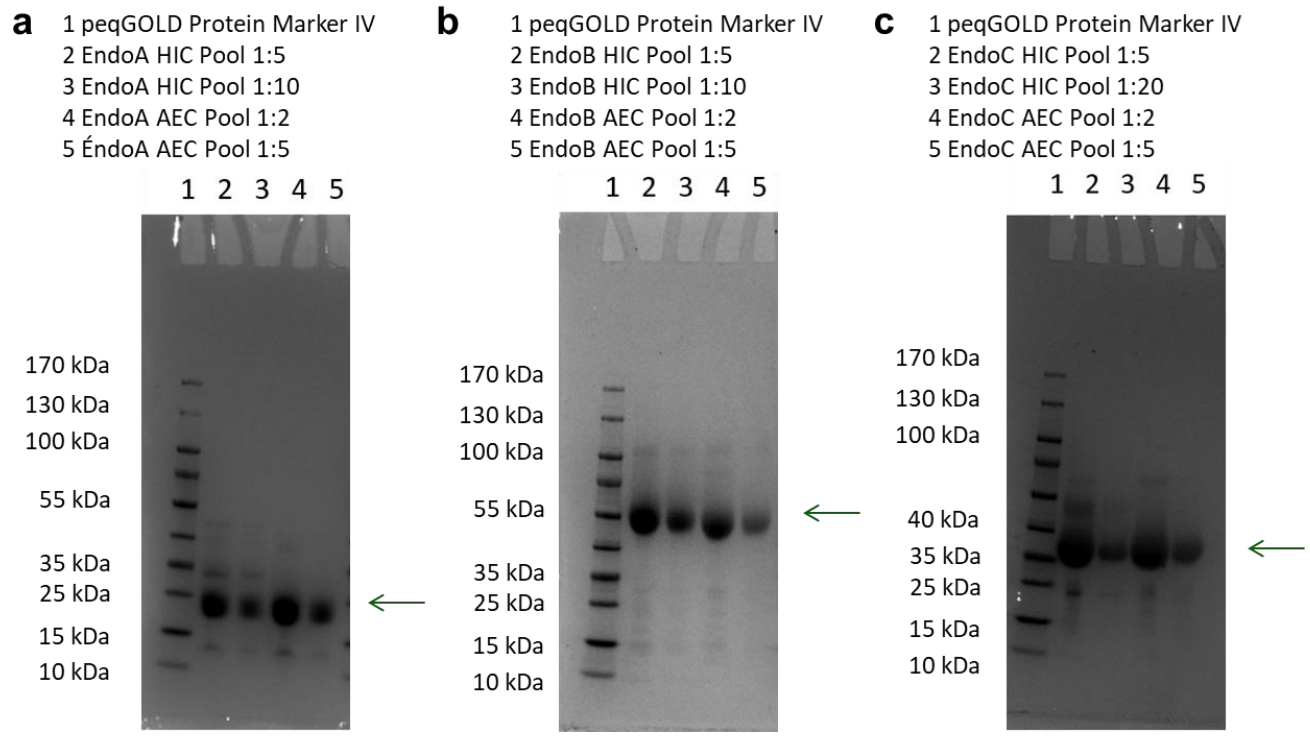

Fig. 14 SDS-PAGE analysis of endoglucanases purified from refining enzyme cocktails after HIC and AEC (size of putative endoglucanases is indicated with arrows). EndoA showed a size of 20 kDa (a), EndoB a size of 55 kDa (b) and EndoC a size of 37.5 kDa (c). EndoA had a protein content of 10.9 mg ml^{-1} after AEC, EndoB 7.1 mg ml^{-1} and EndoC 10.5 mg ml^{-1}

3.4.2.1 Activity of purified endoglucanases

EndoA and EndoC had similar endoglucanase activities on the endoglucanase specific CellG5 substrate while the activity of EndoB was about ten times lower (**Fig. *15***). The difference in activities was more pronounced on CMC as substrate (**Fig. *15*)**, with slightly higher values for EndoB in comparison to the other two purified endoglucanases. No significant β-glucosidase activity could be detected for all purified endoglucanases (all measured values below 1.0 nkat ml^{-1} or 0.2 nkat mg^{-1}, respectively). Xylanase activities were determined using the specific XylX6 substrate and showed an activity of 0.38 nkat mg^{-1} for EndoA, 20.6 nkat mg^{-1} for EndoB and 0.070 nkat mg^{-1} for EndoC. EndoB showed still a rather high xylanase activity, indicating a possible secondary xylanase activity of this enzyme which has been reported for several endoglucanases [48], [49]. The performance of the purified enzymes was also measured on filter paper. EndoC showed the lowest activity in agreement with the lowest β-glucosidase which contributes to the release of reducing sugars from filter paper. The synergistic action of endoglucanases with β-glucosidases is required for accurate values, as endoglucanases can lead to the accumulation of large amounts of the inhibitory cellobiose [50]. When looking at the

specific activity values related to the protein concentration, EndoA showed significantly higher activities on filter paper than the other two purified endoglucanases, which was not the case for carboxymethylcellulose. Determined volumetric activities in nkat ml^{-1} of the enzyme formulations are listed in **Table S*2*** of the Online Resource.

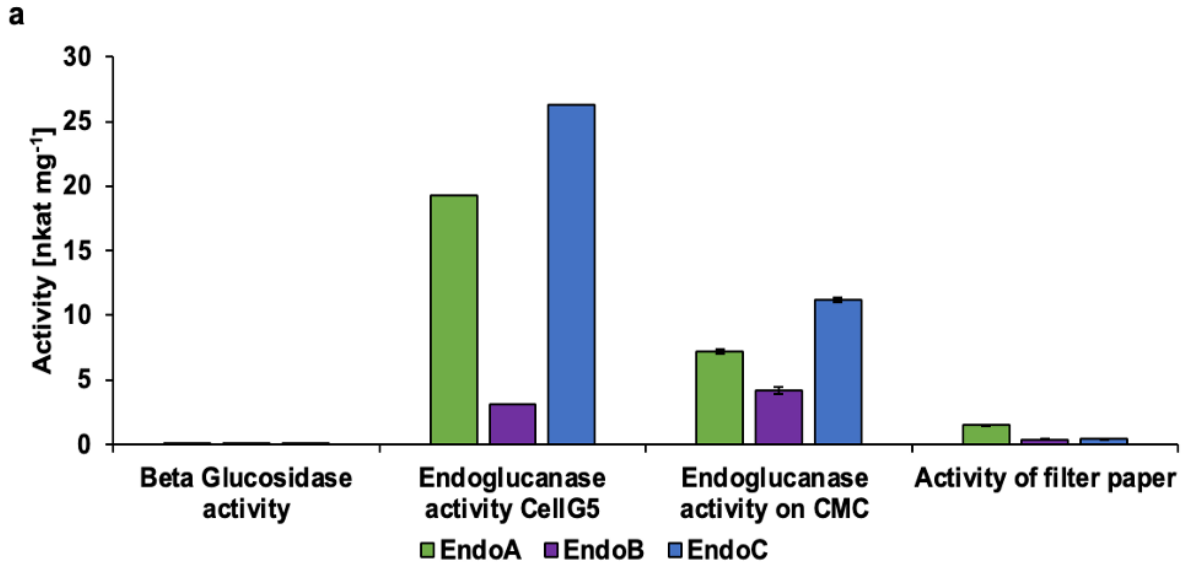

Fig. 15 Activities of endoglucanases purified from enzyme formulations used for refining. (a) shows β-glucosidase activities as well as the endoglucanase activities on the CellG5 substrate, carboxymethylcellulose or on filter paper in nkat mg^{-1}

The capability of the purified endoglucanases and enzyme formulations to reduce the viscosity of the model substrate carboxymethylcellulose was investigated using the rheometer at 45 °C. In total 900 datapoints were recorded for each enzyme, corresponding to 900 seconds. When dosed at the identical activity of 0.83 nkat ml^{-1} according to the endoglucanase specific CellG5 substrate, a similar decrease in viscosity is expected. However, despite reaching comparable viscosity curves in the enzyme formulations and purified endoglucanases, minor discrepancies between the different enzymes could be observed. A faster viscosity decrease should indicate a hydrolysis preferably attacking the middle of the cellulose chain. FiberCare R showed the fasted viscosity decrease followed by both EnzB and the endoglucanase EndoB purified therefrom (**Fig. 4**). The hydrolysis of cellulose is also dependent on the catalytic centre, some enzymes could have only a narrow tunnel, which leaves not much space for substituted side-groups, as is was suggested in a research study examining the hydrolysis of carboxymethylcellulose by EGIII or Cel6a on the

rheometer, the latter showing no endoglucanase activity despite a minor activity was expected [51]. Another important factor to consider is also the ability of enzymes like xylanases to degrade carboxymethylcellulose as well, which could be the reason for the higher activity of EnzB, while FiberCare R also contains a carbohydrate binding module aiding the binding of endoglucanase to its substrate. The ability of carbohydrate binding modules to improve the degree of refining was already demonstrated in a recent research study [52].

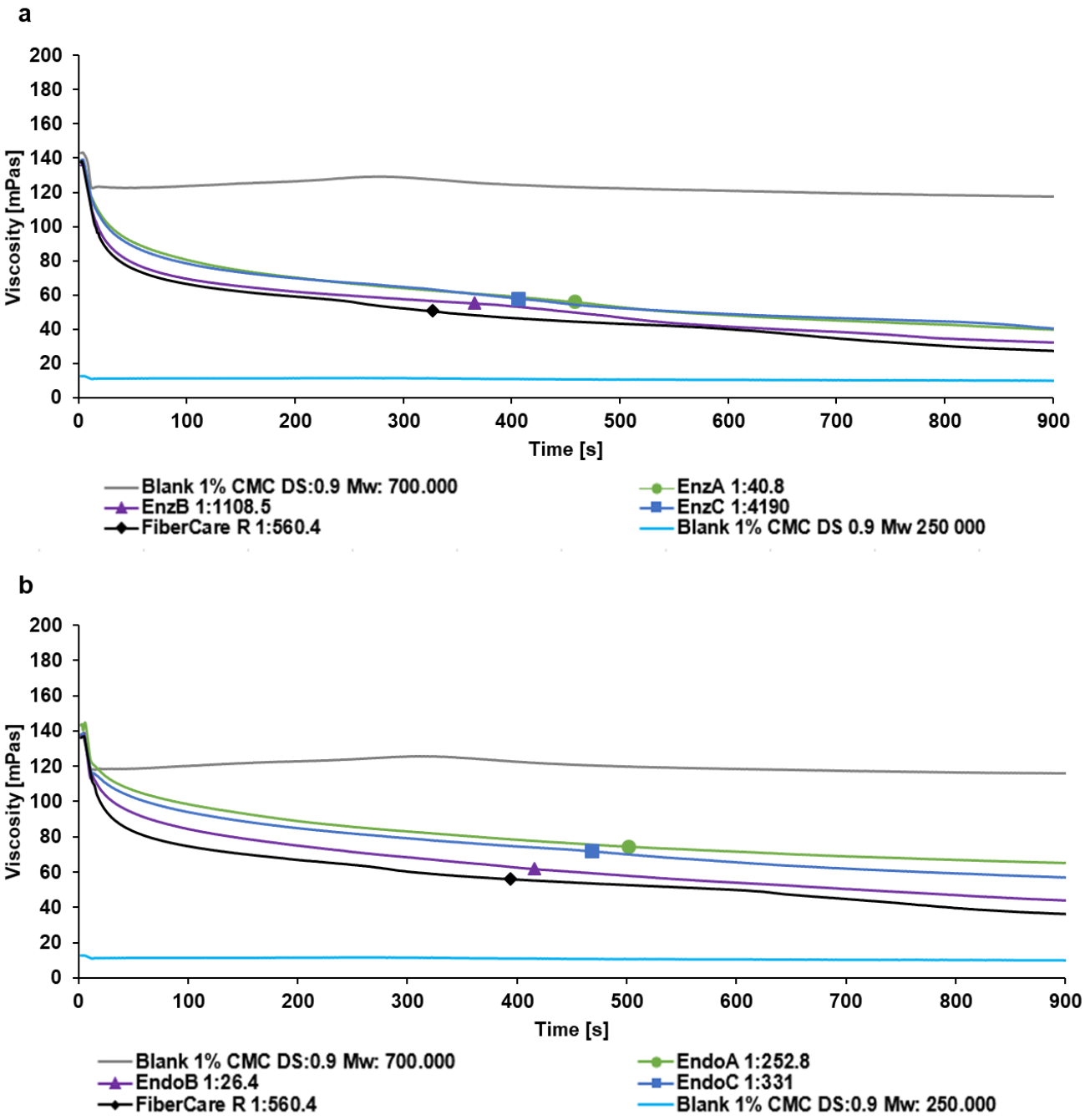

Fig. 16 Viscosity measurements using carboxymethylcellulose with a degree of substitution of 0.9 and an average molecular weight of 700.000 g/mol. Measurements were conducted at 45°C and 900 datapoints were recorded in total, corresponding to 900 s total measurement time. All enzyme formulations and purified endoglucanases were adjusted to the same activity of 0.003 nkat ml^{-1} according to the CellG5 Cellulase assay. (a) shows the results of the enzyme formulations, (b) the results of the purified endoglucanases

3.4.3 Activity of enzyme formulations and purified endoglucanases under refining conditions

To allow activity-based dosing of the enzymes, all activities were assessed again under the conditions used for laboratory refining trials, which were conducted using 50% tap water + 50% deionized water at pH 7. Compared to the standard assay conditions at pH 4.8, EnzB and FiberCare R showed quite comparable endoglucanase activity on the endoglucanase specific CellG5 substrate within this pH range while EnzA and EnzC were less active (Figure 5). Noticeable is also a slightly increase in β-glucosidase activity of EndoC (0.088 nkat mg^{-1} or 0.510 nkat ml^{-1}) and FiberCare R (0.0087 nkat mg^{-1} or 0.353 nkat ml^{-1}) while the other two endoglucanase purifications EndoA and EndoB showed similar values. Determined volumetric activities in nkat ml^{-1} of the enzyme formulations and purified endoglucanases at refining conditions are listed in **Table S*3*** of the Online Resource.

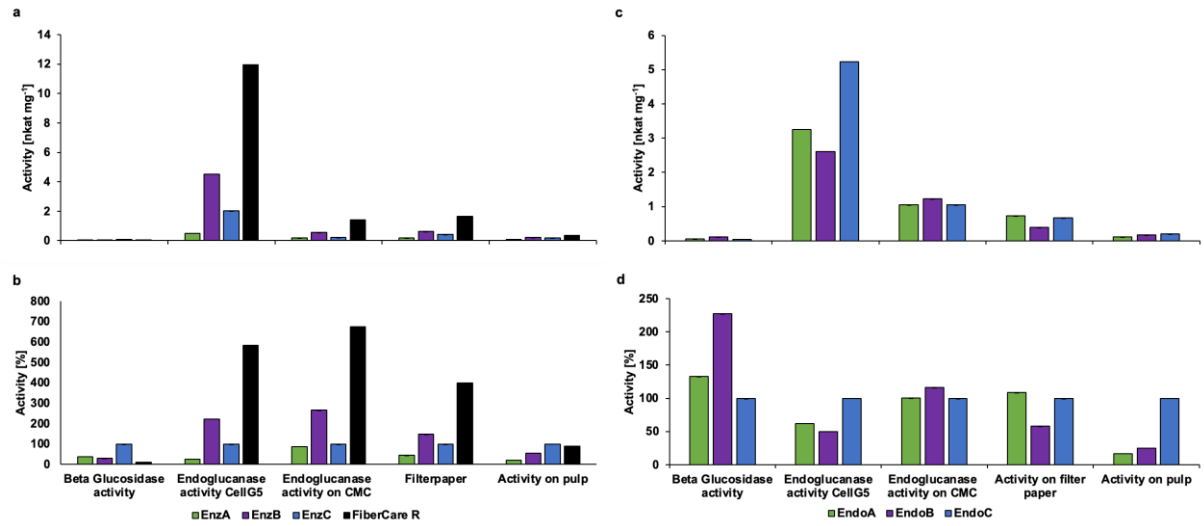

Fig. 17 Activities of enzyme formulations and purified endoglucanases related to the protein content [nkat mg^{-1}] or related to EnzC or EndoC at refining conditions (50% tap water + 50% deionized water at pH 7). (a,b) show the results of the enzyme formulations, (c,d) of the purified endoglucanases. The panels (b) and (d) show the relative activities in % in relation to EnzC (b) or EndoC (d) for better visualization of the differences between the enzymes.

3.4.3.1 Activity on pulp

Apart from filter paper as a standard substrate, release of reducing sugars from the short fibre SF Sulfate pulp by the different enzyme formulations and purified endoglucanases was studied. EnzA showed a slightly lower activity on pulp ($8.24*10^{-2}$ nkat mg^{-1}) than EnzB (0.232 nkat mg^{-1}) and EnzC (0.188 nkat mg^{-1}) while the commercial endoglucanase FiberCare R was the most active (0.371 nkat mg^{-1}) amongst the tested enzymes (**Fig. 17**). In addition to endoglucanases, many other

enzymes are present in the used formulations, synergistically contributing to the hydrolysis of pulp. Consequently, the relative activity of EnzB was higher on pulp than on filter paper indicating the contribution of the xylanase fraction contained in this preparation.

For the purified endoglucanases, EndoA could reach a value of 0.110 nkat mg^{-1}, EndoB of 0.168 nkat mg^{-1} while EndoC reached an activity of 0.199 nkat mg^{-1}. The results are also diverging from the corresponding activities on filter paper, where Endo A achieved 0.726 nkat mg^{-1}, EndoB only 0.390 nkat mg^{-1} and EndoC 0.668 nkat mg^{-1}. It is important to highlight how all purified endoglucanases exhibited higher activity on filter paper. Pulps are obtained through the pulping process which removes lignin and, to some extent, hemicelluloses with only residual amounts of both that might remain, depending on the process [53]. Lignin and hemicelluloses can affect cellulase accessibility [54]. For example, one study showed an increase of cellulase accessibility on sugarcane bagasse after lignin removal [55]. Haske-Cornelius already showed varying content of hemicelluloses between different pulps using NMR, although a different short fibre pulp was used in our study [6]. Like in the filter paper activity assay, the activity assay on pulps is also depended on enzymes like β-glucosidases that further degrade oligosaccharides released from endoglucanases to glucose.

3.4.4 Effect of enzyme formulations and purified endoglucanases on refining

The performance of the enzyme formulations and the corresponding purified endoglucanase was assessed in laboratory PFI mill refining experiments. All enzyme preparations were adjusted to the identical endoglucanase activity of 5.0 nkat using the specific CellG5 substrate which showed promising results for EnzC in preliminary experiments. In the refining experiments, the degree of refining was measured with increasing number of revolutions. For example, at 6000 revolutions, a degree of refining of 45.0 [°SR] was measured in the presence of EnzC while it was only 35.5 [°SR] for the control (**Fig. 18a**). In other words, addition of enzymes leads to a certain degree of refining with a lower number of revolutions. When compared to EnzC, the effect of EnzB was less pronounced despite the identical endoglucanase activity dosed, while EnzA exhibited the lowest degree of refining of 40.7 [°SR] at 6000 revolutions. The use the commercial endoglucanase FiberCare R led to a similar degree of refining as EnzC with 44.8 [°SR] at 6000 revolutions. The significant improvement in the degree of refining caused by the endoglucanase FiberCare R

indicates their importance in this process while there seem to be differences between the individual endoglucanases, as EnzA and EnzB led to slightly lower values regarding the degree of refining. Besides the degree of refining, following properties were measured: fiber length and tensile strength. The results are highlighted in **Fig. 18**. Additionally, fibril area and air resistance according to Gurley was determined (shown in **Fig. S20** of the Online Resource), which also showed a similar trend as the discussed fiber and paper properties.

The tensile index of the resulting paper sheets is an important factor for the assessment of the refining process and generally increases with the number of revolutions due to denser paper structure through increased bonding of fibres [56]. The increase in inter-fibre bonding during refining is related to an increase of the total fiber surface area, caused by the formation of fibrils on the surface of the fibres [57]. At 6000 revolutions, EnzC led to the greatest improvement of the tensile index to 76.0 Nm g^{-1} compared to a value of 72.1 Nm g^{-1} in the absence of enzymes. In agreement with a lower effect in refining, EnzB and EnzA lead to a lower tensile index. Likewise, the tensile index improvement was lower for the endoglucanase FiberCare R, which is, however, in contrast one of the highest effects in the degree of refining. Overall similar tensile index properties could be obtained, which is another indication that the enzyme dosage according to the new endoglucanase specific CellG5 assay is a major improvement compared to the dosage based on reducing sugar release.

Another factor for the assessment of the enzyme performance is the fibre length, which was 804 µm for the control and decreased to values between 794 µm and 766 µm in the presence of enzymes (**Fig. 18b).** Fibre shortening can have negative impacts on the tensile strength [58]and on tear index [58]. However, despite shorter fibers caused by EnzA, the resulting tensile strength was higher compared to FiberCare R. Therefore, enzymes contained in EnzA could have formed more fibrils, leading to a more intense bonding.

The tear index increased for all enzymes at 0 U when compared to the control without enzymes, however, at higher refining levels the tear index was decreasing for all enzyme formulations (**Fig. 18d**). Similar behaviour was already observed in a study that also used eucalyptus pulp in cellulase-assisted refining experiments, showing first an increase of the tear index during enzyme treatment, followed by an decrease at

higher refining stages [59]. This effect was more pronounced for EnzC and FiberCare R, which showed a high increase of the tear index already at 0 and 1500 U, while EnzA and EnzB exhibited this increase of tear index at 4000 U and therefore at a later stage, where the tensile index values of EnzC and FiberCare R were already decreasing. The highest decrease in tear index could be observed for EnzC, which also showed the highest decrease in fiber length. Overall, the usage of derivatized cellopentaose also led to similar tear index values, but also indicate that through the vast variety of available endoglucanases, some differences still remain, which may be attributed to differences in the catalytic domains of the enzymes.

The amount of sugars released from the short fiber pulp under refining conditions was quantified. Interestingly, values measured for the individual enzymes varied in a wide range between 0.157 nmol s^{-1} and 0.835 nmol s^{-1} (**Fig. 18e**) and even more for the purified endoglucanases with values between $7.51*10^{-3}$ nmol s^{-1} and 0.450 nmol s^{-1} (Fig. ***18***H). Moreover, the enzyme causing the lowest release of reducing sugars lead to the highest degree of refining (**Fig. 18e**). Therefore, since dosed at identical endoglucanase activities based on cellopentaose, this indicates the important role of this activity in refining in accordance with some previous studies [28]. Furthermore, the fact that the release of reducing sugars cannot be used as a parameter for dosing refining enzymes was confirmed similarly to what was previously reported in several studies [60], [61].

In addition to enzyme formulations, the effect of the purified endoglucanases on refining was assessed. In the presence of EndoC even a slightly higher degree of refining of 47.9 [°SR] was reached compared to any of the enzyme preparations **(Fig. 18f**). Also, a higher degree of refining was seen for EndoA (42.4 [°SR] after 6000 U) compared to the corresponding enzyme formulation EnzA (40.7 [°SR]), suggesting additionally components in this formulation that may negatively affect the refining process. All purified endoglucanases led to similar tensile index values, despite similar differences in the degree of refining as in the enzyme formulations, suggesting the degree of refining is not the only factor for the prediction of the suitability of endoglucanases. Similar tensile index values were expected due to the adjustment of the endoglucanases to the same activity according to the endoglucanase specific derivatized cellopentaose (CellG5) substrate. When compared to the corresponding

enzyme formulations, EndoA led to a much higher tensile index (**Fig. 18h**). This again may indicate excessive degradation of fibres by synergistic action of enzymes (xylanases, β-glucosidases, cellobiohydrolases, LPMO) present in the formulations. The opposite behaviour could be observed for EnzC, where additional components in the enzyme formulation may have supported the refining process. Indeed, EnzB and EnzC showed by far the highest xylanase activity which can have a positive effect on paper properties [62]. Regarding fibre length, the reduction was expectedly less pronounced with the pure enzyme when compared to the enzyme formulations (**Fig. 18g**). The tear index data show similar tendencies as their industrial enzyme formulations, however, now all purified endoglucanases including EndoA and Endo B show an increase in tear index at 0 and 1500 U, in contrast to their corresponding enzyme formulations, which could be related to additional components in the enzyme formulation affecting the tear index, but differences at 0U and 1500 U are not that pronounced and could be simply within the standard deviation. However, at higher refining levels (4000 U and 6000 U), tear index values began to decrease, with the highest decrease observed for EndoC, but this decrease was less pronounced than with its corresponding enzyme formulation (EndoC:7.78 $mNm^2\ g^{-1}$, EnzC: 7.41 $mNm^2\ g^{-1}$), suggesting additional enzyme components in EnzC affecting the tear index negatively.

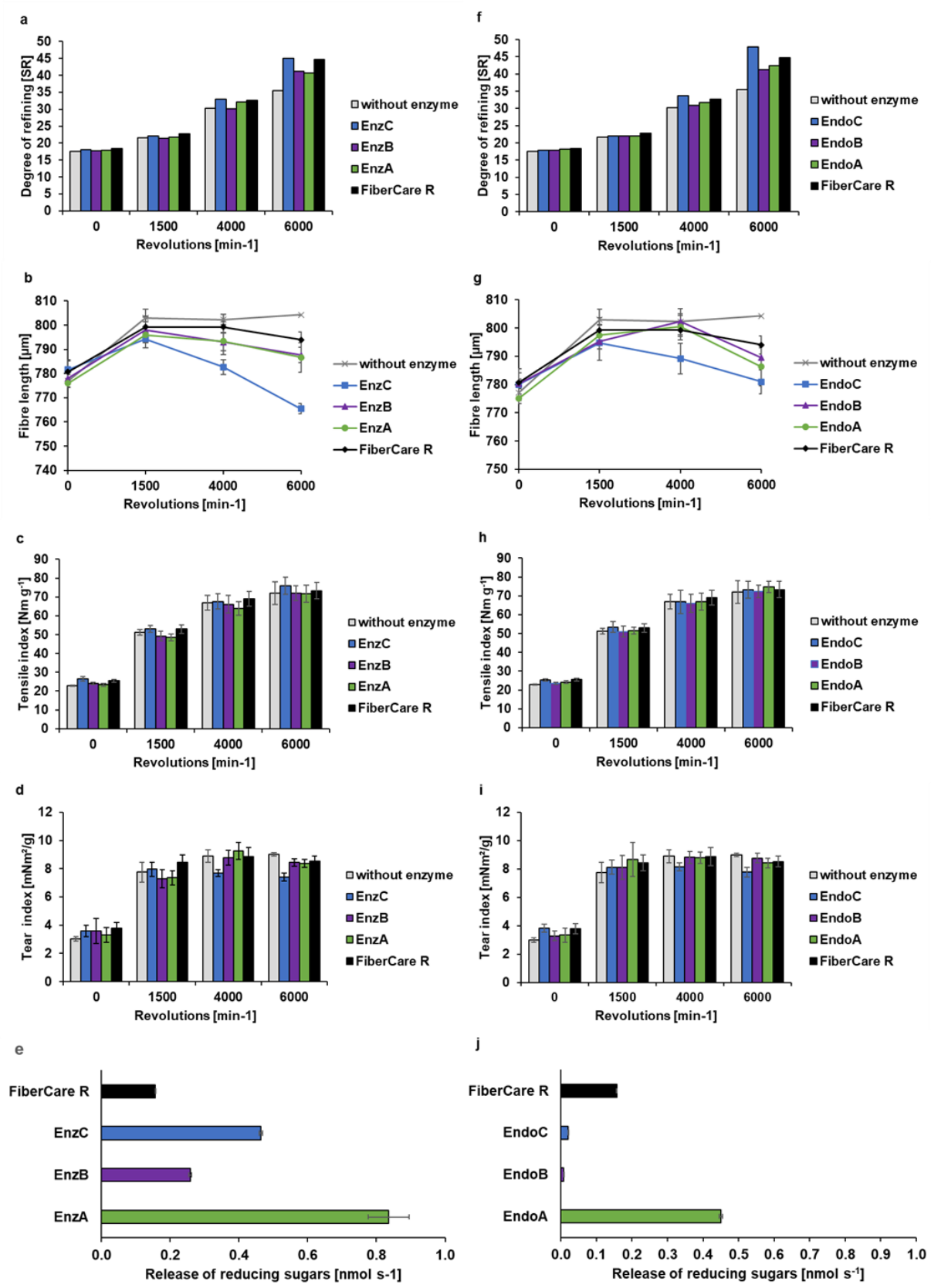

Fig. 18 Effect of enzyme preparations and purified endoglucanases on refining of short fibre sulfate pulp and resulting paper properties as well as release of reducing sugars. (a-e) shows the results for the enzyme formulations, (f-j) the results for the purified endoglucanases

3.5 Conclusions

Investigation of three different enzyme formulations and endoglucanases purified thereof clearly confirms that primarily endoglucanases are responsible for refining effects. Yet, in the industry commercial enzyme formulations rather than purified enzymes are used and hence there is a strong need to predict their effect in refining and to define their dosing. Moreover, considerable fibre damage must be avoided potentially occurring when overdosing. Obviously, dosing based on volume or protein is no suitable as enzyme activity may decrease during storage and enzyme content may vary in formulations from batch to batch. Here, we demonstrate that the endoglucanase activity on the derivatized cellopentaose substrate (CellG5) correlates well to the effect in refining after adjustment to the identical activity, while assays conventionally used to include release of reducing sugars from pulp or filter paper are not suitable. Despite the adjustment to the same endoglucanase activity, some differences between the enzymes could still be observed during the laboratory refining trials. This suggests the influence of additional components in the enzyme formulations such as xylanases on tensile strength.

Declarations

Author contributions

Martin Nagl and Lukas Skopek performed the experiments. Martin Nagl, Oskar Haske-Cornelius, Alessandro Pellis, Georg Guebitz and Wolfgang Bauer planned the experiments and analysed the data. Georg Guebitz and Wolfgang Bauer supervised the work. Martin Nagl wrote the manuscript. Oskar Haske-Cornelius, Alessandro Pellis, Wolfgang Bauer, Gibson Stephen Nyanhongo and Georg Guebitz corrected the manuscript. The presented data was discussed by all authors prior to submission and all authors agreed to submit.

Conflicts of interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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Availability of data and material

Additional material related to this article can be found as Online Resource.

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3.7 Supplementary Material

Results of the activity assays of the enzyme formulations and purified endoglucanases in nkat ml^{-1}

Table S2 Results of the activity assays of enzyme formulations and purified endoglucanases in nkat ml^{-1} using 50 mM Citrate Buffer at pH 4.8

Enzyme	Beta Glucosidase [nkat ml^{-1}]	Endoglucanase activity on CellG5 [nkat ml^{-1}]	Endoglucanase activity on CMC [nkat ml^{-1}]	Xylanase activity [nkat ml^{-1}]	Activity on filter paper [nkat ml^{-1}]
EnzA	15.52	34.04	14.52	48.33	3.97
EnzB	55.71	923.93	164.85	10948.78	245.41
EnzC	342.05	3492.42	937.77	2085.13	478.58
FiberCare R	0.28	467.13	78.93	0.96	136.40
EndoA	0.63	210.71	78.87	4.13	16.20
EndoB	0.81	21.96	29.72	145.89	2.88
EndoC	0.11	276.22	117.97	0.74	5.18

Table S3 Results of the activity assays of enzyme formulations and purified endoglucanases in nkat ml^{-1} using 50 % tap water + 50% deionized water, pH 7

Enzyme	Beta Glucosidase [nkat ml^{-1}]	Endoglucanase activity on CellG5 [nkat ml^{-1}]	Endoglucanase activity on CMC [nkat ml^{-1}]	Xylanase activity [nkat ml^{-1}]	Activity on filter paper [nkat ml^{-1}]	Activity on pulp [nkat ml^{-1}]
EnzA	0.89	13.55	5.06	11.05	5.00	2.24
EnzB	4.58	799.42	99.06	6336.44	109.13	40.81
EnzC	22.35	519.44	53.76	437.66	105.96	47.65
FiberCare R	0.35	482.60	57.61	1.92	67.18	15.00
EndoA	0.70	35.51	11.55	5.25	7.92	1.21
EndoB	0.78	18.42	8.67	8.99	2.76	1.19
EndoC	0.51	54.96	11.11	0.22	7.03	2.10

SDS-PAGE of the enzyme formulations

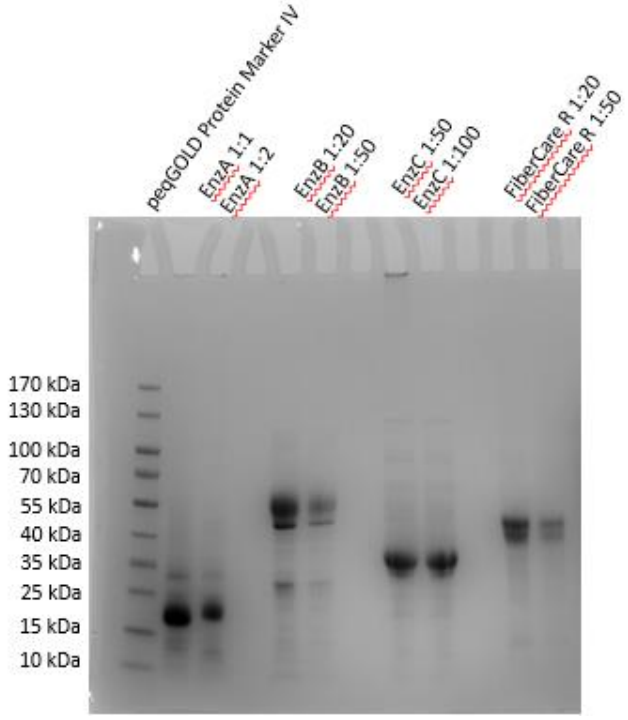

Fig. S19 SDS-PAGE of enzyme formulations including the EnzA, EnzB and EnzC used in the pulp and paper industry and the commercial FiberCare R endoglucanase

EnzA, EnzB and EnC enzyme formulations, obtained from the pulp and paper industry, as well as the commercial endoglucanase FiberCare R were analysed using SDS-PAGE **(Fig. S1)**. SDS-PAGE revealed that every enzyme formulation of the pulp and paper industry has one enzyme fraction with high concentration among a high number of proteins with a lower concentration.

Results of the Cellobiohydrolase I activity assay

Table S4 Results of the Cellobiohydrolase I activity assay of enzyme formulations and purified endoglucanases in nkat ml^{-1}

Enzyme	**Activity [nkat ml^{-1}]**
EnzA	0.0035
EnzB	0.0060
EnzC	0.083
FiberCare R	0.0035
EndoA	0.0024
EndoB	0.0083
EndoC	0.0011

Megazyme Cellobiohydrolase I	4.82

Additional measured parameters of the laboratory refining trials

Besides the degree of refining, fibre length and tensile strength parameters, also the fibril area and the air permeability according to Gurley were measured. The results show the same tendencies, with EnzC giving the highest effect regarding air permeability (increased to 79.2 s at 6000 U) and the caused fibrillation area (increased to 5.15 % at 6000 U), while effects of EnzA and EnzB as well as their purified endoglucanases were lower. A clear distinction from the control without enzyme addition was achieved by all enzymes (air permeability according to Gurley: 56.8 s and fibril area 3.98 % at 6000 U).

Regarding the tensile strength EnzC led to the greatest improvement to 6.05 kN m^{-1} at 6000 U compared to a value of 5.73 kN m^{-1}in the absence of enzymes. In agreement with a lower effect in refining, EnzB and EnzA lead to a lower tensile strength. Likewise, the tensile strength improvement was lower for the endoglucanase FiberCare R, which is, however, in contrast one of the highest effects in the degree of refining. EndoA and EndoB led to a much higher tensile strength when compared to their corresponding enzyme formulations **(Fig. S2)**.

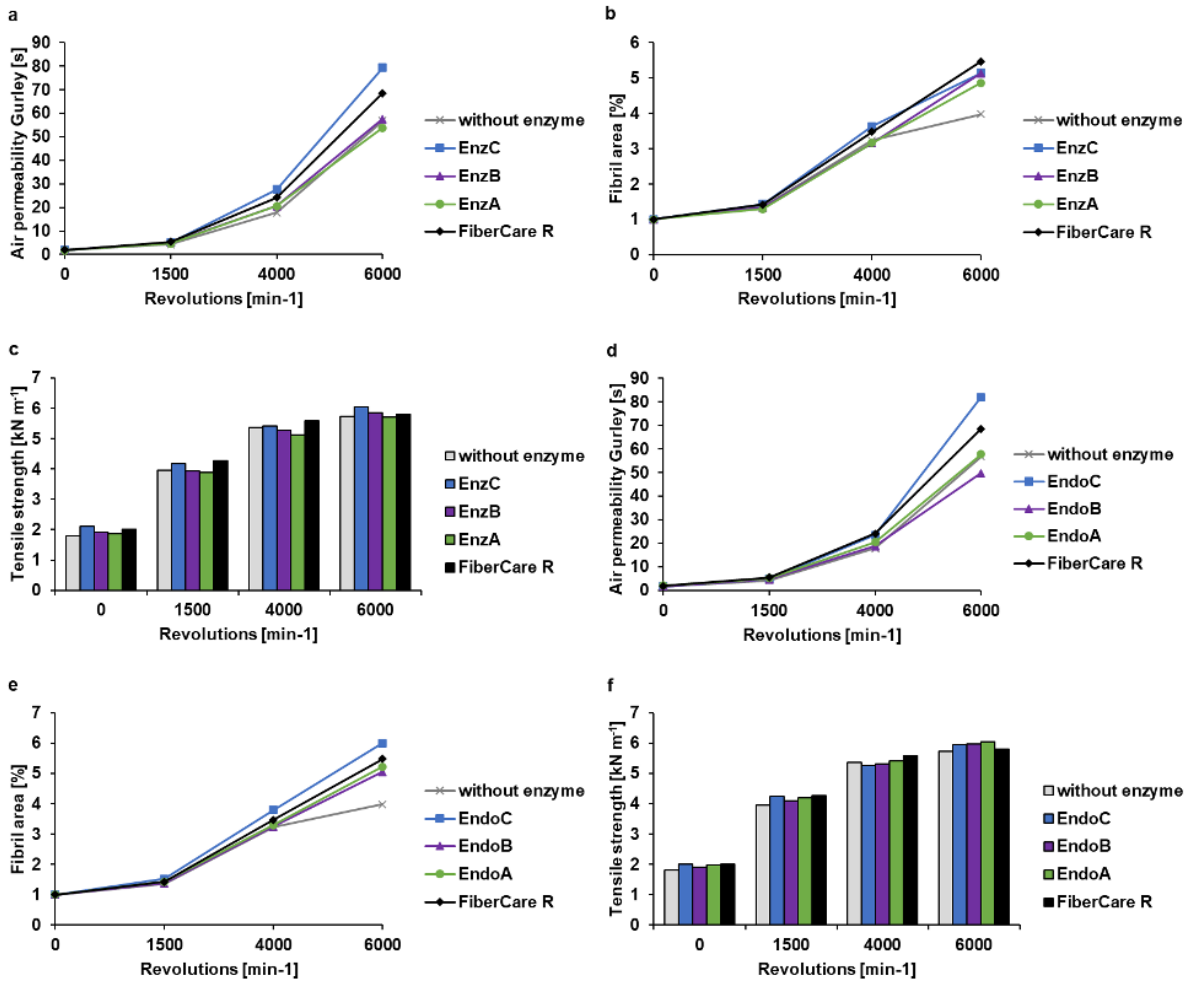

Fig. S20 Determined air permeance values according to Gurley, measured fibril area and tensile strength of the enzyme formulations (a-c) and of the purified endoglucanases (d-f)

Scanning electron microscopy (SEM) pictures of refined sample sheets

Scanning electron microscopy pictures were acquired of the formed handsheets and compared with the control without enzyme treatment and with the results of the commercial endoglucanase formulation FiberCare R (***Fig.* S22**, ***Fig.* S23**, **Fig. S23**, **Fig. S24**). Pictures were acquired at 0, 1500, 4000 and 1500 revolutions to reveal any differences at high refining and low refining levels. Obtained pictures show only minor differences between the enzyme formulations and purified endoglucanases, which was expected due to the adjustment of the enzymes to the same activity according to the novel endoglucanase specific CellG5 substrate.

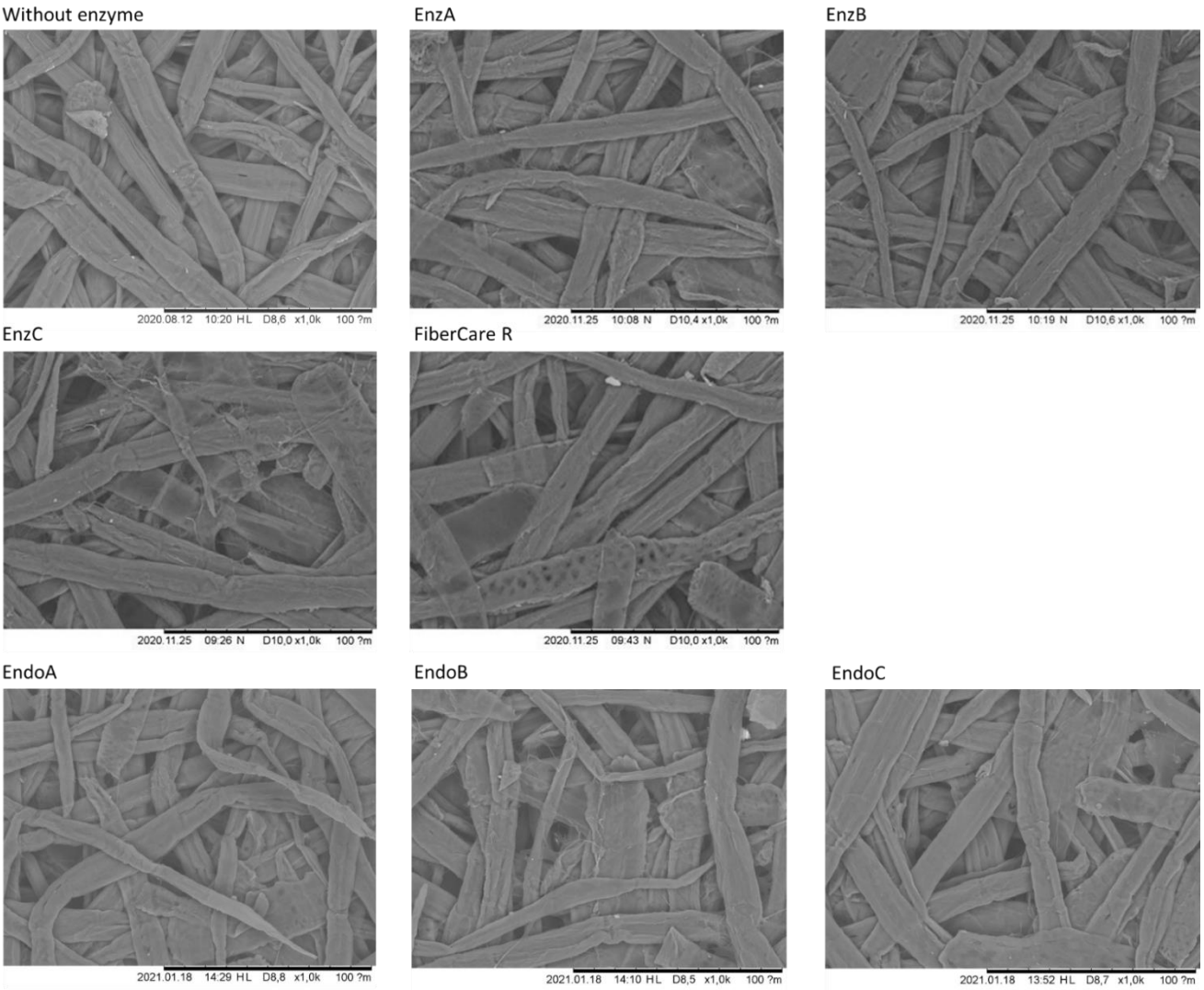

Fig. S21 Scanning electron microscope pictures of sample sheets at 1000x magnification. Comparison of the sample sheets refined at 0 U using the enzyme formulations EnzA, EnzB or EnzC and purified endoglucanases EndoA, EndoB and EndoC with sample sheets without enzyme treatment and with the commercial endoglucanase FiberCare R

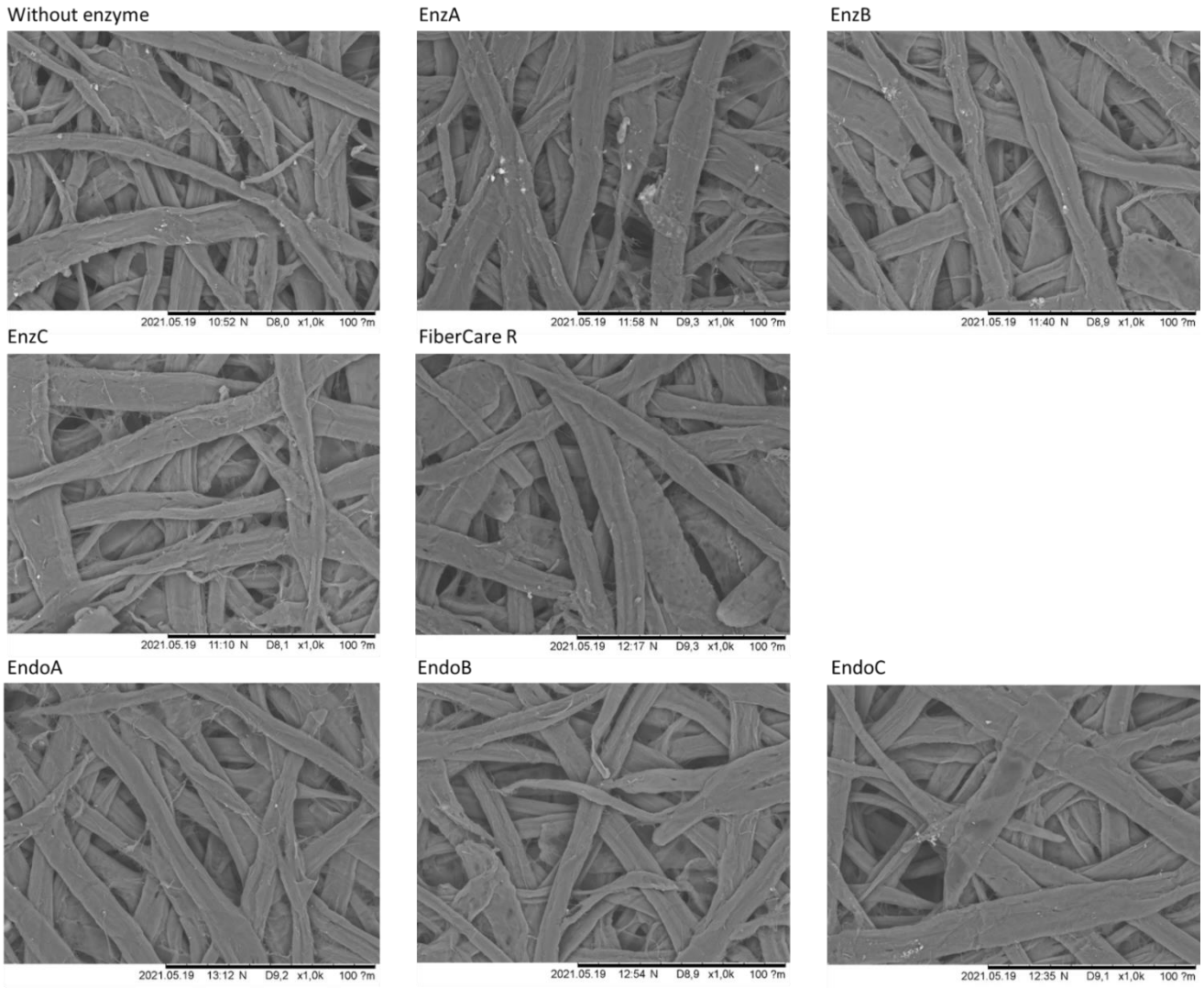

Fig. S22 Scanning electron microscope pictures of sample sheets at 1000x magnification. Comparison of the sample sheets refined at 1500 U using the enzyme formulations EnzA, EnzB or EnzC and purified endoglucanases EndoA, EndoB and EndoC with sample sheets without enzyme treatment and with the commercial endoglucanase FiberCare R

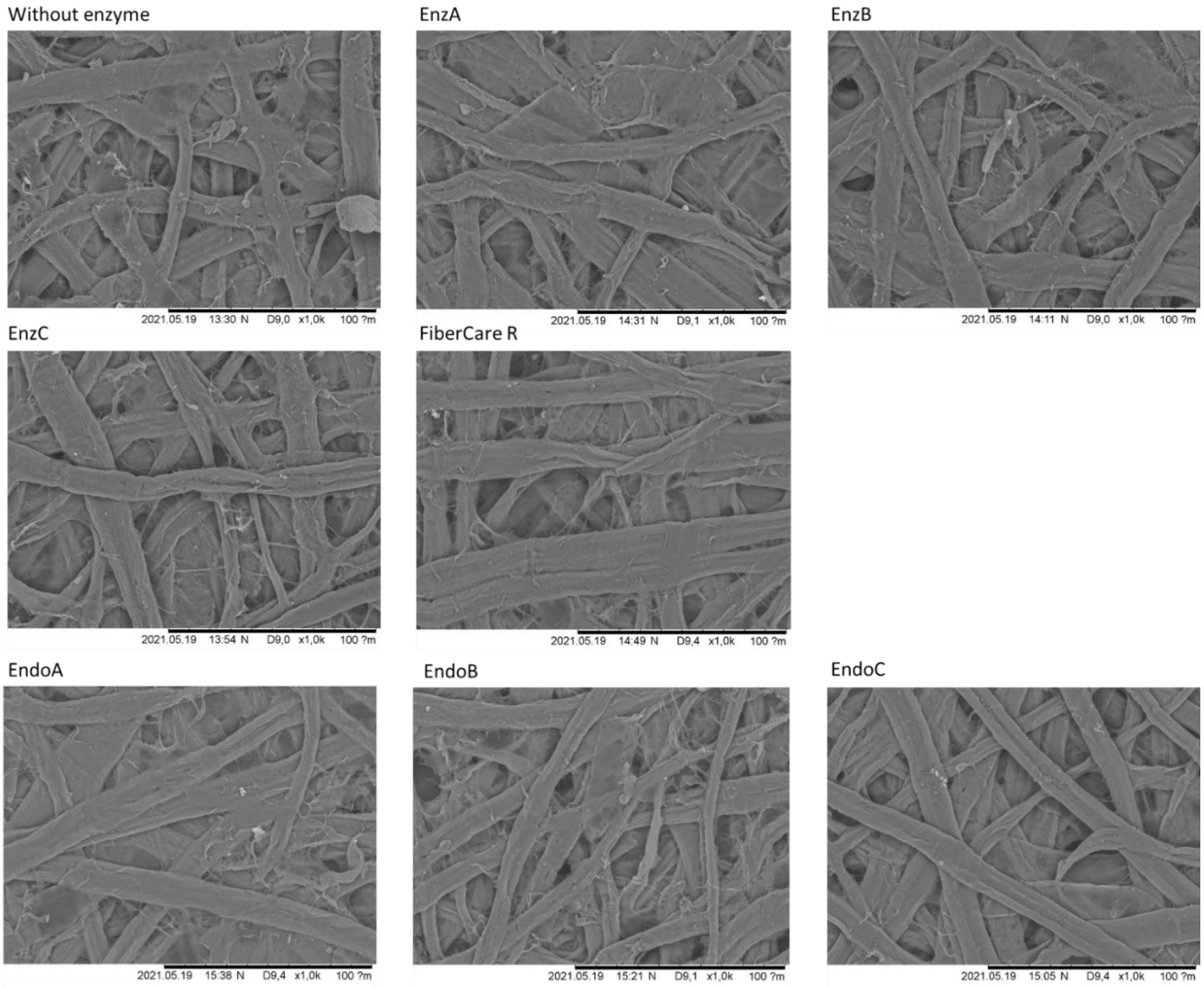

Fig. S23 Scanning electron microscope pictures of sample sheet at 1000x magnification. Comparison of the sample sheets refined at 4000 U using the enzyme formulations EnzA, EnzB or EnzC and purified endoglucanases EndoA, EndoB and EndoC with sample sheets without enzyme treatment and with the commercial endoglucanase FiberCare R

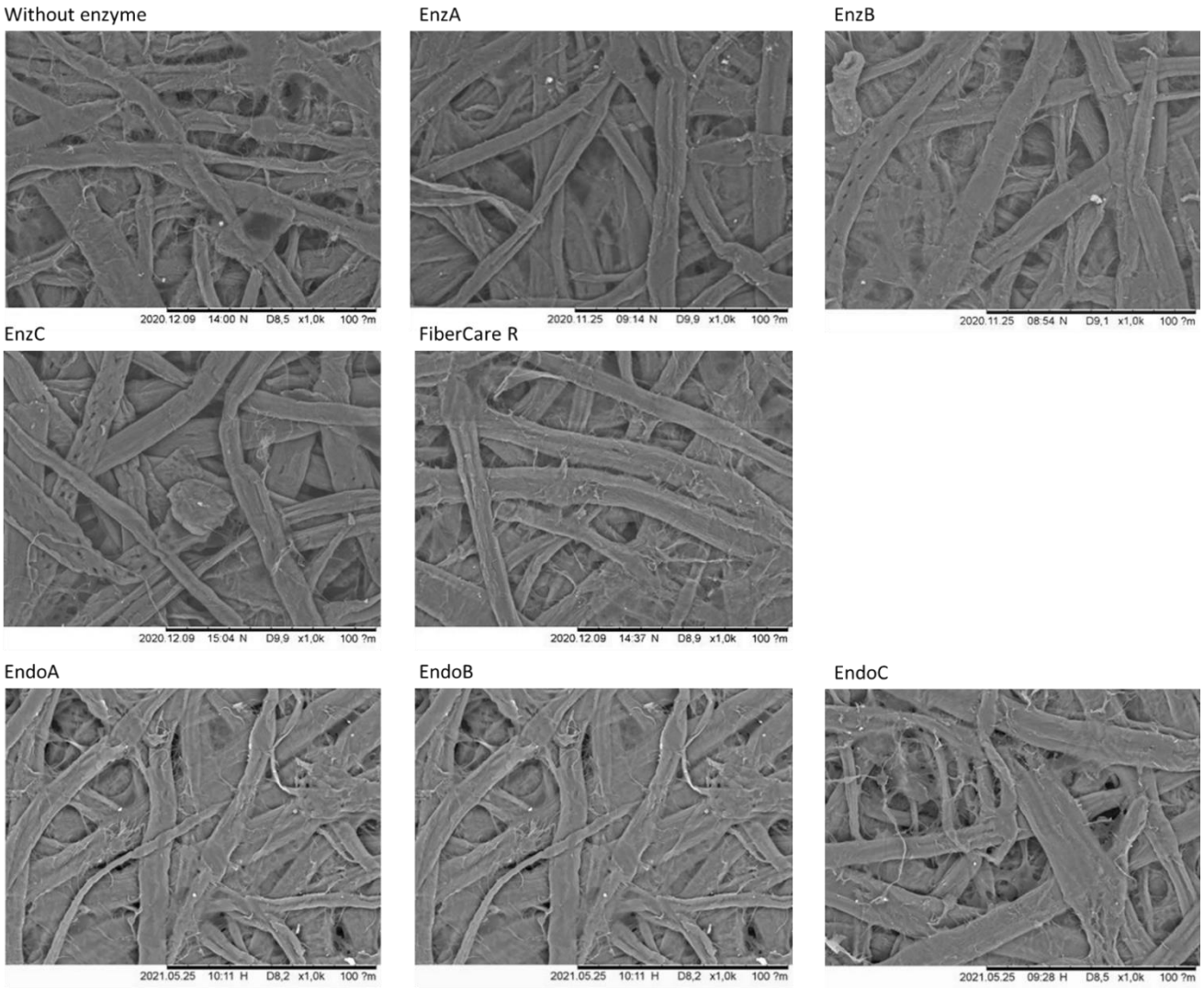

Fig. S24 Scanning electron microscope pictures of sample sheet at 1000x magnification. Comparison of the sample sheets refined at 6000 U using the enzyme formulations EnzA, EnzB or EnzC and purified endoglucanases EndoA, EndoB and EndoC with sample sheets without enzyme treatment and with the commercial endoglucanase FiberCare R

4

Mechanistic investigation of the effect of endoglucanases related to pulp refining

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4.1 Abstract

Endoglucanases are increasingly being touted as the ultimate solution for reducing energy consumption during the refining process in the pulp and paper industry. However, due to the high variety of endoglucanases in different enzyme formulations, these perform heterogeneously when applied to different pulps. In this study, the effect of four endoglucanases on softwood and hardwood pulp was studied using confocal laser scanning microscopy (CLSM) after addition of fluorescently labelled carbohydrate binding modules (CBMs). Nuclear magnetic resonance (NMR) analysis and high-performance liquid chromatography (HPLC) quantification of released oligo- and monosaccharides was performed for in-depth mechanistical investigation. Changes in the crystallinity levels caused by enzymatic degradation of amorphous regions were monitored by incubation with two different CBMs from *Caldicellulosiruptor bescii* and from *Thermobifida fusca* with high preference to either amorphous or crystalline regions of cellulose, respectively. When dosed at identical activity on the endoglucanase specific CellG5 substrate, CLSM analysis indicated the highest decrease of amorphous regions for those endoglucanases which were also most active in laboratory refining trials and which released highest amounts of cellooligomers from pulp. Using ^{13}C-NMR analysis, an increase in para-crystalline cellulose caused by enzyme application was observed. Release of reducing sugars was determined at identical CellG5 dosage, indicating a high variance between the enzymes, especially

when complex enzyme formulations were used. Scanning electron microscopy images were obtained for visualization of the endoglucanase activity. The results of mechanistical studies indicate that reduction of amorphous moieties of pulp by endoglucanases is especially beneficial for the refining process.

Keywords: Endoglucanase, CLSM, Carbohydrate Binding Module, CellG5, HPLC, NMR

4.2 Introduction

Cellulose is a highly abundant material, used for various processes like paper or textile production. Refining of pulps is a key step in the paper making process aimed at increasing fiber-fiber interaction, and positively affects homogeneity, flexibility, tensile index, internal bond, tensile energy absorption and many other characteristics, which result in paper with desired properties[1]–[3]. There are also trade-offs such as a reduction in drainability, specific volume, opacity and air permeability. Traditionally, refining of paper pulps is achieved using either conical, disc or cylindrical refiners to externally and//or internally fibrillate cellulose fibers or in some cases to shorten the fibers. The principal drawback of currently applied refining technologies is the high unit energy consumption, usually ranging from 150 to 500 kWh/ton paper and accounting for 30 to 50% of the total energy used for paper making and generation of high amounts of fines [4]–[6] Therefore, the use of enzymes during pulp refining emerged as a new measure to reduce energy consumption, resulting in energy savings up to 40 % [2], [7]–[9].

Within the cellulose degrading enzyme systems, endoglucanases are thought to be primarily responsible for the energy reduction during refining as previous studies investigating endoglucanases in refining experiments showed [10]–[12]. Endoglucanases attack the cellulose chain internally by cleaving the β- 1,4-linkage between the glucose chains and have a high affinity for the amorphous part of cellulose, thereby increasing the overall crystallinity (Mansfield and Meder, 2003; Miotto et al., 2014; Kamppuri et al., 2016). However, studies showed that not every enzyme formulation used in the pulp and paper industry is leading to a significant increase in the degree of refining or has beneficial effects on paper sheets properties. For example, a study showed an energy reduction of 20% during refining of bleached softwood pulp after testing of two different endoglucanases, while the third

endoglucanase did not show any effect on the refining properties [5].Therefore, there is a need to better understand the role of endoglucanases in the refining process and to investigate which synergistically acting enzymes are required [9]. However, as enzyme formulations work heterogeneously among pulps, paper companies must assess the suitability of each formulation in labour and cost intensive refining trials. One way for the assessment of endoglucanase suitability is the measurement of crystallinity changes caused by the degradation of amorphous regions by endoglucanases [16], [17].

Crystallinity of pulps was until now mainly assessed using X-Ray diffraction or NMR spectroscopy, making the monitoring time consuming and expensive, but the methods offer a quantitative measure of changes on the molecular structure and chemical composition [18]–[20]. For example, X-ray diffraction and NMR were used for determination of the amount of crystalline cellulose in plant cell walls or for the investigation of cellulose crystallinity and allomorphs of different pulps during pulping [21], [22]. Carbohydrate binding modules are non-hydrolytic domains that facilitate the catalytic activity of carbohydrate specific enzymes by binding to their respective substrates [23]–[25]. Based on their amino acid sequence, carbohydrate binding modules are classified into families, with more than 70 different families discovered in 2017 and 88 families in 2021 [26], [27]. Carbohydrate binding modules bring enzymes close to the surface of carbohydrates like cellulose, however, the enzymes themselves do not necessarily have to be active directly on the target carbohydrates of the CBMs, but rather on adjacent substrates. For example, the mannanase Man5A enzyme of *Trichoderma reesei* contains a CBM 1 carbohydrate binding module that binds to cellulose, while the enzymes are only active on mannan, as mannan and cellulose are adjacent in plant cell walls [28], [29].

While there are carbohydrate binding modules derived from cellulases and xylanases that can bind to cellulose and xylan, there are also carbohydrate binding modules that are able to bind to synthetic polymers like PET, thus enhancing hydrolysis when e.g., fused to cutinases [30]–[32]. A study showed that carbohydrate binding modules have a varying tendency to bind crystalline or amorphous (less-ordered) regions of the heterogenous cellulose substrate dewaxed cotton [33]. This characteristic was already used to observe changes in crystallinity when applying an enzyme formulation

designed for total hydrolysis on never-dried pulps [34]. In contrast to NMR, this method only can visualize changes on the surface of cellulose, as it depends on the binding of the carbohydrate binding modules to their substrates [35], [36].

In our previous study: “Biorefining: the role of endoglucanases in refining of cellulose fibers” we tested the endoglucanase specific CellG5 substrate as a new dosage method in laboratory refining trials, which reduced the differences between the enzyme formulations and purified endoglucanases and resulted in similar paper properties. In the present study, the effect of the purified endoglucanases on different pulps was investigated using NMR, HPLC, SEM and confocal laser scanning microscopy (CLSM) analysis after incubation with labelled carbohydrate binding modules that are specific to amorphous or crystalline areas of cellulose fibers to provide a mechanistic insight into the remaining differences between the enzymes.

4.3 Material & Methods

4.3.1 Pulps, enzyme formulations and chemicals

Enzyme formulations (EnzA, EnzB, EnzC) were provided by Austrian paper manufacturers. The commercial endoglucanase FiberCare R was kindly provided by Novozymes A/S (Bagsværd, Denmark). A softwood sulfate long fiber pulp (spruce) and a hardwood sulfate short fiber pulp (eucalyptus) was provided by companies of the pulp and paper industry.

The CellG5 Cellulase assay kit was purchased from Megazyme (Bray, Ireland). Carbohydrate binding modules CBM 28A from *Caldicellulosiruptor bescii* and CBM 2D from *Thermobifida fusca* were purchased from NZYTech (Lisboa, Portugal). The fluorescent dyes Dylight 405 and Dylight 634 were purchased from Thermo Fisher Scientific (Vienna, Austria). All other chemicals were obtained from Sigma-Aldrich (Austria) in HPLC grade if not stated otherwise.

4.3.2 Endoglucanase activity and protein content

Endoglucanase activity was determined using the Megazyme CellG5 Cellulase kit (Bray, Ireland). The mechanism of this assay is explained in a previous study [37]. As buffer 50 mM citrate buffer, pH 4.8 was used. For this purpose, a volume of 100 µl of diluted enzyme solution was mixed with 100 µl of CellG5 substrate in 10 ml glass tubes and incubated at 45°C for 10 minutes. The reaction was stopped by addition of 3 ml 2% (w/v) tris solution (pH 10). The entire volume was then transferred into 3 ml

cuvettes and absorbance was measured using a Hitachi U2900 Photometer (Chiyoda, Japan).

The concentration of the protein content was determined using the NanoDrop NP80 (Implen, Germany) by measuring the absorbance at 280 nm. Before the measurement a volume of 2 µl of 50 mM citrate buffer (pH 4.8) was used a blank. Afterwards, 2 µl of each sample were applied, and the protein concentration was measured in duplicates. For the calculation of the protein concentration, the extinction coefficient of BSA was used: 44.289 [M^{-1}*cm^{-1}].

4.3.3 Endoglucanase purification

Endoglucanases were purified from the provided enzyme formulations (EnzA, EnzB, EnzC) using a combination of hydrophobic interaction chromatography (HIC) and anion exchange chromatography (AEX) as described in a previous study characterizing the activities of these enzymes [11]. The purified endoglucanases were named according to the enzyme formulations they were isolated from: EndoA, EndoB and EndoC.

In brief: enzyme formulations were diluted in binding buffer A (10 mM acetate buffer + 1.5 M $(NH_4)_2SO_4$ at pH 4.8) to reach a final volume of 70 ml. After binding and column washing, elution of the endoglucanases was started. EndoA was eluted using a 30% buffer B (10 mM acetate buffer pH 4.8) step, followed by a linear gradient of 30-80% B, while EndoC was eluted by using a 45% Buffer B step, followed by a linear gradient from 45%-90% B. EndoB was purified using a 50% B step, followed by a linear gradient ranging from 50-100% B. Collected endoglucanase enzyme fractions were identified using SDS-PAGE, pooled and concentrated using a Vivaflow 50 membrane with a 5000 Da cut-off (Sartorius, Germany). The final concentrated volume of 5 ml was applied to PD-10 columns (Cytiva, USA) and eluted in anion exchange buffer A.

For further removal of residual enzymes other than endoglucanases, AEC was applied using a HiTrap DEAE FF 5 ml column. A sample volume of 25 ml was applied and after washing of the column with buffer A (10 mM Tris pH 7.5), elution of the endoglucanases was started using a gradient of buffer B (10 mM Tris pH 7.5 + 1 M NaCl). EndoA and EndoB were purified using a linear gradient ranging from 0 to 7% B, while EndoC was already eluted using a gradient of 0 to 1.5% B. Finally, endoglucanases were identified using SDS-PAGE and pooled again using Vivaspin 20, 3000 Da centrifugal

concentrators (Sartorius, Germany). PD-10 columns (Cytiva, USA) were used to exchange the buffer to 50 mM citrate buffer (pH 4.8).

4.3.4 Confocal Laser Scanning Microscopy (CLSM)

4.3.4.1 Labelling of Carbohydrate Binding Modules:

The buffer of purchased CBM 28A and CBM 2D was exchanged to 1x PBS using Vivaspin 20, 3000 Da centrifugal concentrators, 10 ml fresh PBS was added in steps during centrifugation at 3700 rpm to a final volume of 500 µl. The concentrates containing the CBMs in PBS were subsequently used for labelling. CBM 28A was labelled with DyLight 405, CBM 2D with DyLight 633 using the DyLight Antibody Labelling Kit (Thermo Fisher Scientific, Austria). Labelling was performed according to the supplier's manual. In brief, a volume of 40 µl of 0.67 M borate buffer (pH 8.5) was added to 500 µl of PBS solution (containing the respective CBMs) and 500 µl of the protein solution were then transferred to the vial containing the DyLight dye. After 1-hour of incubation at room temperature, the labelled CBMs were separated from excess dye through addition of the protein solution to 250 µl of supplied purification resin in spin columns. Labelled CBMs were obtained in the flow through by centrifugation for 1 minute at 1000 g.

4.3.4.2 Incubation of pulps with purified endoglucanases:

An amount of 10 mg (dry matter) of pulp (softwood or hardwood) was added into micro centrifugation tubes. The activity of all endoglucanases was adjusted to 0.035 nkat according to the endoglucanase specific Megazyme CellG5 Cellulase kit. The total reaction volume was set to 250 µl using 50 mM citrate buffer (pH 4.8) or 50 % tap water + 50 % deionized water (adjusted to pH 7 with 1 M HCl). Pulps were incubated at 45°C and 500 rpm for 4 hours on a thermomixer (Eppendorf, Austria). For removal of bound enzymes, a similar washing protocol as in a previous study was applied [34]. Therefore, pulps were washed 3x with 250 µl fresh ultrapure water and incubated with 250 µl 1% SDS solution at 99°C, 500 rpm for 10 minutes to remove residual enzymes from the cellulose fibers. Pulps were then washed 1x in 250 µl absolute ethanol, 1x in 250 µl ultrapure water and finally suspended in 250 µl PBS. For the immobilization of single fibers on microscopy glass slides, single fibers were pulled out of the microcentrifugation tubes and immobilized in two separate sections on a microscopy glass slide using nail polish. Nail polish was also used to draw a square around the single fibers to allow CBM addition and washing steps. A volume of 50 µl containing

an equal amount of fluorescently labelled CBM 2D and CBM 28A were added to each section of immobilized single fibers. Fibers were incubated for 10 minutes at room temperature, afterwards the CBM solution was gently removed with a pipette and a volume of 50 µl fresh PBS was added for fiber washing. The PBS solution was again gently removed with a pipette and samples were dried on air until CLSM measurement. An Olympus FV1000 confocal laser scanning microscope was used for image acquisition. Excitation of DyLight 633 was performed at 638 nm, that of DyLight 405 at 400 nm. Emission of fluorescence was recorded at 647 nm at a BF position of 650 nm and a BF range of 100 nm for DyLight 633 and 422 nm at a BF position of 425 nm and BF range of 35 nm for DyLight 405. Colours were assigned arbitrarily to enable a clear distinction between the colours, hence DyLight 633 was displayed as red and DyLight 405 as blue colour. The 10x UPLSAPO 10X2 objective lens was used for the acquisition of all images using following parameters for both colours: 600 hv (intensity), 1.75 gain and 9% offset. Background correction was applied using the background subtraction functionality of ImageJ by applying a rolling ball radius of 50 pixels to all pictures. Three different endoglucanases that were purified from enzyme formulations of the pulp and paper industry as well as the commercial endoglucanase FiberCare R that is also suitable for pulp refining were compared on either a long fiber softwood or a short fiber hardwood pulp in duplicates as enzyme performance may vary between different pulps [9]. In addition, the complex enzyme formulations from which the endoglucanases were purified from were analysed at refining conditions on the hardwood pulp. A blank sample without the addition of any enzymes was measured for comparison. The blue and red colour intensity ratios of the duplicate images were calculated after intensity read-out with ImageJ using the integrated density calculation function and used for semi-quantitative evaluation. The corresponding second set of CLSM images is shown in the Online Resource (**Fig. S*32***, **Fig. S33**).

4.3.5 High-performance liquid chromatography (HPLC) of enzyme treated pulps

For HPLC, 50 mg (dry matter) of pulps were incubated with enzymes at a dosage of 0.83 nkat for 4 hours in 1250 µl 50 % tap water + 50% deionized water (adjusted to pH 7). The enzyme reaction was stopped by incubation at 99°C for 5 minutes on a thermomixer. For analysis of the supernatant after enzyme treatment, the sample was diluted 1:5 to reach a total volume of 960 µl. Precipitation of proteins and lipids, was performed prior to HPLC by addition of 20 µl of 2% potassium hexacyanoferrate(II) trihydrate solution and 20 µl of 2% Zinc sulfate heptahydrate solution, mixing and

centrifugation of the samples for 30 minutes at 12500 rpm. The supernatant was then filtrated through a 0.45 µm filter into glass vials, while the pellets were discarded. Samples were applied to an Agilent 1260 Infinity LC system (Santa Clara, USA), equipped with an ION-300 (Transgenomic Inc) column for the separation of sugars, which were separated for 45 minutes. Data analysis was performed using the Agilent OpenLab software. Cellobiose, cellotriose, cellotetraose, glucose and xylose standards ranging between 0-0.96 mg ml^{-1} were measured along the samples and used for the calculation of sample concentration in mg ml^{-1}. Measurements were performed in duplicates and error bars indicate the standard deviation.

4.3.6 Reducing sugar assays on pulps

Endoglucanase activity in terms of reducing sugar release was tested on different pulps using 50% tap water + 50 % deionized water (adjusted to pH 7 with 1 M HCl) to mimic conditions used in industrial pulp refining. The reaction was conducted in 10 ml glass tubes, using 10 mg (dry matter) of each pulp. A volume of 200 µl 50% tap water + 50 % deionized water (pH 7) was added for suspension of the pulps, followed by addition of the diluted enzymes at different timepoints (0, 10, 20, 40 and 60 minutes). The incubation was stopped with the addition of 250 µl 1 M NaOH. DNS was used for the colour formation and added with a volume of 250 µl, then the tubes were boiled for 5 minutes in a boiling water bath. Absorbance was measured at 540 nm after transferring 200 µl in 96-well plates using an Infinite 200 Pro Tecan Reader (Zurich, Switzerland). Concentration of released reducing sugars was quantified with a glucose standard curve ranging from 0-20 mM. Duplicate measurements were performed, and error bars indicate the standard deviation. The obtained activity values in nkat ml^{-1} were used to calculate reducing sugar release at the conditions applied for HPLC.

4.3.7 ^{13}C-NMR analysis of enzyme treated pulps

For the analysis of enzyme treated pulps with ^{13}C-NMR, a higher amount of pulp was required than for the CLSM measurements. Therefore, reaction conditions were upscaled by the factor 5. An amount of 50 mg (dry matter) of each pulp was incubated with the enzymes at a dosage of 0.175 nkat in 1250 µl 50 mM citrate buffer (pH 4.8) at 45°C. The enzyme reaction was stopped through incubation at 99°C for 5 minutes at 300 rpm. Solid-state NMR experiments were performed on a Bruker Avance III HD 400 spectrometer (resonance frequency of ^{1}H of 400.13 MHz, and ^{13}C of 100.61 MHz, respectively), equipped with a 4mm dual broadband CP-MAS probe. Samples were

swollen in deionized water over night before measurement. ^{13}C spectra were acquired by using the CP-sequence at ambient temperature with a spinning rate of 12 kHz, a cross-polarization (CP) contact time of 2 ms, a recycle delay of 2 s, SPINAL-64 ^{1}H decoupling and an acquisition time of 43 ms. The spectral width was set to 300 ppm. Chemical shifts were referenced externally against the carbonyl signal of glycine with δ = 176.03 ppm. The acquired FIDs were apodized with an exponential function (lb = 11 Hz) prior to Fourier transformation. Peak fitting was performed using the dmfit program [38]. Assignment of crystalline or amorphous fractions was based on Wickholm, Larsson and Iversen, 1998. Peaks between 87.8 ppm and 89.3 ppm were assigned to the crystalline (cellulose Iα: 89.3 ppm, cellulose Iβ: 88.8 ppm, cellulose Iαβ: 87.8 ppm) and para-crystalline regions (88.4 ppm), whereas peaks between 83.2 and 84.2 ppm were considered as amorphous (accessible surface as sum of accessible surface I (84.2 ppm) and accessible surface II (83.2 ppm) as well as inaccessible surface (83.7 ppm)). The crystallinity index was calculated as described in Zuckerstätter et al., 2009.

4.3.8 Scanning electron microscopy (SEM)

For assessment of the effect of the endoglucanases on different pulps with scanning electron microscopy, a higher amount of pulp was required than for the CLSM measurements. An amount of 50 mg pulp (dry matter) was incubated at a dosage of 0.175 nkat in a volume of 1250 µl 50 mM citrate buffer (pH 4.8) for 4 hours in a thermomixer at 45°C and 500 rpm. Enzymes were inactivated by incubation at 99°C for 5 min. Pulps were subsequently washed with fresh 50 mM citrate buffer (pH 4.8) or 50 % tap water + 50 % deionized water (pH 7), the solution was removed, and the pulps dried overnight in a 70°C drying chamber. The extent of the endoglucanase activity was then visualized using the Hitachi TM3030 scanning electron microscope (Chiyoda, Japan). Prior to imaging, samples were coated with 1 nm platinum using the sputter coater Leica EM Ace200 (Wetzlar, Germany) for contrast enhancement. Scanning electron microscopy pictures are available in **Fig. S*34*** of the Online Resource.

4.4 Results and discussion:

4.4.1 Enzyme activity on the endoglucanase specific substrate CellG5

The enzymes compared in this study cause degradation of pulp fibers as visualised by SEM analysis **(Fig. S*34*)** and led at identical refining conditions to an increase of the degree of refining in terms of the Schopper-Riegler (°SR) number in our previous study [11]. In brief, the enzyme formulations EnzA, EnzB and EnzC at 6000 refiner revolutions led to a degree of refining of 40.7 °SR, 41.2 °SR, 45.0 °SR and 44.8 °SR for FiberCare R, respectively. The purified endoglucanases EndoA, EndoB and EndoC, reached degree of refining values of 42.4, 41.3 and 47.9 °SR. For comparison, without enzymes, only a degree of refining value of 35.5 °SR was reached at 6000 revolutions. In this study, the differences in the refining performance seen for these enzymes and their effect on softwood and hardwood pulps was mechanistically studied more in detail. In a first step, endoglucanase activities on the endoglucanase specific derivatized cellopentaose (CellG5) were compared in citrate buffer (pH 4.8) and subsequently in 50% tap water + 50% deionized water (adjusted to pH 7) simulating refining conditions (**Fig. 25)**. EndoC showed the highest endoglucanase activity of 26.3 nkat mg^{-1} while EndoB had the lowest value of 3.1 nkat mg^{-1} using citrate buffer. Interestingly, the commercial endoglucanase FiberCare R showed similar activities on both citrate buffer (11.6 nkat mg^{-1}) and water (12.0 nkat mg^{-1}), as well as EndoB (2.6 nkat mg^{-1}), while the activities of EndoA (3.3 nkat mg^{-1}) and EndoC (5.2 nkat mg^{-1}) decreased significantly at pH 7. Additionally, the corresponding enzyme formulations from which the endoglucanases were purified from, were analysed using the CellG5 substrate at refining conditions (50% tap water + 50% deionized water (pH 7)). The commercial endoglucanase FiberCare R formulation showed the highest activity (11.9 nkat mg^{-1}), followed by EnzB (4.5 nkat mg^{-1}) and EnzC (2.0 nkat mg^{-1}), while EnzA exhibited the lowest activity with 0.5 nkat mg^{-1} [11].

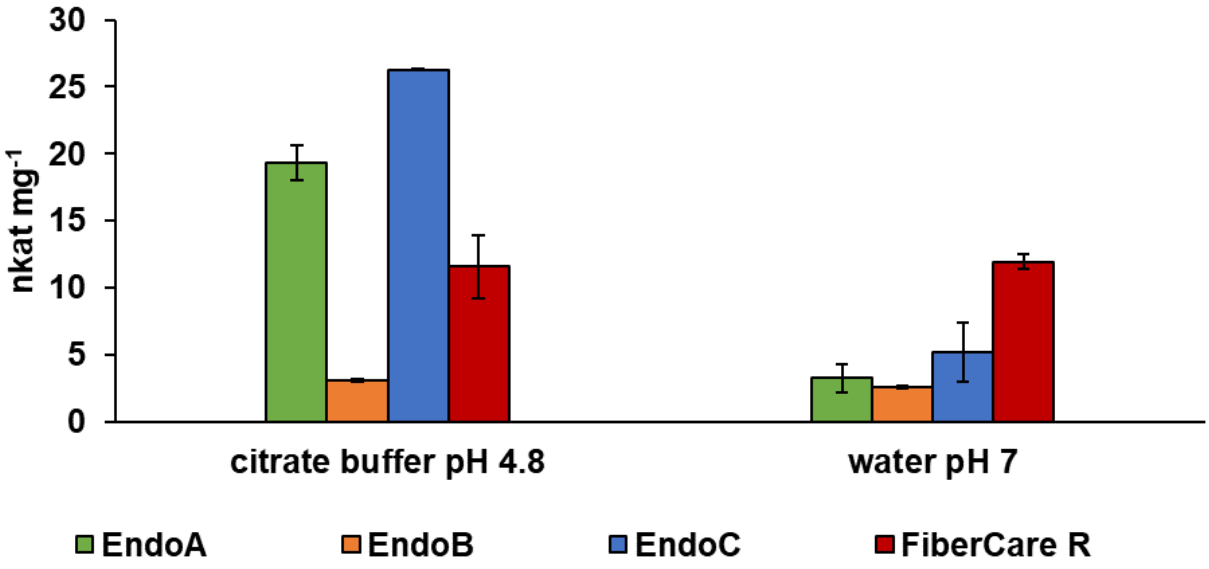

Fig. 25 Activity of purified endoglucanases and of the commercial endoglucanase formulation FiberCare R on derivatized cellopentaose (CellG5). Endoglucanase activity was measured using 50 mM citrate buffer (pH 4.8) or 50% tap water + 50% deionized water (adjusted to pH 7) simulating refining conditions. Measurements were conducted in duplicates and error bars indicate the standard deviation.

4.4.2 Quantification of mono-/oligosaccharides released from pulps using HPLC

Long fiber softwood and short fiber hardwood pulp were treated with purified endoglucanases and the commercial endoglucanase FiberCare R at simulated refining conditions (50 % tap water + 50 % deionized water at pH 7) and the supernatant was analysed using HPLC **(Fig. 26).** On the softwood pulp, when dosed at identical endoglucanase activity, EndoC led to the highest release of cellobiose (0.073 mg ml^{-1}), cellotriose (0.056 mg ml^{-1}) and cellotetraose (0.058 mg ml^{-1}). The second highest cellobiose concentration was released by FiberCare R (0.036 mg ml^{-1}), while the other purified endoglucanases released much lower concentrations (lowest with EndoA: 0.020 mg ml^{-1}). Expectedly, glucose was not found with most purified endoglucanases except for EndoB which released 0.027 mg ml^{-1} glucose and EndoA (0.0012 mg ml^{-1}), suggesting a low amount of β-glucosidase enzyme that could not be entirely removed during purification **(Fig. 26a)**. As expected, the enzyme formulations showed a different pattern when compared to the purified enzymes, as there are also other enzymes than endoglucanases present like β-glucosidases, xylanases or cellobiohydrolases **(Fig. 26b).** Lower concentrations of oligosaccharides suggest their conversion into glucose which was present in higher amounts when compared to the purified enzymes. However, cellotetraose was released by the EnzA formulation while the respective

endoglucanase purification did not, which could hint to a synergistical effect in junction with other enzymes in this enzyme formulation that increased enzyme activity. Interestingly, the glucose levels of EnzB (0.0071 mg ml^{-1}), were lower than in the respective enzyme purification, while the cellobiose (0.039 mg ml^{-1}) and cellotriose (0.035 mg ml^{-1}) levels increased. The higher glucose and xylose levels can be related to the higher volume of the EndoB endoglucanase that had to be added to reach the same activity as the other purified endoglucanases. While EndoA and EndoC had similar endoglucanase activities (EndoA: 35.5 nkat ml^{-1}, EndoC: 55.0 nkat ml^{-1}), EndoB only showed an activity of 18.4 nkat ml^{-1}. Therefore, also impurities like the residual recalcitrant β-glucosidase in EndoB were added in a higher amount as well, overall, a 7.3 times higher β-glucosidase activity was added when compared to the EnzB formulation. In addition, there are also endoglucanases that have a wider substrate specificity and are active on hemicelluloses like xyloglucan, xylan and arabinoxylan such as the endoglucanase family Cel7, which could explain the low amount of xylose released by EndoB[41], [42]. The levels of the oligosaccharides were higher for the formulations, except for EndoC, which hints to synergistical action of the endoglucanase enzymes with other components of the enzyme formulations like β-glucosidases as reported in previous studies [43]–[45].

On the short fiber hardwood pulp, the purified endoglucanases showed a similar pattern as for the long fiber softwood pulp, but a much lower amount of the different sugars was released **(Fig. 26c)**. The cellobiose concentrations of EndoC (0.036 mg ml^{-1}) and FiberCare R (0.013 mg ml^{-1}) were much higher than those of the other two purified endoglucanases (EndoA: 0.0021 mg ml^{-1}, EndoB: 0.0035 mg ml^{-1}). These findings are in agreement both with the decrease of amorphous regions seen with CLSM as well as with the results of the laboratory refining effect previously reported [11]. Similar behaviour was reported for endoglucanases like Cel9A from *Thermobifida fusca* or an GH12 endoglucanase from *Aspergillus niger*, releasing mainly cellobiose and only a minor amount of the other cellooligosaccharides[46]–[48]. In contrast, another study investigating endoglucanases showed that endoglucanases Cel5A and Cel12A produced cellotriose and even small amounts of glucose in addition to cellobiose, while the endoglucanase Cel45A released cellotetraose as the main product, with no cellobiose or glucose formation [49]. Cellotriose was produced by all purified endoglucanases, but when compared to the softwood pulp, EndoC released a

much lower concentration (0.016 mg ml^{-1}) very similar to FiberCare R and EndoB, while EndoA showed the lowest concentration (0.0072 mg ml^{-1}). Cellotetraose could only be quantified for EndoC and FiberCare R, showing comparable values, thus EndoB did not release cellotetraose on the short fiber pulp, in contrast to the long fiber pulp. The enzyme formulations showed similar behaviour as obtained from the softwood pulp, but much lower concentrations of sugars were released **(Fig. 26d)**. For cellobiose, EnzB and EnzC showed comparable concentrations (EnzB: 0.037 mg ml^{-1}, EnzC: 0.044 mg ml^{-1}), while EnzA also showed a release of cellobiose, but below quantifiable levels. However, the rather high cellobiose concentration of the EnzB formulation could be explained by the rather low β-glucosidase activity as expressed by the low glucose concentration (0.010 mg ml^{-1}). EnzB produced the highest amount of cellotriose (0.049 mg ml^{-1}), while EnzA had the lowest value (0.01 mg ml^{-1}). Cellotetraose was only released by EnzA (0.019 mg ml^{-1}), however, some β-glucosidases are also able to degrade cellotetraose, thus cellotetraose could already have been converted to glucose [50], [51]. Xylose was released by all formulations in a similar way with the highest concentration for EnzA (0.11 mg ml^{-1}) and the lowest for EnzB (0.01 mg ml^{-1}). Overall, higher amounts of glucose and cellooligosaccharides were released from softwood than from hardwood pulp, thus suggesting a better accessibility for this pulp. Interestingly, the enzyme formulation EnzA released the highest concentration of monosaccharides despite showing the lowest degree of refining in the laboratory refining trials [11].

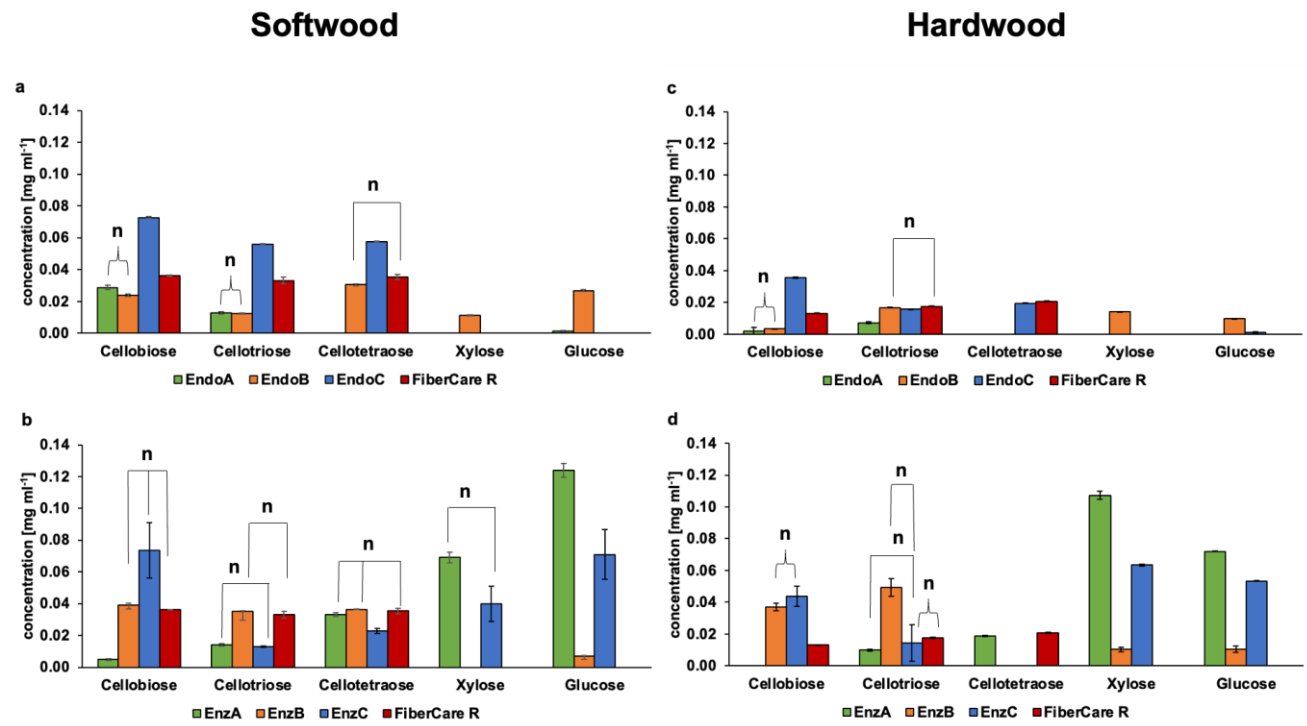

Fig. 26 Quantification (HPLC) of oligo- and monosaccharides released from softwood (a, b) and hardwood pulp by endoglucanases (a,c) and enzyme cocktails (b, d) . Long fiber softwood or short fiber hardwood pulp were treated using 50% tap water + 50% deionized water at pH 7 (simulating refining conditions) at a dosage of 0.83 nkat at 45°C for 4 hours and subsequently inactivated at 99°C for 5 minutes. Measurements were performed in duplicates and error bars indicate the standard deviation. Bars marked with the letter n are not significant to each other according to a performed statistical ANOVA test using a significance level of 0.1.

4.4.3 Release of reducing sugars from softwood and hardwood pulp

The adjustment of enzyme activities for the refining process and for total hydrolysis is often achieved by using reducing sugar assays [4], [12]. This assay is based on quantification of reducing sugars released from the pulps themselves or from model substrates like carboxymethylcellulose or filter paper. The characteristic red colour indicating the concentration of released reducing sugars is developed by the addition of dinitrosalicylic acid [52]. However, for enzyme formulations the release of reducing sugars is not only attributed to endoglucanase activity but also other enzyme activities such as cellobiohydrolases, β-glucosidases or xylanases [53]. In this study, the adjustment of the endoglucanase activity was performed according to derivatized cellopentaose (CellG5), as this substrate is modified to be specific to endoglucanases only. Although CellG5 is a model substrate it offers a higher accuracy through its endoglucanase specificity, when compared to reducing sugar assays on the pulps themselves, which are also influenced by other enzymes like xylanases, cellobiohydrolases and even enzymes like amylases or pectinases, with no role in pulp

refining, but that are often present in complex enzyme formulations of the pulp and paper industry. To allow a comparison with the results of the HPLC quantification, it was calculated how much reducing sugars were released at the identical dosage level of 0.83 nkat that was used for HPLC analysis. Despite the adjustment to the same CellG5 activity, the release of reducing sugars was different for the purified endoglucanases on the softwood as well as hardwood pulp. Softwood and hardwood pulps often contain a varying amount of hemicelluloses and lignin, both of which can influence the activity of cellulases [9], [54], [55]. EndoB showed the highest release of reducing sugars on the softwood and hardwood pulp (3.91 mg/g on both pulps). EndoB still exhibited a rather high β-glucosidase and xylanase activity, which could explain the high release of reducing sugars. However, in contrast, FiberCare R (2.5 mg/g reducing sugar release on hardwood pulp) and EndoC (1.5 mg/g) showed the best results during laboratory refining trials followed by EndoA (1.8 mg/g), which clearly indicates that the release of reducing sugars does not correlate with enzyme performance during pulp refining [11] The differences were even higher with the respective enzyme formulations, which might be related to other components present in the enzyme formulations like β-glucosidases and xylanases, which also contribute to the release of reducing sugars (EnzA: 4.7 mg/g, EnzB: 2.3 mg/g, EnzC: 3.8 mg/g on hardwood pulp). Although, the proportions of the formulations were similar on both pulps, there are differences in detail as the amount of released reducing sugars was lower with the EnzB formulation, which showed a lower concentration on the softwood pulp (1.4 mg/g)**.** Overall, the release of reducing sugars was higher for EnzA (6.7 mg/g), EnzC (4.6 mg/g) and FiberCare R (2.8 mg/g) on the softwood pulp when compared to the hardwood pulp, which suggests a better accessibility for this pulp, thus matching the results of the HPLC measurements. A detailed overview over the calculated reducing sugar release is highlighted in **Fig. S*31*** of the Online Resource.

4.4.4 Visualization of labelled carbohydrate binding modules on pulps by use of confocal laser scanning microscopy (CLSM) as a proxy for endoglucanase activity

CLSM has been demonstrated to be a powerful tool to visualize the binding of cellulases to cellulose e.g. to cellulose filter paper, revealing their preference to bind to dislocations of cellulose [56], [57]. On the other hand, labelled isolated carbohydrate binding modules can also be used to identify changes in crystallinity upon enzyme treatment [34], [58]. In this study, the mode of action of the individual endoglucanases

on diverse pulps was assessed by using two different carbohydrate binding modules, that bind with a high affinity either to amorphous (CBM 28A) or crystalline areas (CBM 2D) of cellulose. Endoglucanases preferentially degrade the amorphous parts of the cellulose chains, thus leading to an increase of the crystalline areas [16]. CBM 28 from *Caldicellulosiruptor bescii* was chosen due to its ability to bind strongly to amorphous substrates like amorphous (less-ordered) cellulose or xylan, while no binding to chitin was reported [59]. On the other hand, the carbohydrate binding module CBM 2 from *Thermobifida fusca* was chosen to detect crystalline regions of cellulose. This CBM binds strongly to microcrystalline cellulose, alpha and beta chitin as well as filter paper and is an important component for exoglucanases, that are degrading crystalline cellulose [60]–[62]. Despite the ability of CBM 2 to bind to crystalline cellulose, CBM 1 would have even a higher affinity to crystalline areas, but CBM 2 was chosen because of a commercial variant was already available. The selection of the CBMs was based on the study by Fox et al., 2013, which classified carbohydrate binding modules depending on their ability to bind to amorphous or crystalline regions based on photoactivated localization microscopy measurements on cotton, thereby introducing the order parameter value Ω (the lower the value, the higher the affinity to amorphous parts). CBM 2 exhibited an Ω value of 0.27, while CBM 28 showed a value of 0.16, thus indicating the binding to more amorphous regions of cellulose.

In contrast to the study by Novy et al., 2019 the elucidation of the effect of different endoglucanases was the main target, hence softwood or hardwood pulps were incubated with purified endoglucanases or their corresponding enzyme formulations and not with the hydrolytic enzyme cocktail CTec3 that is commonly used for total hydrolysis of cellulose containing a wide range of different enzymes like cellulases (including cellobiohydrolases) as well as hemicellulases [63]–[65]. Another difference to Novy et al., 2019 was the fixation of individual fibers on microscopy slides, which was accomplished by pulling single fibers out of the microcentrifuge tubes and subsequent fixation in a square of nail polish, which introduced a boundary for the subsequent CBM solution and enabled washing steps. For the visualization of the binding of the carbohydrate binding modules, each CBM was coupled with a different fluorescent dye. The binding of CBM 28 to amorphous regions of cellulose was arbitrarily assigned to a blue colour, while the binding of CBM 2 to crystalline regions was expressed by a red colour. Through an overlay of both colours, dominating amorphous or crystalline regions of cellulose fibers were visualized using confocal

laser scanning microscopy. Endoglucanases have a high preference for degradation of amorphous regions of cellulose, thus reducing the area for binding of CBM 28 to endoglucanase treated cellulose fibers [66], [67]. With increasing degradation of amorphous regions, the crystalline core of the cellulose fibers lying beneath the amorphous surface is revealed, thus enhancing the binding of CBM 2 indicated by an increase in red colour in this study. [68], [69].

CLSM images of the softwood pulp (**Fig. 27a**) indicated the highest decrease of amorphous regions upon treatment pulp by EndoC and the commercial endoglucanase FiberCare R as indicated by a more intense red colour caused by binding of CBM 2. This effect was less pronounced for EndoA, while EndoB showed the lowest effect. A similar behaviour was measured on the hardwood pulp with EndoC showing the highest decrease of amorphous areas (as indicated by increase of red colour intensity), followed by FiberCare R **(Fig. 27b)**. To confirm this assessment, a semi-quantitative analysis of the colour intensities was performed with ImageJ, the results are highlighted in **Fig. 29**. The hardwood pulp was also evaluated in laboratory refining trials using a PFI mill, showing a similar trend, resulting in EndoC and the commercial endoglucanase formulation FiberCare R leading to the highest degree of refining values when dosed at an identical activity on the derivatized cellopentaose substrate CellG5 [11]. The softwood pulp also expressed a high level of bends and twists, which were not present when compared to the hardwood pulp, a similar observation was already reported earlier [70]. Another factor for varying enzyme performance between the softwood and hardwood pulp is the amount of hemicellulose, which was determined with NMR to be 6.6 % for the softwood and 12.3 % for the hardwood pulp. Hemicellulose is known to inhibit cellulases and therefore can explain the lower amount of released sugars from the hardwood pulp [71]–[73].

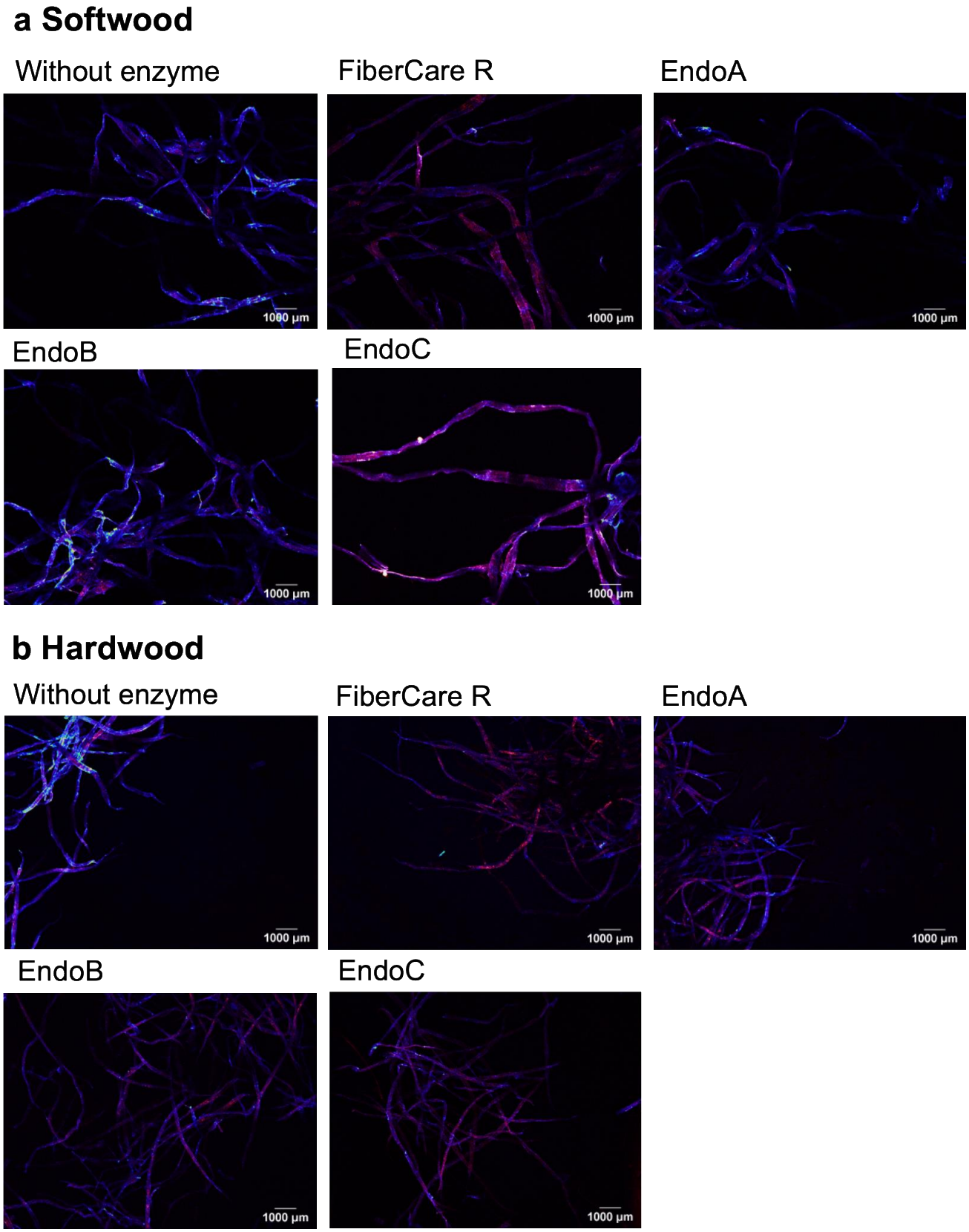

Fig. 27 CLSM images (10x magnification) on pulps incubated before and after endoglucanase treatment with two different carbohydrate binding modules for probing crystallinity changes. Duplicates of each combination of enzyme and pulp were measured. Specific binding of CBM 28 from *Caldicellulosiruptor bescii* to amorphous regions is indicated by an increase in blue colour. Specific binding of CBM 2 from *Thermobifida fusca* to crystalline regions is indicated by an increase in red colour. Long fiber softwood (a) and short fiber hardwood (b) pulps were incubated

with purified endoglucanases as well as the commercial endoglucanase FiberCare R for 4h in 50 mM citrate buffer (pH 4.8).

4.4.4.1 CLSM pictures under refining conditions

To simulate industrial refining conditions, 50% tap water + 50 % deionized water (pH 7) was used for the incubation of hardwood pulp with purified endoglucanases to allow comparison with laboratory refining results. Since the enzyme activities were comparably low at these conditions, more volume of each endoglucanase was added to reach the same activity of 0.035 nkat. CLSM analysis indicated the highest effect for EndoC and FiberCare R, leading to the most pronounced decrease of amorphous regions of cellulose, similar to the results in citrate buffer **(Fig**. **28a)**. These measurements nicely match with the laboratory refining trials, where EndoC and FiberCare R caused the highest degree of refining when dosed at identical endoglucanase activity using CellG5 [11].

In a next step, the effect of the enzyme formulations EnzA, EnzB and EnzC, from which the endoglucanases were purified from was studied on hardwood pulp using the same method (**Fig**. **28b)**. Additional enzymes like β-glucosidases or xylanases are present in these enzyme formulations and could lead to different results either by affecting the fibers positively or negatively. EnzC showed the highest decrease of amorphous regions as indicated by the decrease of blue colour intensity, followed by EnzA and EnzB. All these enzyme formulations had previously been investigated for refining when dosed based on identical activity using CellG5 [11]. In these previous results the highest degree of refining values was found for EndoC and FiberCare R correlating to the most pronounced decrease of amorphous regions seen in this study. This CLSM based method therefore allows to predict the effect of enzyme formulations in refining without the need of enzyme purification steps. A semi-quantitative analysis of the colour intensities was performed with ImageJ under refining conditions as well as to enable an additional way for assessment, the results are highlighted in **Fig. 29**.

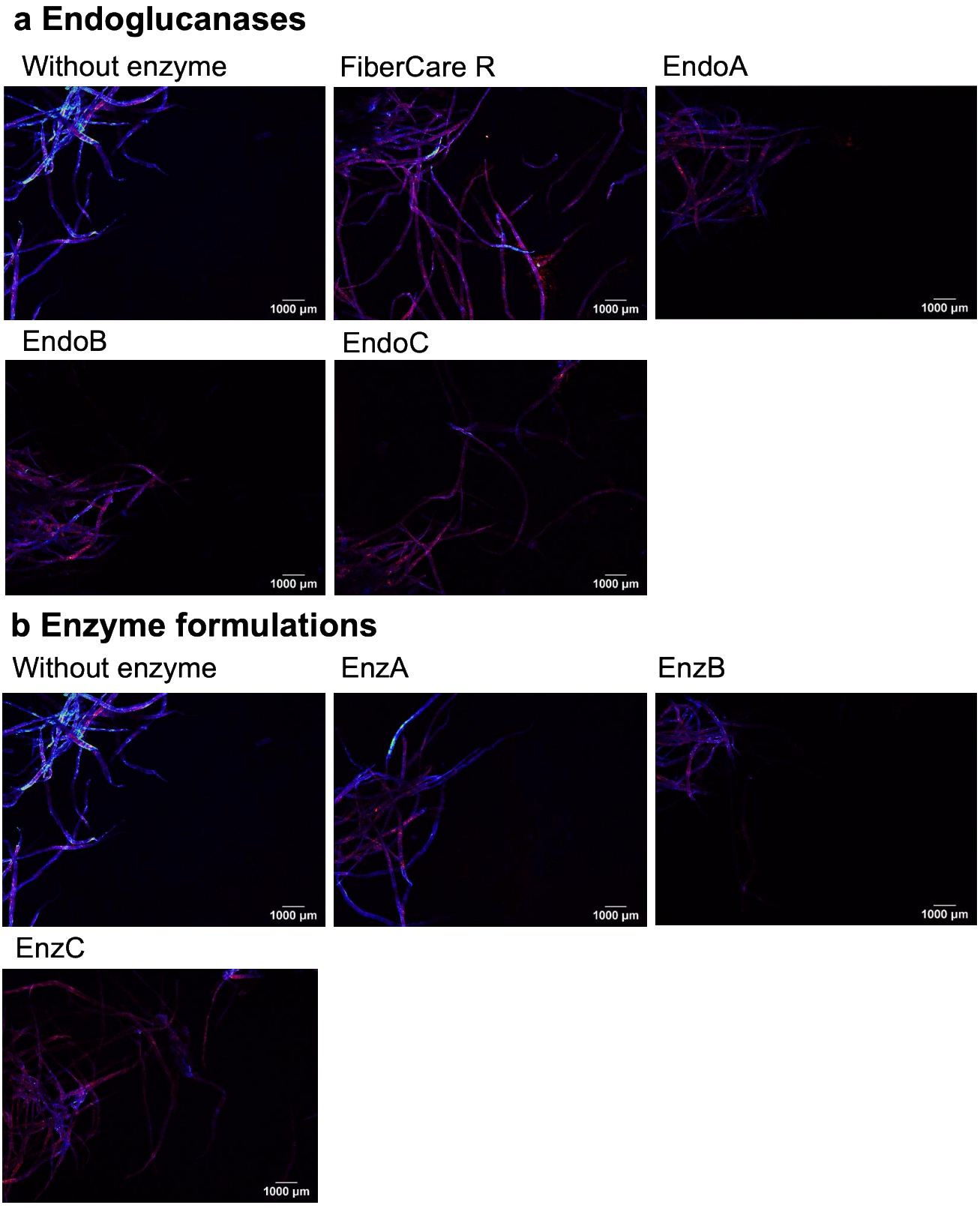

Fig. **28** CLSM images (10x magnification) on short fiber hardwood pulp incubated before and after endoglucanase treatment at refining conditions (50% tap water + 50% deionized water at pH 7) with two different carbohydrate binding modules for probing crystallinity changes. Duplicates of each combination of enzyme and pulp were measured. Specific binding of CBM 28 from *Caldicellulosiruptor bescii* to amorphous regions is indicated by an increase in blue colour. Specific binding of CBM 2 from *Thermobifida fusca* to crystalline regions is indicated by an increase in red colour. (a) shows the results for purified endoglucanases on short fiber hardwood pulp, (b) the

results of the original enzyme formulations, from which the endoglucanases were purified from on the hardwood pulp.

4.4.4.2 Quantification of the fluorescent colour intensity

The intensities of the blue (amorphous) and red (crystalline) colours were quantified with Image J to provide a (semi)-quantitative evaluation of the CLSM images. The calculated blue/red colour ratios are provided in **Fig. 29**. Compared to the sample without enzyme addition, the blue/red colour ratio was decreasing for all enzymes, regardless of the pulp and buffer type. The most pronounced decline could be measured with EndoC and FiberCare R on the softwood pulp (**Fig. 29a),** which is in accordance with the results of the HPLC measurements, which showed a higher release of fragments on the softwood pulp. EndoC and FiberCare R exhibited also the highest decrease on the hardwood pulp using citrate buffer (**Fig. 29b)** and 50% tap water + 50% deionized water **(Fig. 29c**), although to a much lower extent. When using 50% tap water + 50% deionized water (pH 7) as it was used for the laboratory refining trials, EndoC and FiberCare R could achieve 52.4 % and 49.1 % lower blue/red ratio, respectively, when compared to the blank without enzyme addition. For comparison, EndoA and EndoB only reached a reduction of 46.7 % and 37.5 %, respectively. These results are in accordance with the results of the laboratory refining trials. When using citrate buffer, the differences were lower (**Fig. 29b**), with EndoB even showing a slightly higher reduction of the ratio (27.9 %) than EndoC (26.6%), while EndoA still was the enzyme with the lowest reduction (26.1 %) and FiberCare R with the highest (40.5 %). However, the quantification of the colour intensity is only semi-quantitative and strongly depends on the amount of fibers on the CLSM image, the fiber heterogeneity and occurring artifacts on the CLSM images which all contribute to a higher standard deviation and therefore the quantification should only complement visual assessment. The pulp and paper industry often uses complex enzyme formulations rather than costly commercial endoglucanase formulations. After application of the corresponding enzyme formulations, EnzA achieved a 45.7 %, EnzB 52.6 % and EnzC 57.2 % reduction of the red/blue colour ratio in relation to the blank, which is also in accordance with the laboratory refining trials and shows that the CLSM method using carbohydrate binding modules can also be used for prediction of the behaviour of complex enzyme formulations without the use of any purification steps. Finally, the ANOVA test was applied for the evaluation of non-significant results following statistics. According to these tests, most enzymes could significantly lower the blue/red colour ratios

compared to the blank, except for EndoA and EndoB on the softwood pulp. However, Endo C exhibited also a significantly better result than EndoA and EndoB. On the hardwood pulp using citrate buffer, FiberCare R could significantly reduce the ratio compared to EndoA. When mimicking refining conditions (water pH7), all endoglucanases reduced the colour ratios compared to the blank, but here the purified endoglucanase EndoC could also achieve a better ratio compared to EndoA. After application of the corresponding enzyme formulations, all tested formulations could achieve lower colour ratios than the blank but were not significantly different to each other. Overall, the results of the statistical testing showed that the adjustment to the enzyme activity according to the endoglucanase specific CellG5 substrate achieved similar results, especially for the enzyme formulations containing a whole set of different enzymes, but also confirmed the semi-quantitative nature of this method. However, also small differences between the enzymes like for EndoC and FiberCare R were revealed, which match the results of the HPLC and reducing sugar analysis.

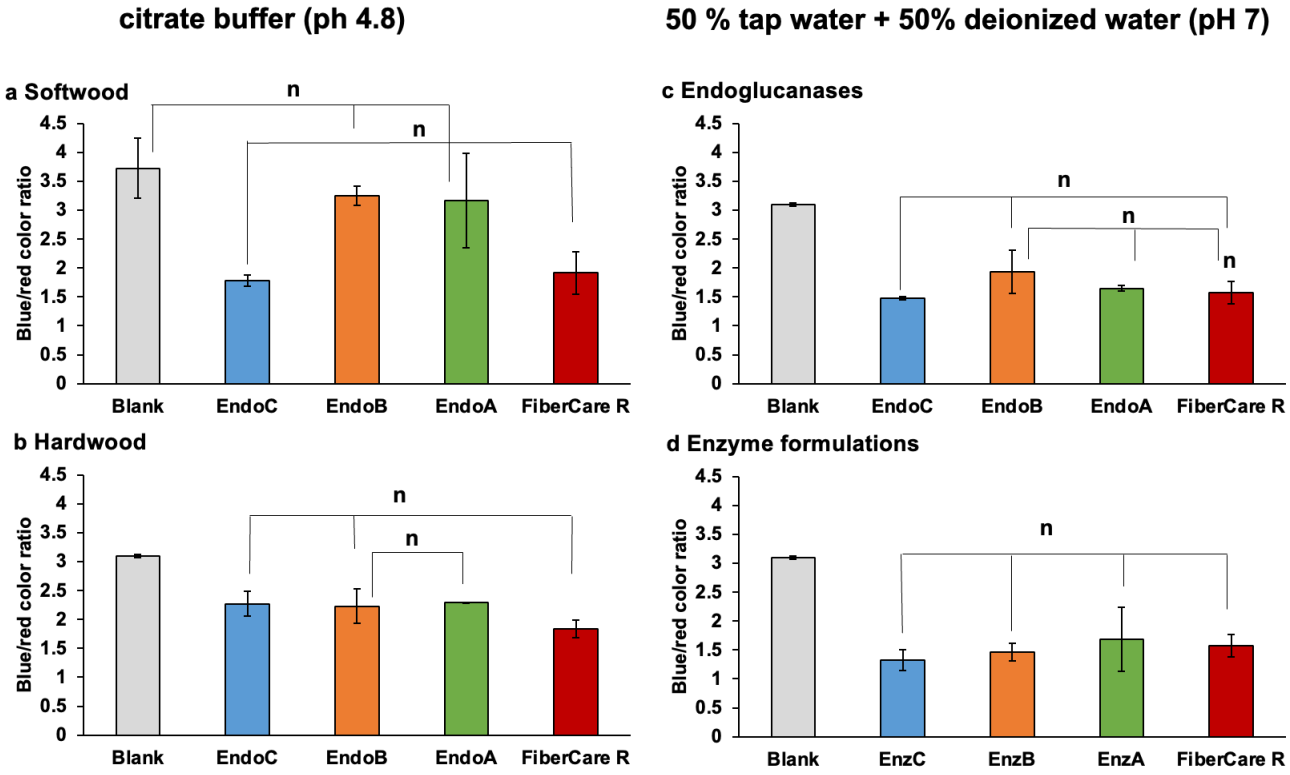

Fig. 29 Calculated blue/red colour ratios of the CLSM images after quantification of the blue and red colour intensities using ImageJ. The average ratios of the duplicate images were calculated, error bars indicate the standard deviation. (a) and (b) show the ratios of the purified endoglucanases (EndoA, EndoB and EndoC) together with the commercial endoglucanase FiberCare R using 50 mM citrate buffer (pH 4.8) on softwood and hardwood pulp, respectively, while (c) shows the ratios of the purified endoglucanases on hardwood pulp using 50% tap water + 50% deionized water (pH 7) and (d) the ratios of the enzyme formulations (EnzA, EnzB, EnzC) on hardwood pulp using 50% tap water + 50% deionized water (pH 7). Bars marked with the letter n are not significant to each other according to a performed statistical ANOVA test using a significance level of 0.1.

4.4.5 ^{13}C-NMR of enzyme treated pulps

Changes of the pulps properties upon enzyme treatment were investigated using ^{13}C-solid state NMR after incubation of the pulps with the respective enzymes in 50 mM citrate buffer (pH 4.8) for 4 h. For the long fiber softwood pulp, the amount of para-crystalline cellulose increased after treatment with all endoglucanases in comparison to the untreated pulp sample **(Fig. 30a)**. Para-crystalline cellulose is less ordered than crystalline cellulose and is composed of thin layers on the surface of the crystallites. Para-crystalline layers lead to weakening of the crystallites and aid in the dissolution of cellulose [74], [75]. The commercial endoglucanase FiberCare R (+ 27.0 %) and EndoC (+ 20.1 %) led to the highest increase in para-crystalline fraction when compared to the blank without enzyme treatment, although all investigated endoglucanases could considerably increase the para-crystalline fraction. These two endoglucanases also showed a high effect in the CLSM images using carbohydrate binding modules. The increase in para-crystalline cellulose was also observed in a study investigating lytic polysaccharide monooxygenases which also have a high affinity for amorphous cellulose, suggesting a weakening of the fibers by the enzymes, causing fibrillation and disruption of the crystalline structure, which becomes less ordered or para-crystalline [76]. Although FiberCare R and EndoC were the enzymes with the highest effects in NMR as well as in the CLSM results, a comparison between the two methods is still difficult, as NMR gives information about the ultrastructure and chemical environment of cellulose, while the fluorescently labelled carbohydrate binding modules rely on the binding to the cellulose surface after enzyme treatment and cannot monitor changes beneath the cellulose surface [21], [77], [78]. Amorphous fractions are divided into accessible surface as well as inaccessible surface fractions. Accessible surface is in contact with water or solvent, while inaccessible surface fractions consist of fibril-fibril contact surface that are inaccessible to solvents in the fibril interior [79]. The accessible surface fraction was considerably reduced by most endoglucanases (highest for EndoC: -13.6 %) except for the commercial endoglucanase FiberCare R, which increased this fraction by 1.2 %. However, the inaccessible surface was also decreased by all endoglucanases. The highest decrease was observed for FiberCare R (-26.9 %). The reduction of inaccessible fraction could indicate the introduction of dislocations and disruptions within the fibrils by the enzymes. Enzymes like cellulases often contain carbohydrate binding modules that bring the enzymes in proximity to their substrates, which have the ability to disrupt the

packaging of the cellulose structure with one study even showing that the bulk structure of the investigated amorphous cellulose films was affected [80], [81]. The theory that the packing of cellulose is weakened is also supported by the amount of cellulose Iαβ, as this fraction was decreasing slightly for all endoglucanases, the highest decrease was caused by FiberCare R (-29.5 %). Interestingly, the fraction of Iα cellulose increased while the Iβ cellulose fraction decreased, although being the thermodynamically more stable form [82]. However, endoglucanases often contain non-catalytic components like carbohydrate binding modules, that are able to destabilize or disrupt the structure of cellulose, even releasing small fragments, without having any hydrolytic activity [83]–[85]. It was shown that for example the carbohydrate binding module of endoglucanase II binds to the crystalline areas of cellulose, while the catalytic domain works on the nearby amorphous parts [16]. A similar observation was made with endoglucanase III from *Trichoderma reesei*, disrupting the structure of crystalline cellulose through the breakage of hydrogen bonds [86]. Overall, the crystallinity index increased for all enzyme treated samples, giving a value of 69.0 % for FiberCare R, 68.3 % for EndoB and EndoC and 66.7 % for EndoA when compared to the control (62.2 %).

For the short fiber hardwood pulp similar results were obtained, although the differences were less pronounced **(Fig. 30b**). This fits with the results obtained by the reducing sugar assays and HPLC, showing a much lower release of products for this pulp. This was also expressed in the crystallinity index, which remained constant or improved only marginally for all observed endoglucanases. This may be attributed to differences between softwood and hardwood pulps like softwood pulps having bends and twists as well as studies showing a different mode of action for softwood pulps leading to fragmentation and increased fiber shortening for softwood pulps [70], [87]. However, the crystallinity index is not always the appropriate measure to assess suitability of the enzymes, which was also suggested by studies dealing with its interpretation and it was found that with progressed hydrolysis, extensive changes to the fiber structure are occurring, making it impossible to attribute changes to the crystallinity index alone [34], [88]. Refining trials on hardwood pulp using a PFI laboratory mill showed that even due to identical dosage, EndoC and FiberCare R achieved a higher degree of refining than EndoA and EndoB [11]. This suggests a different mode of action of the endoglucanases, which might also be attributed to

differences in the catalytic domain, as for example *Trichorerma reesei* exhibits at least 5 different endoglucanases, each showing distinct catalytic domains [89], [90]. The para-crystalline cellulose fraction increased the most with EndoC (+ 6.4 %), followed by EndoB (+ 6.2 %) and FiberCare R (+ 1.1 %), while EndoA led to a minor decrease of 0.94 %. The amount of cellulose I αβ decreased considerably with EndoC (-32.6 %), suggesting a slight degradation and disordering of the crystalline structure upon enzymatic treatment. This was not observed for the other endoglucanases, with the purified EndoA showing a higher amount of cellulose Iα and Iβ than in the untreated sample, thus not being able to affect these fractions. The accessible surface fraction was decreased considerably by most endoglucanases with the highest effect caused by EndoC (-18.8 %), followed by EndoB (- 9.7 %) and FiberCare R (-2.6 %), while it was increased by 14.4 % with EndoA. These findings suggest that EndoA showed a different enzyme mechanism, leading to a lower degree of refining in laboratory refining trials than EndoC or FiberCare R. Another difference to the long fiber softwood pulp is the inaccessible surface fraction, which is influenced by the introduction of distortions and dislocations within the fibrils. While this fraction decreased for the softwood pulp by all endoglucanases, the same behaviour could only be reported for EndoA (- 5.1 %) and FiberCare R (-0.68 %), while this fraction increased for EndoC (+ 4.2 %) and EndoB (+ 1.5 %), suggesting that EndoC and EndoB, could not affect these fiber areas.

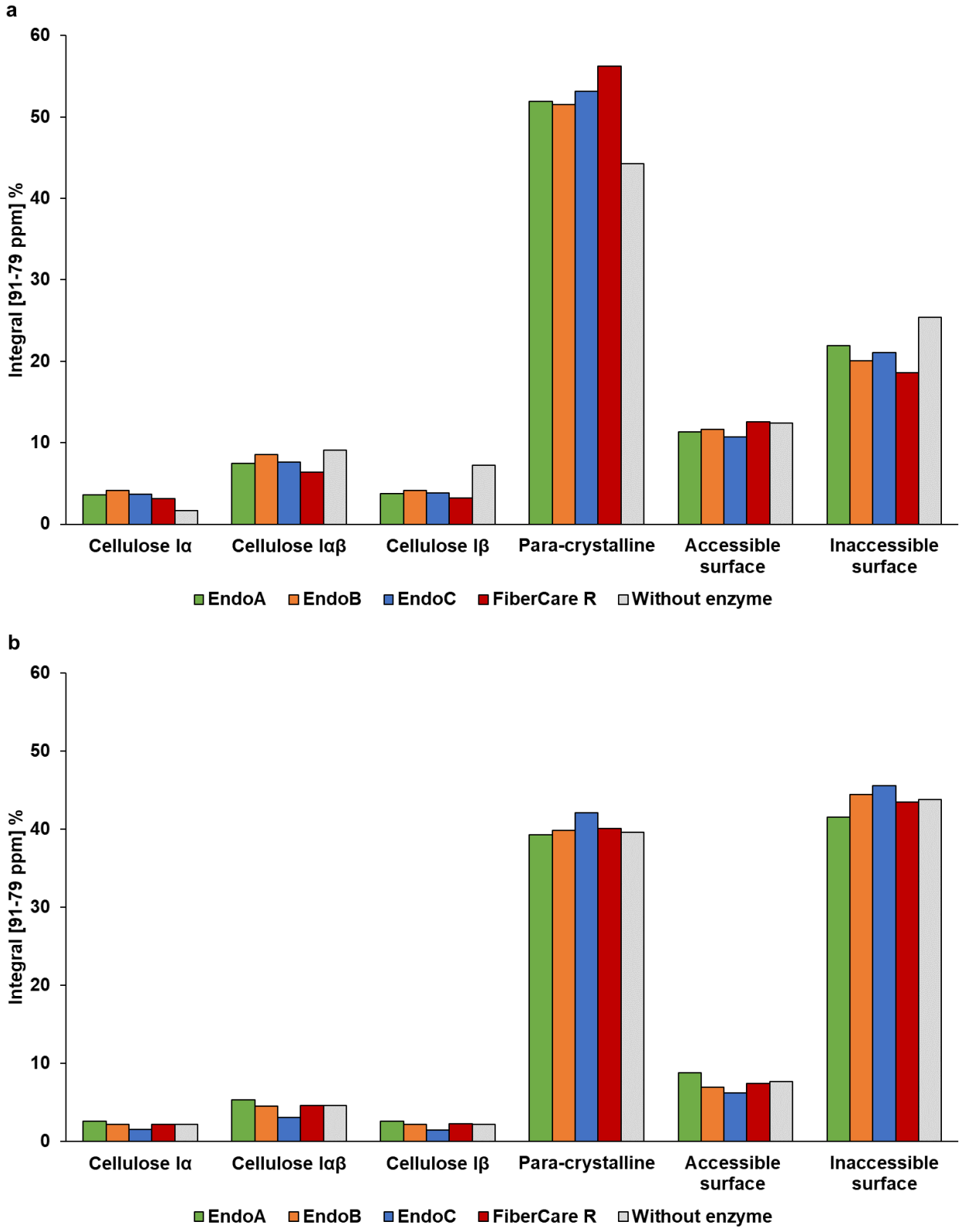

Fig. 30 ^{13}C-NMR analysis of long fiber softwood or short fiber hardwood pulp after treatment with purified endoglucanases for 4 hours in 50 mM citrate buffer (pH 4.8). NMR measurements were performed without replicates. Differences in the amorphous and crystalline fractions caused by the purified endoglucanases EndoA, EndoB, EndoC as well as the commercial endoglucanase FiberCare R are highlighted in percent related to the integral of 91-79 ppm. (a) shows the NMR results of the softwood pulp (b) the results of the hardwood pulp, which was also already tested in laboratory refining trials.

4.5 Conclusion

The action of various endoglucanases which were beneficial in refining of softwood and hardwood pulp was mechanistically studied. Generally, the enzymes were more active on softwood pulp suggesting a better accessibility. Carbohydrate binding modules were successfully labelled with fluorescent dyes and used to assess the crystallinity changes upon endoglucanase treatment. By incubating pulps with purified endoglucanases as well as with a commercial endoglucanase formulation, even small differences in the action of the endoglucanases on pulp could be visualized despite adjustment to the same endoglucanase activity according to the endoglucanase specific CellG5 substrate. EndoC and the commercial endoglucanase formulation FiberCare R showed the highest decrease of amorphous moieties and were also most active in previous laboratory refining trials. ^{13}C-NMR analysis of the pulps treated with purified endoglucanases from the respective enzyme formulations, revealed further mechanistic insights, as the best working enzymes during refining also mainly led to an increase in the para-crystalline cellulose fraction, indicating the degradation of amorphous regions. HPLC analysis of the released products at refining conditions of these enzymes revealed high concentrations of cellobiose and cellotetraose especially by those enzymes which showed the most pronounced refining effect. In summary, differences in refining behaviour seen for various endoglucases correlate to a different extent of crystallinity changes and oligomers liberated from softwood and hardwood pulps.

Declarations

Author contributions

Martin Nagl and Lukas Skopek performed the experiments and analysed the data. Martin Nagl, Oskar Haske-Cornelius, Alessandro Pellis, Georg M. Guebitz and Wolfgang Bauer planned the experiments and participated in the conceptualization of the experiments. Florian Bausch performed the formal analysis of the ^{13}C-NMR data. Georg Guebitz and Wolfgang Bauer supervised the methodology. Martin Nagl wrote the manuscript. Alessandro Pellis, Wolfgang Bauer, Gibson S. Nyanhongo and Georg M. Guebitz corrected the manuscript. The presented data was discussed by all authors prior to submission and all authors agreed to submit.

Conflicts of interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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Availability of data and material

Additional material related to this article can be found as Online Resource.

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4.7 Supplementary material

Release of reducing sugars from softwood and hardwood pulp

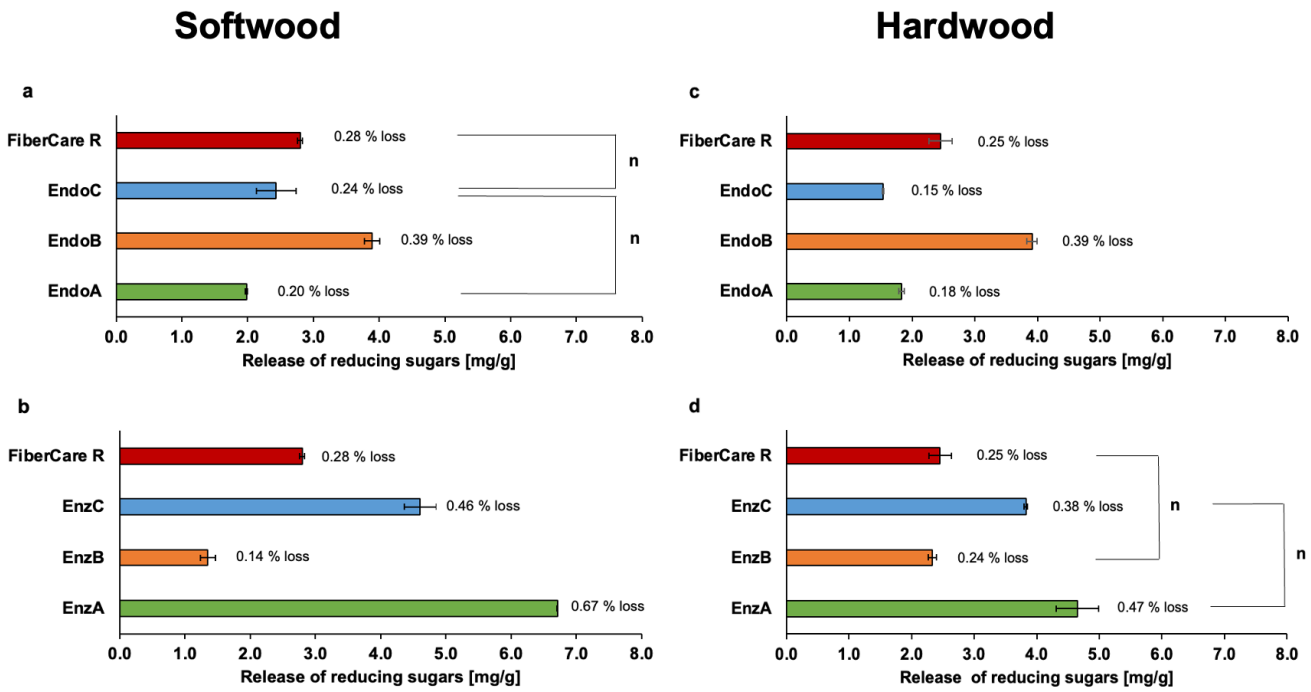

Fig. S31 Release of reducing sugars by different purified endoglucanases and the corresponding enzyme formulations from softwood and hardwood pulp. Compared to [11], new measurements on the hardwood pulp were performed. Reducing sugar release at the same dosage level of 0.83 nkat (according to the CellG5 activity assay) was calculated based on the determined nkat ml^{-1} values of the activity assays conducted on either the softwood or the hardwood pulp. Activity assays were performed in duplicates, error bars indicate the corresponding standard deviation. In addition, the loss related to the pulp amount before enzyme addition is given in percent. (a) shows the calculated release of reducing sugars of the purified endoglucanases for the softwood pulp, (b) for the corresponding enzyme formulations on the softwood pulp, (c) the calculated release of reducing sugars of the purified endoglucanases for the hardwood pulp and (d) the calculated release of the reducing sugars of the enzyme formulations for the hardwood pulp.). Bars marked with the letter n are not significant to each other according to a performed statistical ANOVA test using a significance level of 0.1.

Duplicate set of CLSM images using carbohydrate binding modules:

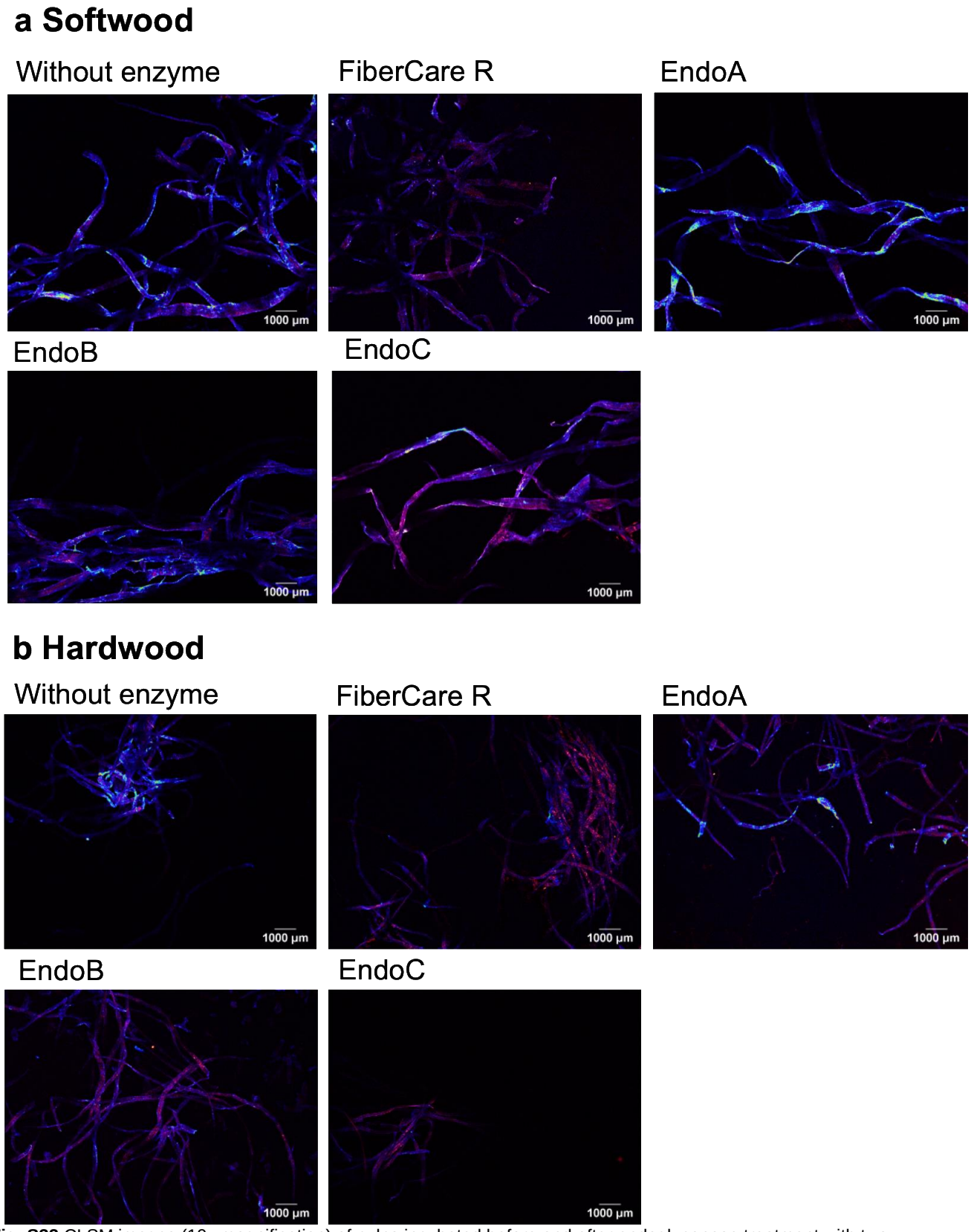

Fig. S32 CLSM images (10x magnification) of pulps incubated before and after endoglucanase treatment with two different carbohydrate binding modules for probing crystallinity changes. Duplicates of each combination of enzyme and pulp were measured. Specific binding of CBM 28 from *Caldicellulosiruptor bescii* to amorphous regions is indicated by an increase in blue colour. Specific binding of CBM 2 from *Thermobifida fusca* to crystalline regions is

indicated by an increase in red colour. Long fiber softwood (a) and short fiber hardwood (b) pulps were incubated with purified endoglucanases as well as the commercial endoglucanase FiberCare R for 4h in 50 mM citrate buffer at pH 4.8.

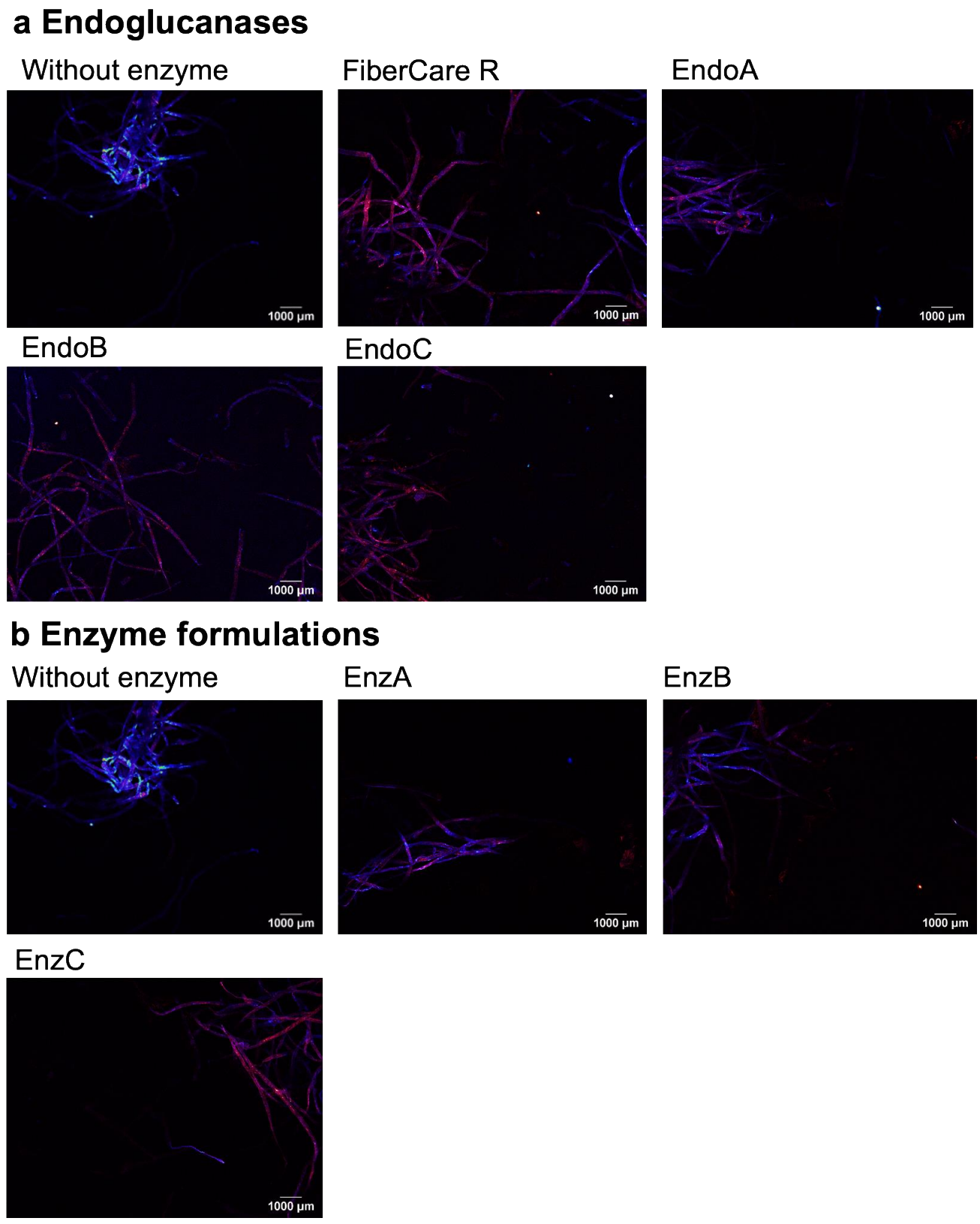

Fig. S33 CLSM images (10x magnification) of short fiber hardwood pulp incubated before and after endoglucanase treatment at refining conditions (50% tap water + 50% deionized water at pH 7) with two different

carbohydrate binding modules for probing crystallinity changes. Duplicates of each combination of enzyme and pulp were measured. Specific binding of CBM 28 from Caldicellulosiruptor bescii to amorphous regions is indicated by an increase in blue colour. Specific binding of CBM 2 from Thermobifida fusca to crystalline regions is indicated by an increase in red colour. (a) shows the results for purified endoglucanases on short fiber hardwood pulp, (b) the results of the enzyme formulations, from which the endoglucanases were purified from.

Visualization of endoglucanase efficiency using the scanning electron microscope (SEM)

Measurements using scanning electron microscopy (SEM) could confirm that all purified endoglucanases as well as the commercial endoglucanase FiberCare R have caused damage to the fibers (Error! Reference source not found.). Thereby, all purified endoglucanases should lead to fibrillation during pulp refining.

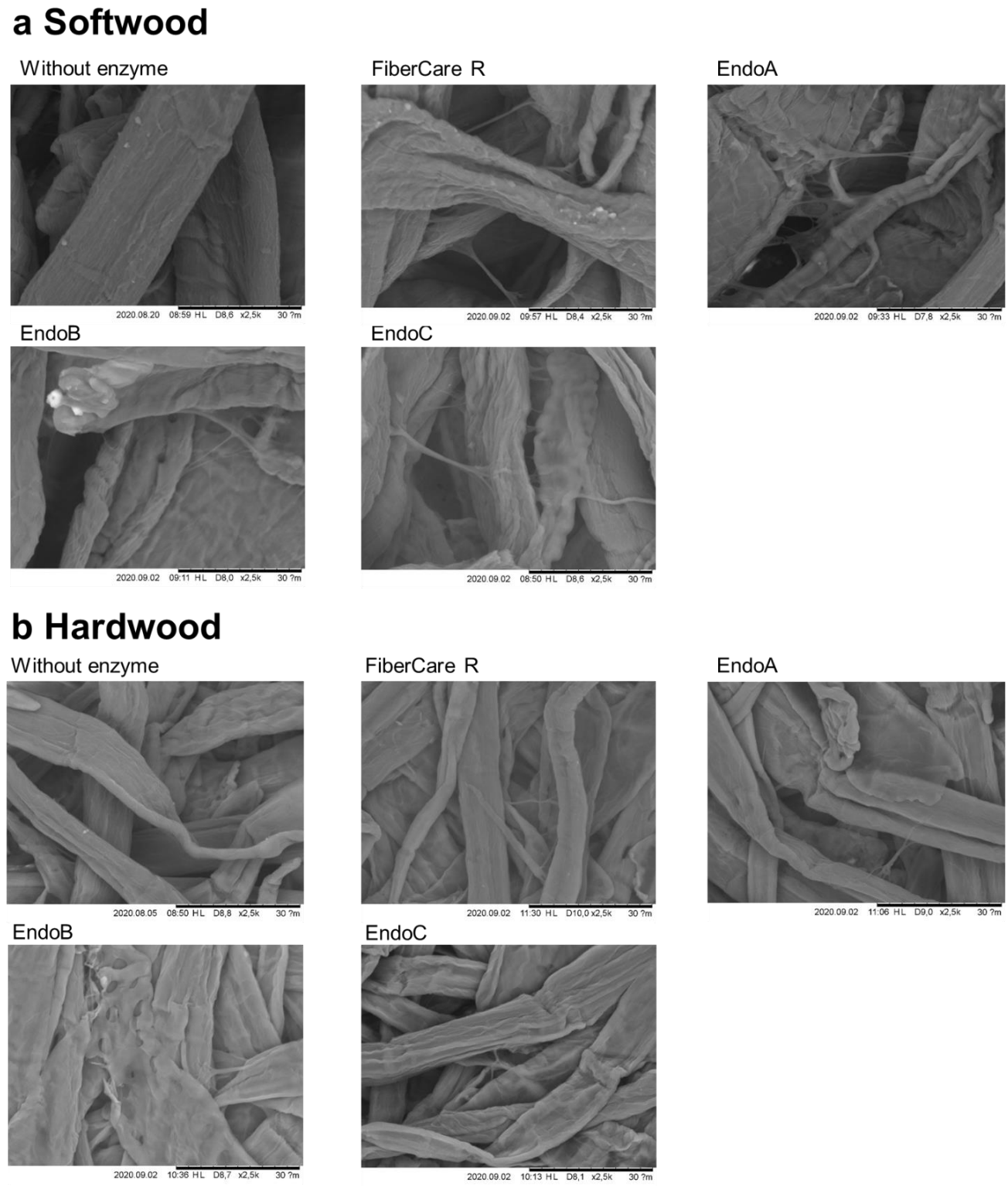

Fig. S34 Scanning electron microscopy (SEM) pictures of enzyme treated pulps at 2500 x magnification. Long fiber softwood or short fiber hardwood pulp was treated for 4 hours at a dosage of 0.035 nkat at 45°C with EndoA, EndoB, EndoC or the commercial endoglucanase FiberCare R. Sample were sputter coated with 1 nm of platinum prior to SEM analysis. (a) shows the results of the softwood pulp, (b) the results of the hardwood pulp

Table S5. ^{13}C-NMR results of the long fiber softwood pulp. Amount of cellulose Iα, Iβ, Iαβ, para-crystalline fraction, accessible surface and inaccessible surface is given in percent.

Long fibre Pulp	**Without enzyme addition**	**EndoA**	**EndoB**	**EndoC**	**FiberCare R**
Cellulose Iα	1.7	3.6	4.2	3.7	3.1
Cellulose Iαβ	9.1	7.5	8.5	7.6	6.4
Cellulose Iβ	7.2	3.7	4.2	3.8	3.2
Para-crystalline	44.3	51.9	51.5	53.1	56.2
Inaccessible surface	25.3	21.9	20.1	21.1	18.6
Accessible Surface	12.4	11.4	11.6	10.7	12.5
Crystallinity index	62.2	66.7	68.3	68.3	69.0

Table S6. ^{13}C-NMR results of the short fiber hardwood pulp. Amount of cellulose Iα, Iβ, Iαβ, para-crystalline fraction, accessible surface and inaccessible surface is given in percent.

Short fibre Pulp	**Without enzyme addition**	**EndoA**	**EndoB**	**EndoC**	**FiberCare R**
Cellulose Iα	2.2	2.6	2.2	1.5	2.2
Cellulose Iαβ	4.6	5.3	4.5	3.1	4.6
Cellulose Iβ	2.2	2.6	2.2	1.5	2.2
Para-crystalline	39.6	39.2	39.8	42.1	40.0
Inaccessible surface	43.7	41.5	44.4	45.6	43.5
Accessible Surface	7.7	8.8	6.9	6.2	7.5
Crystallinity index	48.6	49.7	48.7	48.2	49.1

5

Towards a better understanding of synergistic enzyme effects during refining of cellulose fibers

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5.1 Abstract

Refining of cellulose fibers is essential for reaching desired paper properties, yet highly energy demanding. Enzymes like endoglucanases (e.g. EndoC) are increasingly used to reduce energy consumption during pulp refining. However, prediction of the enzyme effect is still a major concern, considering the high variety of commercially available enzyme formulations, containing a range of different enzymes. In this study, synergisms of xylanases and β-glucosidases in combination with endoglucanases purified from enzyme formulations were studied and related to their refining performance. Size exclusion chromatography with multi-angle laser light scattering (SEC-MALLS) of carboxymethylcellulose revealed that a narrow size distribution and a high reduction in molecular weight are beneficial characteristics for refining. SEC-MALLS of hardwood pulp resulted in pronounced formation of low molecular weight fractions (log MW 4.3) for most efficient refining enzymes. Application of enzyme formulations and combinations of endoglucanase EndoC with β-glucosidase or xylanase using Fourier-transform infrared spectroscopy (FTIR) revealed synergistic effects that promoted degradation of amorphous parts of cellulose. Laboratory refining trials on hardwood pulp confirmed the increase in degree of refining and tensile index after addition of xylanase and β-glucosidase. Surface plasmon resonance (SPR) analysis resulted in strong binding of endoglucanases to regenerated cellulose, which correlated to refining performance.

Keywords: Endoglucanases, Paper Production, Refining, SEC-MALLS, FTIR, SPR

5.2 Introduction

Refining of cellulose fibers involves mechanical treatment and is an integral part of paper production with the aim to increase paper properties such as fiber-fiber bonding or tensile index. The refining process is highly energy intensive, requiring up to 27 % of the total energy for paper production. However, pre-treatment using enzymes such as endoglucanases can reduce the required energy for pulp refining by up to 40% (Lecourt et al., 2010; Fleiter et al., 2012; Buzala et al., 2016). While enzymes have proven to reduce the energy consumption, the prediction of their effect is still challenging, due to the high variety of enzymes that play a role in lignocellulose hydrolysis in nature such as endoglucanases, xylanases or β-glucosidases which are present in commercial preparations to different extents [3]–[5].

Endoglucanases (E.C. 3.2.1.4) cleave β-1,4 linkages internally and in amorphous regions of cellulose [6]. A common substrate for the determination of endoglucanase activity is water soluble carboxymethylcellulose [7], [8]. Cellobiohydrolases are exo-1,4-β-glucanases that are releasing cellobiose from the ends of the cellulose chains, while β-glucosidases (E.C. 3.2.1.21) cleave cellobiose into the main building block glucose [9]. In nature, decomposition of cellulose also involves the synergistic action of oxidoreductases (e.g. lytic polysaccharide monooxygenases or cellobiose dehydrogenases) that aid in the oxidative cleavage of glycosidic bonds [10], [11].

Commercial enzyme formulations are commonly obtained from organisms such as *Trichoderma reesei*, which produce a great variety of enzymes acting on lignocellulose [12]–[14]. However, not all of these enzymes may be beneficial to the pulp refining process. For example, production of glucose by β-glucosidases is not desired from an industrial point of view due to increases in the chemical oxygen demand requiring extensive wastewater treatment [15]–[18]. On the other hand, β-glucosidases may be required to prevent product inhibition of endoglucanases, which leads to a loss of enzyme activity by accumulation of cellobiose [19], [20]. Besides cellulose, pulp may still contain considerable amounts of xylans which can be hydrolysed by endoxylanases, β-xylosidases among other enzymes acting on side groups like

arabinose or glucuronic acid. Obviously, (partial) hydrolysis of xylans may have an effect on refining of pulp [21], [22].

One method for the investigation of enzyme mechanisms on pulp is the application of size exclusion chromatography coupled with a multiangle laser light scattering detector (SEC-MALLS), which enables to determine molecular weight distributions of cellulose [23], [24]. SEC-MALLS can be applied on soluble carboxymethylcellulose, but also on water-insoluble pulp which needs to be dissolved in dimethylacetamide/lithium chloride (DMAc/LiCl) prior to analysis. Enzyme treatment of pulp is often quantified by analysis of released water-soluble fragments (e.g. cellooligosaccharides) using methods like HPLC, however, for pulp refining, changes to the insoluble part of pulp are of interest, as it is transformed to the final product, in contrast to processes requiring complete hydrolysis [25], [26]. However, SEC-MALLS on pulp requires extensive dissolution steps, specialized SEC-MALLS equipment and for some pulp types dissolution may not be possible, which still underlines the need for soluble substrates like carboxymethylcellulose for comparison [27]–[29]. Another method for the characterization of enzyme mechanisms is surface plasmon resonance (SPR), which measures the adsorption of biological molecules onto surfaces and therefore enables the study of enzyme binding to their substrates [30], [31]. However, for the analysis of the binding of enzymes to cellulose, dissolution in dimethylacetamide/lithium chloride (DMAc/LiCl) and subsequent coating onto the sensor chip is required [32], [33]

In this study, we investigated the effect of complex enzyme formulations used for pulp refining and of individual enzymes purified thereof to elucidate potential synergisms that are beneficial for the refining process. In previous studies the use of derivatized cellopentaose (CellG5) for enzyme dosage in pulp refining was introduced and carbohydrate binding modules were applied as probes for prediction of the most efficient refining enzymes. However, there is still a lack of information regarding the changes caused to the insoluble part of cellulose, synergistic effects of xylanases and β-glucosidases in conjunction with endoglucanases and the binding behaviour of endoglucanases when dosed according to activity based on derivatized cellopentaose (CellG5). Comparability between different enzymes intended for refining was complicated through lack of specificity for endoglucanases of previously used reducing sugar assays, especially when complex formulations are applied that include

xylanases and β-glucosidases. We hypothesized that differences in enzyme performance after adjustment to identical endoglucanase activity (CellG5) are related to diverging enzyme mechanisms and hence can enable new ways for prediction as well as the investigation of synergistic enzyme effects. Therefore, a variety of analytical techniques including size exclusion chromatography (SEC-MALLS), FTIR, and SPR were applied, and the results correlated to enzyme performance in laboratory refining trials using a PFI type refiner.

5.3 Materials and methods

5.3.1 Pulps, enzyme formulations and chemicals

Bleached hardwood sulfate pulp (eucalyptus) as well as commercial enzyme formulations (EnzA, EnzC) were provided by companies of the Austrian pulp and paper industry. The commercial endoglucanase FiberCare R was kindly provided by Novozymes A/S (Bagsværd, Denmark). All other chemicals were obtained from Sigma-Aldrich (Austria) in HPLC grade if not stated otherwise. The β-glucosidase E-BGLUC from *Aspergillus niger* as well as the CellG5 and XylX6 activity assays were obtained from Megazyme (Bray, Ireland).

5.3.2 Enzyme purification

XylC xylanase was purified from the EnzC formulation using size exclusion chromatography (SEC) after hydrophobic interaction chromatography (HIC). EnzC formulation was suspended in 70 ml binding buffer A (10 mM acetate buffer + 1.5 M $(NH_4)_2SO_4$ at pH 4.8) for HIC and loaded onto three in series connected HiTrap Phenyl HP 5 ml HIC columns from Cytiva (Marlborough, MA, USA). Elution was performed after a 45% step of buffer B (10 mM acetate buffer pH 4.8), followed by a linear gradient of 45–90% B. SEC was subsequently used to purify the xylanase from the HIC concentrate. Therefore, a HiPrep Sephacryl S-100 HR (Cytiva, USA) 120 ml SEC column was used and equilibrated with running buffer (10 mM acetate buffer, pH 4.8 + 150 mM NaCl). A volume of 1.2 ml enzyme solution was loaded onto the column at a flow rate of 0.5 ml min^{-1}. Elution was performed using 1 column volume of the same buffer at a flow rate of 0.5 ml min^{-1}. Desired fractions were pooled after SDS-PAGE and concentrated using Vivaspin 20 (3000 Da cut-off) centrifugal concentrators (Sartorius, Germany). The buffer was subsequently exchanged to 50 mM citrate buffer

(pH 4.8) using PD-10 columns (Cytiva, USA) and the solution concentrated with Vivaspin 20 concentrators (Sartorius, Germany).

Endoglucanases were purified from the enzyme formulations EnzA and EnzC using consecutive steps of hydrophobic interaction (HIC) and anion exchange chromatography (AEX) as described in previous studies (Nagl et al., 2021, 2022). In brief, for HIC, elution of endoglucanases was performed by addition of different amounts of buffer B (10 mM acetate buffer pH 4.8). A 30 % buffer B step followed by a linear gradient of 30–80% B was used for the elution of EndoA and a 45% B step, followed by a linear gradient of 45–90% B for EndoC. Desired fractions were pooled after SDS-PAGE, concentrated using Vivaflow 50 crossflow concentrators (Sartorius, Germany) and buffer exchanged to anion exchange buffer A (10 mM Tris pH 7.5) using PD-10 columns (Cytiva, USA). For anion exchange, a sample volume of 25 ml was applied to a HiTrap DEAE FF 5 ml column (Cytiva, USA). EndoA was eluted using a 0–7 % and EndoC a 0–1.5 % linear gradient of buffer B (10 mM Tris pH 7.5 + 1 M NaCl). Endoglucanase fractions were pooled after SDS-PAGE, concentrated using Vivaspin 20 centrifugal concentrators (Sartorius, Germany) and buffer exchanged to 50 mM citrate buffer (pH 4.8) using PD-10 columns (Cytiva, USA).

5.3.3 Activity Assays

5.3.3.1 Endoglucanase activity on CellG5 (derivatized cellopentaose):

Endoglucanase activity was determined using the endoglucanase specific CellG5 activity assay from Megazyme (Bray, Ireland). The principle of this assay is outlined in Mangan et al., 2016. Endoglucanase samples were diluted in 50% tap water + 50% deionized water (adjusted to pH 7.0) to simulate the conditions used for pulp refining or in 50 mM citrate buffer pH 4.8. A volume of 100 µl diluted enzyme solution was added to 50 µl of CellG5 substrate in 10 ml glass tubes and incubated for 10 minutes at 45°C in a water bath. The reaction was stopped by addition of 3 ml of 2% Tris solution (pH 10). The solution was then transferred into cuvettes and the absorbance was measured at 400 nm using a Hitachi U2900 photometer (Chiyoda, Japan). Measurements were performed in duplicates and endoglucanase activity was calculated in nkat ml^{-1}.

5.3.3.2 β-glucosidase activity

β-glucosidase activity was determined using the substrate 4-nitrophenyl β-D-glucopyranoside (Sigma Aldrich, Austria). The assay was performed similar as described in Parry et al., 2001. A volume of 200 µl of 2 mM 4-nitrophenyl β-D-glucopyranoside (suspended in 50% tap water + 50% deionized water adjusted to pH 7) was transferred into 10 ml glass tubes and equilibrated in a 45°C water bath. Enzymes were diluted in 50% tap water + 50% deionized water (pH 7.0) and a volume of 50 µl was added to the substrate. The reaction was incubated for 0, 10, 20, 30, 40, 50 and 60 min and stopped by addition of 500 µl of methanol. Finally, 750 µl of 500 mM $NaPO_4$ buffer (pH 7.0) was added for colorization. A volume of 200 µl was transferred into a 96 well plate and the absorbance was measured at 410 nm using an Infinite M200 Pro plate reader (Tecan, Switzerland). Measurements were performed in duplicates and the β-glucosidase activity was calculated in nkat ml^{-1}. Determined β-glucosidase activity of Megazyme E-BGLUC is outlined in Table S1 of the supplementary material.

5.3.3.3 Xylanase activity

Xylanase activity was determined using the xylanase specific XylX6 activity assay from Megazyme (Bray, Ireland). The principle of this assay is outlined in Mangan et al., 2017. Therefore, the xylanase containing sample was diluted in 50% tap water + 50% deionized water (adjusted to pH 7.0) to simulate the conditions used for pulp refining. A volume of 50 µl diluted enzyme solution was added to 50 µl of XylX6 substrate in 10 ml glass tubes and incubated for 10 min at 45°C in a water bath. The reaction was stopped by addition of 1.5 ml of 2% Tris solution (pH 10). The solution was then transferred into cuvettes and the absorbance was measured at 400 nm using a Hitachi U2900 photometer (Chiyoda, Japan). Measurements were performed in duplicates and xylanase activity was calculated in nkat ml^{-1}. Determined xylanase activity of XylC xylanase is outlined in Table S2 of the supplementary material.

5.3.4 Laboratory refining trials using hardwood pulp

Laboratory refining experiments were conducted using 130 g oven-dry hardwood sulfate pulp. Pulps were pre-soaked overnight in 50% tap water + 50% deionized water (adjusted to pH 7.0) and disintegrated for 10 min. Enzyme treatment was conducted at 4% (w/w) pulp consistency in 50% tap water + 50% deionized water (adjusted to pH 7.0) at 45°C for 30 min under continuous agitation. An enzyme dosage of 5.06 nkat

according to the endoglucanase specific CellG5 assay was added of the endoglucanases. For the study of potential synergistical effects of additional enzymes in the enzyme formulations, purified XylC xylanase was added at a dosage of 8.3 nkat together with EndoC, while β-glucosidase from Megazyme was added using a dosage of 16.7 nkat. The enzyme reaction was stopped by the addition of 30% hydrogen peroxide. Refining experiments were subsequently performed using a PFI laboratory mill type Mark IV (Hamjern Maskin AS, Norway) at 1500, 4000 and 6000 revolutions according to ISO 5264–2:2011 and the achieved degree of refining was determined using the Schopper-Riegler method (ISO 5267 - 1:1999). A L&W Fiber Tester Plus with an optical resolution of 3.3 µm/pixel from ABB (Kista, Sweden) was used for characterization of length weighted fiber length (using 10.500 – 15.000 fibers) and fibril area. Paper sheets were formed using a RK4-KWT sheet former from Frank PTI (Austria) according to the Rapid–Köthen method (ISO 5269 - 2:2004). Tensile strength of the paper sheets was tested according to ISO 1924–2:2008 and air permeance Bendtsen according to ISO 5636–3:2013 after conditioning the samples for at least 24 h in the climate room at 23°C and 50% relative humidity. 13-15 paper samples were tested, and error bars indicate the standard deviation.

5.3.5 SEC-MALLS of enzyme treated carboxymethylcellulose

A volume of 250 µl of 0.5 % carboxymethylcellulose (average molecular weight: 700.000 g/mol, degree of substitution: 0.9) was suspended in 50 mM citrate buffer (pH 4.8) and treated using enzymes at a dosage of 0.21 nkat (CellG5) at 45°C for 2 h. Furthermore, samples with addition of 50 mM citrate buffer (pH 4.8) instead of enzymes were prepared as blanks. The reaction was stopped by incubation at 99°C for 15 minutes on a thermomixer. A volume of 725 µl MQ water was added, and the reaction mixtures were filtrated through 0.45 µm filters. A 1260 Infinity II liquid chromatography system equipped with a quaternary/binary pump, an autosampler, a diode array detector and a refractive index detector from Agilent Technologies (Waldbronn, Germany) was used in combination with a MALLS HELIOS DAWN II detector from Wyatt Technology (Santa Barbara, US). As pre-column a PL aquagel-OH MIXED Guard (PL1149-1840, 8 µm, 7.5 × 50 mm^2) was applied, followed by the separation column PL aquagel-OH MIXED H (PL1549-5800, 4.6 × 250 mm^2, 8 µm), both from Agilent Technologies (Waldbronn, Germany). As running buffer 50 mM $NaNO_3$ + 3 mM NaN_3 was used. A volume of 100 µl sample was injected and run for 90 minutes at a flow rate of 0.3 ml min^{-1} of eluent. Data analysis was performed using ASTRA 7 and

SigmaPlot 12.5. Bovine serum albumin with molecular weight of 66.430 kDa and a concentration of 1 mg ml^{-1} was used for calibration of the MALLS detector. Duplicate measurements of all samples were performed. Recorded refractive index (RI) values are highlighted in **Fig. S*40*** of the supplementary material.

5.3.6 SEC-MALLS of enzyme treated hardwood pulp

An amount of 50 mg hardwood pulp was incubated with enzymes for 48 h at a dosage of 0.83 nkat on a thermomixer at 45°C and 500 rpm using 1250 µl 50 mM citrate buffer (pH 4.8). The reactions were stopped by incubation at 99°C for 5 minutes at 500 rpm. The supernatant was removed, the pulps washed using 1 ml 50 mM citrate buffer (pH 4.8) and dried on air. Pulp dissolution in DMAc/LiCl (9% w/v) was performed according to a standard protocol [38]. Briefly, samples were activated through a solvent exchange (EtOH to DMAc), followed by overnight stirring in DMAc. After the activation, samples were filtered and approx. 25 mg were dissolved in 2 ml DMAc/LiCl (9%, w/v) overnight at room temperature. Dissolved samples were diluted with DMAc, filtered through a 0.45 µm syringe filter and analysed using a GPC/MALLS-RI system consisting of a G1312B HPLC pump and an G1367B autosampler from Agilent Technologies (Waldbronn, Germany), a Dawn DSP200 MALLS detector from Wyatt Technology (Santa Barbara, US), and a Shodex RI-71 refractive index detector from Showa Denko (New York,US). Four serial GPC columns, Styragel HMW6E, 20 µm, 7 × 300 mm (Waters GmbH, Vienna, Austria) were used as stationary phase. A sample volume of 100 µl was applied at a flow rate of 1.00 ml min^{-1} of eluent and run for 45 minutes. DMAc/LiCl (0.9%, w/v) filtered through a 0.02 µm filter was used as an eluent. Data were evaluated with Astra4.7, Grams 7, Access and OriginPro 2020 software. Duplicate measurements of all samples were performed.

5.3.7 Fourier-transform infrared spectroscopy (FTIR) of enzyme treated pulps

An amount of 10 mg hardwood pulp was incubated with purified endoglucanases or enzyme formulations in microcentrifuge tubes at a dosage of 0.035 nkat for 4 h at 45°C on a thermomixer at 500 rpm using 50% tap water + 50% deionized water (adjusted to pH 7.0) and a volume of 250 µl. For the analysis of potential synergistic effects 0.12 nkat of E-BGLUC β-glucosidase or 0.056 nkat XylC xylanase was added to EndoC. The reactions were stopped through incubation for 5 minutes at 99°C and 500 rpm on a thermomixer. The supernatant was discarded, and any bound enzymes were

removed by washing 3× in MQ water, subsequent incubation in 1 % SDS at 99°C for 10 min, washing in 99 % ethanol and finally MQ water. Afterwards, pulps were dried overnight at 70°C. Pulps were subsequently analysed using the Perkin Elmer (Waltham, USA) Spectrum 100 Fourier-transform infrared (FTIR) spectrometer. A spectrum between 4000 and 600 cm^{-1} with a total number of 40 scans was acquired for each sample with a resolution of 4 cm^{-1}. The spectra were normalized in the region 1200 cm^{-1} using the Spectrum 10 software. Average values of duplicate measurements were calculated.

5.3.8 Surface Plasmon Resonance of microcrystalline cellulose

For SPR, microcrystalline cellulose from Merck (Darmstadt, Germany) was prepared similarly as stated in a previous study [39]. Therefore, 2 g of the microcrystalline cellulose was placed in 100 ml MQ water for 18 h at 22°C for swelling, treated 2× in 25 ml methanol for 45 min at 40°C and finally swollen 4× in 25 ml anhydrous DMAc for 45 min at 22°C. Fibers were recovered using filter paper to enable the transfer between different solvents. The cellulose fibers were subsequently vacuum dried for 24h at 60°C and suspended in DMAc/LiCl (7% w/v). Gold chips from the SIA kit Au (Cytiva) were cleaned using $NH_4OH/H_2O_2/H_2O$ (1:1:3 ratio) at 75°C for 15 min, subsequently rinsed with MQ water and dried at 80°C. The chips were treated with 100 mg l^{-1} Poly(diallyldimethylammoniumchloride) solution (PDADMAC) and 0.01 M NaCl for 30 min, rinsed with MQ water and finally dried at 80°C. Modified gold chips were coated with 80 µl of 0.5 % (wt) cellulose (dissolved in DMAc/LiCl) at 3000 rpm for 3 min using a spin coater, dried at 80°C and washed several times with MQ water for removal of LiCl. Finally, the coated SPR sensor chips were dried at 80°C and assembled according to the supplier's manual. A Biacore T200 (Cytiva) SPR was used at a flow rate of 10 µl min^{-1} at 45°C with 50 mM citrate buffer (pH 4.8) as running buffer. Multi-cycle kinetic measurements were performed using endoglucanase enzymes at three different enzyme activities (0.18, 0.73 and 2.83 nkat ml^{-1} according to the endoglucanase specific CellG5 activity assay). Association was conducted for 60s and dissociation for 180s. Sensor chips were regenerated after each run using 4 M $MgCl_2$ for 60s and 10 mM glycine/HCl (pH 2.5) for 30s. Three runs using running buffer were measured as blank. Measurements were performed in duplicates on two separate channels. Data analysis was performed using the BiaEvaluation Software.

5.3.9 Scanning electron microscopy of refined paper sheets

Paper sheets formed after laboratory refining experiments were visualized using scanning electron microscopy (SEM). Therefore, pieces were cut off and sputter coated with 1 nm platinum using a Leica EM ACE 200 sputter coater (Wetzlar, Germany) for contrast enhancement. Sputter coated samples were subsequently analysed using a Hitachi 3030 scanning electron microscope (Chiyoda, Japan). Each sample sheet was analysed in duplicates. Pictures were obtained using a 1000× magnification and are highlighted in the supplementary material (**Fig. S*41***, **Fig. S*42***, **Fig. S*43***).

5.4 Results and discussion

5.4.1 Hydrolysis of carboxymethylcellulose

Our recent studies have indicated that activity of enzyme formulations and endoglucanases on derivatized cellopentaose (CellG5) shows a much higher correlation to their performance in refining of pulps when compared to other activities assays based on the release of reducing sugars [34], [40]. Yet, even when dosed at identical activity on CellG5, some differences in refining performance were still observed. Therefore, enzymatic hydrolysis of carboxymethylcellulose (CMC) was studied by using SEC-MALLS to reveal potential differences in the hydrolysis mechanism of endoglucanases after adjustment to identical activity on derivatized cellopentaose (CellG5). Carboxymethylcellulose is likewise a classical substrate for endoglucanase characterization, which are sometimes even termed CMCases [41], [42]. Because of the good water solubility, endoglucanase activity on CMC is often used for determination of required enzyme dosage in pulp refining [43], [44].

All purified endoglucanases decreased of the molecular weight after 2 hours of treatment relative to the blank (1.64 * 10^6 g mol^{-1}), with FiberCare R showing the highest decrease (3.77 * 10^5 g mol^{-1}), followed by EndoC (4.98 * 10^5 g mol^{-1}) and EndoA (9.64 *10^5 g mol^{-1}). These results are in accordance to previously conducted refining trials on hardwood pulp, with FiberCare R and EndoC also showing the highest efficiency in terms of achieved degree of refining [34]. The observed behaviour of the endoglucanases would also fit to the mode of action of the endoglucanases, which randomly cut within the cellulose chains, leading to a rapid decrease in molecular

weight [45], [46]. Furthermore, SEC-MALLS also revealed differences in the size distribution. Expectedly, CMC was cleaved into lower MW fractions by all endoglucanases after 2-hour incubation (**Fig. 35a**). However, EndoC formed higher amounts of fractions at log MW 5.2, while FiberCare R and EndoA showed the highest release of fractions at log MW 5.0, although FiberCare R to a much higher extent than EndoA. In addition, EndoC and FiberCare R expressed a very narrow size distribution as indicated by a lower dispersity value when compared to EndoA. While EndoA exhibited a dispersity value of 3.73, the dispersity values were indeed lower for FiberCare R (3.22) and EndoC (3.39). EndoA was also among the endoglucanases, with the lowest performance in previously conducted refining experiments [34] Therefore, endoglucanases exhibiting a wide size distribution might be disadvantageous for pulp refining purposes. Similar observations were conducted in a study investigating the effect of several endoglucanases using SEC-MALLS on methyl cellulose, in which endoglucanases showed the degradation of cellulose into lower molecular weight fractions, while some endoglucanases like HiCel7B led to a very broad distribution and others like BaCel5A to a much narrower distribution, the latter resulting in a similar pattern as EndoC [47].

However, for complete hydrolysis of cellulose, additional enzymes like cellobiohydrolases or β-glucosidases are necessary, - cellobiohydrolases can attack cellulose chains either from the reducing or the non-reducing end, while β-glucosidases may enhance endoglucanase activity through prevention of product inhibition [19], [48], [49]. Therefore, the corresponding complex enzyme formulations EnzA and EnzC from which the endoglucanases were purified from were also analysed using SEC- MALLS. These enzyme formulations contain additional enzymes like β-glucosidases, which could contribute to a different cutting pattern.

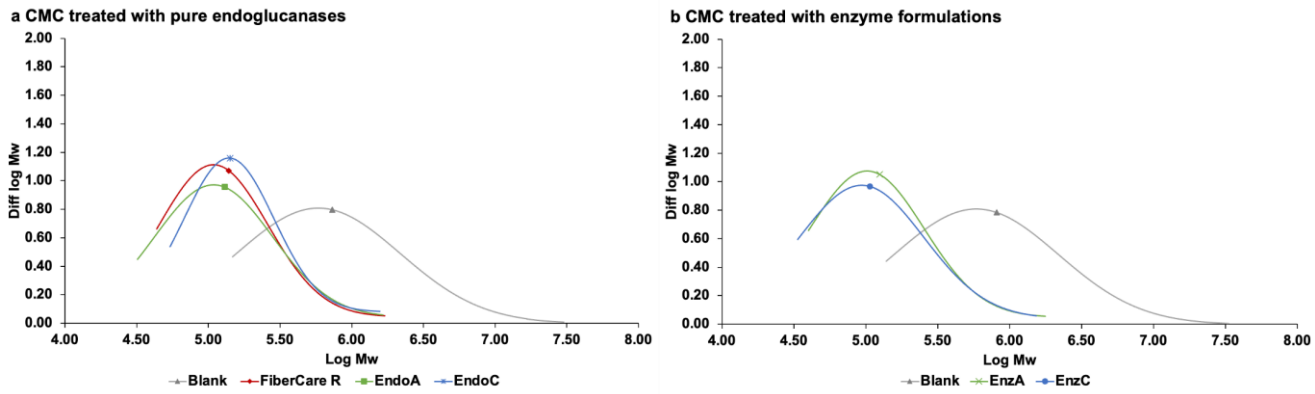

Fig. 35 Size exclusion chromatography of enzyme hydrolyzed carboxymethylcellulose (degree of substitution: 0.9, average molecular weight 700.000 g mol^{-1}). CMC was hydrolyzed at identical CellG5 endoglucanase activity with pure endoglucanases or complex enzyme formulations. Chromatograms of average values of duplicate measurements highlight changes in differential weight fraction after 2-hour enzyme hydrolysis. (a) shows the chromatograms for the purified endoglucanases EndoA and EndoC as well as the commercial endoglucanase FiberCare R, (b) for the corresponding complex enzyme formulations EnzA and EnzC.

Indeed, the enzyme formulations resulted in an even lower molecular weight after 2-hour treatment than for the respective pure endoglucanases at identical CellG5 activity, with EnzC showing the highest reduction (3.36 * 10^5 g mol^{-1}) while the EnzA formulation exhibited a much lower effect (3.71 * 10^5 g mol^{-1}). As observed for the pure endoglucanases, the enzyme formulation with the highest reduction in molecular weight, also exhibited the highest efficiency in previously conducted laboratory refining trials on hardwood pulp [34]. However, compared to the pure endoglucanases, no additional increase in the refining performance could be observed. The strong reduction of molecular weight by EnzC suggests that parts of CMC were degraded through synergistic action with enzymes like β-glucosidases that are present in this formulation along with endoglucanases. The resulting size distributions give an insight regarding synergistical effects by additional enzymes in the complex enzyme formulations **(Fig. 35b)**. The EnzA formulation showed the highest formation of fragments at log MW 5.01, while the EnzC formulation cut CMC in even lower fragments as indicated by the peak position of log MW 4.97. Therefore, EnzC resulted in the generation of lower molecular weight fractions than EnzA, which may be related to its higher β-glucosidase activity. Indeed, EnzC showed the highest β-glucosidase activity (55.7 nkat ml^{-1}), while EnzA showed only an activity of 15.5 nkat ml^{-1} [34].

The EnzC enzyme formulation led to a much wider size distribution with a dispersity value of 3.53 compared to the purified endoglucanase EndoC (3.39), which might be attributed to additional enzymes like β-glucosidases that cleave the products of

endoglucanases into smaller fragments [50]. Indeed, EnzA exhibited a 3.6 times lower β-glucosidase activity and showed a much narrower size distribution than EnzC. The resulting size distribution of EnzA (dispersity: 3.33) was even narrower than for its purified EndoA counterpart (dispersity: 3.73), possibly caused by additional enzymes in this formulation in combination with a considerably low β-glucosidase activity. A detailed overview of the characteristic values after SEC-MALLS of CMC is highlighted in ***Table 7***.

Table 7 Determined molecular weight and dispersity values using SEC-MALLS after 2-hour enzyme treatment on carboxymethylcellulose

Enzyme	**Molecular weight (g mol^{-1})**	**Dispersity Đ**
Blank	1.64 * 10^6	3.20
EnzA	3.71 * 10^5	3.33
EnzC	3.36 * 10^5	3.53
FiberCare R	3.77 * 10^5	3.22
EndoA	9.64 * 10^5	3.73
EndoC	4.98 * 10^5	3.39

5.4.2 SEC-MALLS of enzyme treated hardwood pulp

As a next step, SEC-MALLS was applied directly on enzyme treated hardwood pulp, which requires dissolution of the pulp in DMAc/LiCl and specialized SEC-MALLS equipment. Hence, comparison with the results of CMC is of interest, as prediction of enzyme behaviour using dissolved pulps involves labour-intensive steps and is not possible for all pulp types [27], [29]. Indeed, differences between the applied enzymes could be observed. Compared to the measurements on CMC, the molecular weight was expectedly decreased as well by all enzymes. While the blank showed an average molecular weight of 4.58 * 10^5 g mol^{-1}, it was reduced the most by the commercial endoglucanase FiberCare R (2.97 *10^5 g mol^{-1}), followed by EndoA (3.13 * 10^5 g mol^{-1}) and EndoC (3.58 * 10^5 g mol^{-1}). In addition, the complex enzyme formulation EnzC, containing additional enzymes like β-glucosidases and xylanases was analysed, which resulted in the lowest measured molecular weight of 2.87 * 10^5 g mol^{-1}, suggesting that parts of cellulose were degraded through synergistic action. In contrast to the SEC-MALLS measurements on CMC, no correlation to the refining performance could be

deduced from the changes in molecular weight despite the adjustment to identical enzyme activity.

However, the molecular weight distribution gives further insight into the cleaving pattern of the different enzymes (**Fig. 36**). Untreated hardwood pulp resulted in two major size populations, one smaller at log MW 4.39 and one with a larger fraction at log MW 5.48. Enzymatic hydrolysis resulted in the degradation of higher molecular weight fractions, while smaller molecular weight fractions from log MW 5.5 to log MW 4.4 increased. Most investigated enzymes degraded the high molecular weight fractions from log MW 6.5 to log MW 6 in a similar manner, except for EndoC, which affected this area the least. However, enzyme treatment also shifted the lower molecular weight fractions at log MW 4.4. EndoC (4.32) and FiberCare R (4.33) resulted in a pronounced shift to lower molecular weight, while EndoA (4.41) and EnzC (4.41) shifted to slightly higher molecular weight fractions. EndoC and FiberCare R were also those enzymes with the highest performance in laboratory refining trials on the same hardwood pulp [34]. Interestingly, EndoA and EnzC resulted in a similar cleaving pattern, despite EnzC including additional enzymes like β-glucosidases or xylanases. However, both exhibit a narrower molecular weight distribution, expressed by a lower dispersity value (Blank: 8.35, EndoA: 6.29, EndoC: 7.80, FiberCare R: 7.02 and EnzC: 5.36) suggesting that higher amounts of cellulose were degraded. As the molecular weight distribution was narrower for both EndoA and EnzC in the area between log MW 4.5 and 3.5, these fractions were more affected by these enzymes, when compared to EndoC and FiberCare R. Similar observations were reported in a study investigating GH45, GH7 and GH5 endoglucanases using SEC-MALLS on softwood pulp, resulting in a comparable variety of dispersity values as well as a similar increase of lower molecular weight fractions as in the current study [51]. A detailed overview of the characteristic values after SEC-MALLS of CMC is highlighted in ***Table 8***.

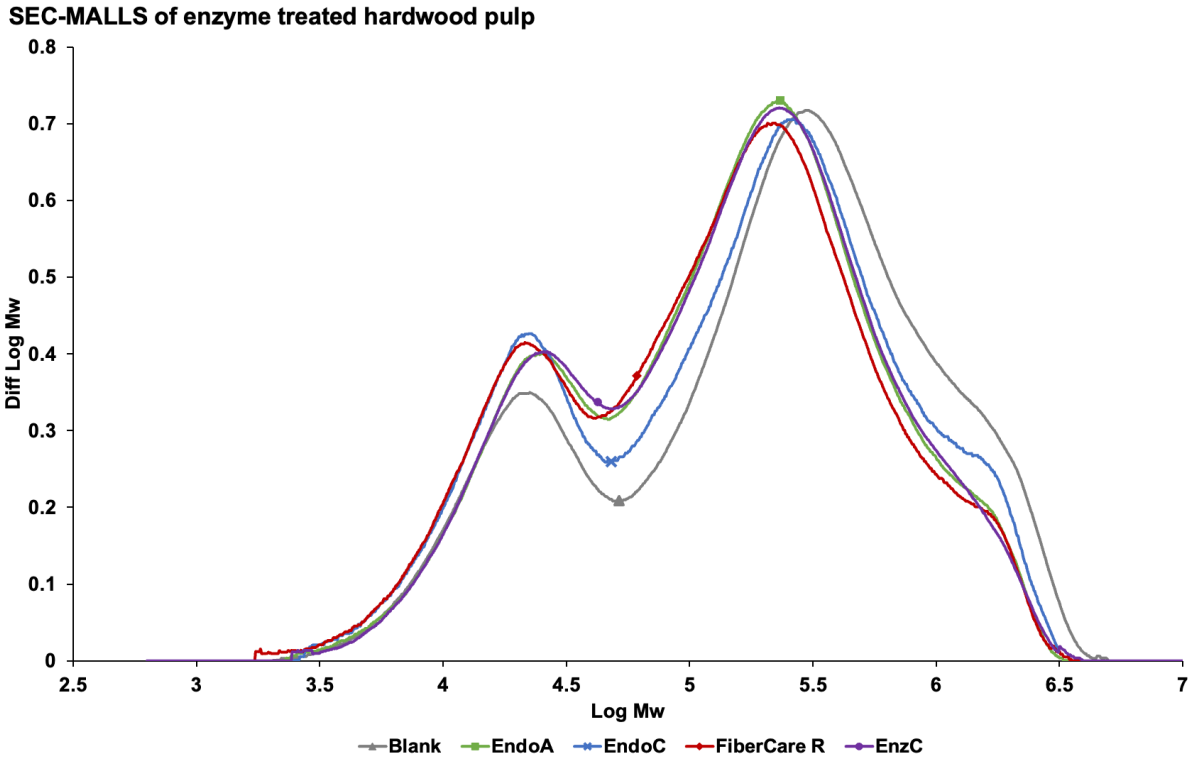

Fig. 36 Average molecular weight distribution of enzyme treated hardwood pulp after SEC-MALLS analysis. Hardwood pulp was treated for 48 hours with the pure endoglucanases EndoA, EndoC and FiberCare R as well as the complex enzyme formulation EnzC and subsequently measured with SEC-MALLS. Measurements were performed in duplicates and average values were calculated for plotting the curves.

Table 8 Determined molecular weight and dispersity values using SEC-MALLS after 2-hour enzyme treatment on hardwood pulp used for refining

Enzyme	**Molecular weight (g mol^{-1})**	**Dispersity Đ**
Blank	4.58 * 10^5	8.345
EnzC	2.87 * 10^5	7.795
FiberCare R	2.97 * 10^5	7.015
EndoA	3.13 * 10^5	6.290
EndoC	3.58 * 10^5	7.795

5.4.3 FTIR on hardwood pulp treated with purified endoglucanases and enzyme formulations

FTIR measurements were performed on hardwood pulp after enzyme treatment with pure endoglucanases at identical endoglucanase activity (based on CellG5). FTIR allows the identification of changes in functional groups, crystallinity, amorphous regions and properties such as the hydrogen bonding intensity of cellulose [52], [53]. One important characteristic property for the assessment of endoglucanase induced changes is the amorphous region of cellulose, which is assigned to the wavenumber 897 cm^{-1}. Endoglucanases preferentially hydrolyse the amorphous regions of cellulose, therefore the investigation of this area can provide a valuable insight into the function of the endoglucanases [6], [54], [55]. The sample without enzyme treatment showed an absorbance of 5.50 at the amorphous region (897 cm^{-1}). In contrast, EndoC treatment led to the highest reduction at this region to 4.98, followed by FiberCare R showing a value of 5.00 and EndoA, which exhibited a value of 5.53 **(Fig. 37a).** These results are in accordance with previously conducted laboratory refining trials on hardwood pulp showing the highest degree of refining values for EndoC (47.9 °SR) and FiberCare R (45.0 °SR), while EndoA showed a value of 42.4 °SR at 6000 refiner revolutions [34]. A high reduction of amorphous regions could therefore also translate to good refining performance.

The total crystallinity index (TCI) according to Nelson and O'Connor is calculated as the ratio of the absorbance at 1372 cm^{-1} and 2900 cm^{-1}[56]. The TCI increased for all investigated endoglucanases. While the sample without enzyme treatment showed a crystallinity index of 1.53, it was increased the most by EndoA (1.90), followed by EndoC (1.67) and FiberCare R (1.54). This is in accordance with other studies investigating the effect of cellulases, which also resulted in an increase of the total crystallinity index [57], [58]. Another important factor for the assessment of changes to the cellulose structure is the lateral crystallinity index (LOI), which is defined as the ratio between the absorbance at 1428 and 897 cm^{-1} [54], [59], [60]. The sample without enzyme treatment exhibited a lateral crystallinity index of 0.45, which was decreased to 0.42 by EndoA. However, it remained constant for FiberCare R (0.45) and even slightly increased for EndoC (0.46). Finally, the hydrogen bond intensity (HBI) as the ratio between the absorbance of 3400 and 1320 cm^{-1} was calculated, which gives valuable information about the intramolecular structure as well as the hydrogen

bonding between hydroxyl groups [54], [61], [62]. While the reference without enzyme addition exhibited a value of 0.89, it was reduced by all investigated endoglucanases with EndoA showing the lowest value of 0.56, followed by EndoC (0.67) and FiberCare R (0.86). Similar observations were conducted in a study that observed the effect of cellulases on microcrystalline cellulose, showing a reduction of HBI for the processive endoglucanase Cel9A, while the classic endoglucanase Cel5A exhibited a much lower decrease [57]. As EndoA also resulted in poor performance in laboratory refining trials on hardwood pulp, endoglucanases showing a high effect on the HBI could be disadvantageous for the refining of cellulose fibers.

Complex enzyme formulations also contain additional enzymes like β-glucosidases and xylanases. For example, the EnzA formulation exhibited a xylanase activity of 11.7 nkat ml^{-1}, while EnzC showed an activity of 437.7 nkat ml^{-1} at refining conditions [34]. Therefore, the effect of these enzyme formulations was analysed as well to study whether they could modify cellulose even further.

The respective enzyme formulations were also tested in laboratory refining trials on hardwood pulp in a previous study with EnzA showing a degree of refining of 40.7 °SR and EnzC a value of 45.0 °SR [34]. The absorbance of the amorphous region at 897 cm^{-1} was decreased by both enzyme formulations, with EnzA showing an absorbance of 4.54 and EnzC a value of 4.48, when compared to the sample without enzyme treatment that exhibited a value of 5.50 **(Fig. 37b)**. The total crystallinity index increased for both enzyme formulations, with EnzC showing the highest increase (1.70), followed by EnzA (1.67) in comparison to the sample without enzyme treatment (1.53). The crystallinity index was therefore similar to results of the pure endoglucanases and suggest that the crystallinity of the cellulose fibers was not increased any further by the additional enzymes in the formulations. A similar observation was made in a study investigating cellulases, proposing that cellulose chains from the crystalline core become surface chains during enzymatic degradation, disordered and amorphous through contact with the solvent, thus stopping the increase in crystallinity [63]. Furthermore, the lateral crystallinity index increased for all enzyme formulations as well when compared to the reference (0.45) but was identical for both formulations (0.49). However, the HBI was decreased by both formulations, although in a similar manner (EnzA: 0.74, EnzC: 0.75) when compared to the sample without

enzyme addition (0.89). Overall, the enzyme formulations demonstrated the highest effect in the degradation of amorphous regions, which was decreased even further than with their corresponding endoglucanase counterparts.

However, it is difficult to trace the effect of additional enzymes present in the enzyme formulations back to individual enzymes, which is required for the elucidation of synergistic effects. Therefore, the addition of pure β-glucosidase or xylanase to purified endoglucanase EndoC was studied. The addition of purified XylC reduced the absorbance of the amorphous region at 897 cm^{-1} to 4.78, while β-glucosidase from *Aspergillus niger* (β-glu) reduced the absorbance to only 5.28 **(Fig. 37a).** These results indicate that addition of xylanase contributes to the degradation of amorphous regions, probably by improving accesses of endoglucanases due to partial removal of xylan. The addition of β-Glu led to much lower decrease of the absorbance when compared to the blank (5.50). The addition of β-glucosidase could therefore lead to partial degradation of surface chains, thus exposing the crystalline core of cellulose, which becomes more disordered and eventually reverting the intensity of the amorphous region to the "starting value" as previously proposed [63]. The TCI improved after XylC addition (1.56) but decreased considerably after β-glucosidase addition to endoglucanase (1.37), which indeed suggests a disruption of the crystalline structure through enzymatic degradation. The lateral crystallinity index increased to a value of 0.47 after xylanase addition and decreased to 0.43 after β-glucosidase addition, compared to the blank without enzyme treatment (0.45). Finally, the HBI was calculated, which decreased to 0.82 after xylanase addition and increased to a value of 0.98 after β-Glu addition, when compared sample without enzyme addition (0.89). Overall, the addition of xylanase and β-Glu enhanced the degradation of amorphous regions (highest with XylC), while the addition of β-glucosidase also decreased the crystallinity index, supporting the theory that parts of the crystalline core got exposed through degradation of the cellulose fibers. In addition, no significant peak shifts could be observed, thus enzymes in the enzyme formulations did not convert cellulose into other forms but rather changed the content of amorphous cellulose or successively degraded („shaved") layers of cellulose chains from the surface. Furthermore, application of high enzyme dosages was avoided to better mimic conditions used for pulp refining. Although the highest effect could be observed at the amorphous region (897 cm^{-1}), prediction of enzyme behaviour for the refining process using FTIR is still

challenging if complex enzyme formulations with a whole set of different enzymes are applied, because of simultaneous modification of cellulose through synergistic action of all enzymes.

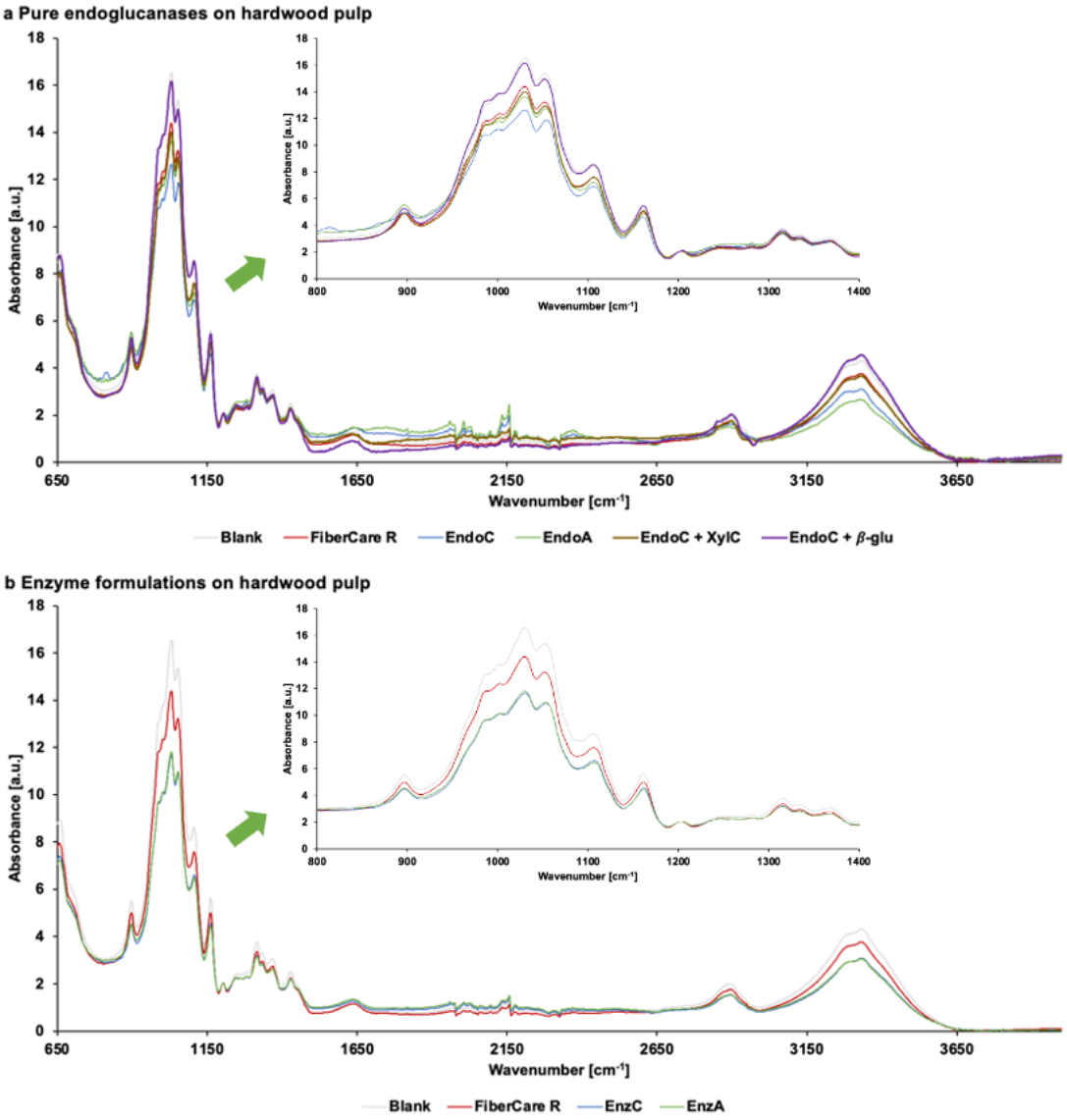

Fig. 37 FTIR analysis of hardwood pulp treated with enzymes at identical activity (according to CellG5). (a) shows the results of hardwood pulp treated with purified endoglucanases (EndoA, EndoC) and the commercial endoglucanase FiberCare R as well as the addition of xylanase or β-glucosidase to EndoC (b) of the corresponding enzyme formulations EnzA and EnzC compared with FiberCare R. In addition, the area between 800 and 1400 cm^{-1} is shown enlarged for better visualization of the differences in the amorphous region (897 cm^{-1}).

5.4.4 Surface Plasmon Resonance of endoglucanases on microcrystalline cellulose

The binding of the purified endoglucanases EndoA, EndoC and the commercial endoglucanase FiberCare R was analysed using SPR on amorphous regenerated cellulose (dissolved in DMAc/LiCl). A similar approach was used in a previous study

investigating lytic polysaccharide monooxygenases [6], [39]. While SPR was already applied in previous studies to measure the effect of carbohydrate binding modules of endoglucanases or for monitoring the degradation of cellulose by cellulase mixtures, the binding of endoglucanases to cellulose at identical activity was of interest to mimic conditions used in pulp refining that is often based on enzyme activity [32], [64], [65]. The purified endoglucanases EndoA, EndoC and the commercial endoglucanase FiberCare R were measured at three different activities using multi-cycle kinetics mode. SPR analysis revealed that the commercial endoglucanase FiberCare R bound with the highest affinity to amorphous regenerated cellulose, followed by EndoC, while EndoA showed only minor binding at highest enzyme activity with a response slightly higher than the blank run **(Fig. 38)**. For example, after 65 seconds during the association phase, the blank run exhibited a response of 96 RU, which increased to 1026 RU after addition of the commercial endoglucanase FiberCare R at the highest activity of 2.83 nkat ml^{-1}, while EndoC exhibited a response of 602 RU, followed by EndoA with a response of 106 RU at identical enzyme activity. Therefore, FiberCare R showed a 1.7 times higher binding signal than EndoC and even 9.7 times higher binding signal when compared to EndoA (**Fig. *38*c**). Similarly, the endoglucanase EndoC, exhibited 5.7 times higher binding than the endoglucanase EndoA. Interestingly, binding of the enzymes to amorphous regenerated cellulose correlated with the performance of these enzymes in previously conducted refining trials on hardwood pulp where the highest degree of refining values measured for EndoC (47.9 °SR) and FiberCare R (44.8 °SR) and the lowest for EndoA (41.3 °SR) at 6000 revolutions of the refiner [34]. However, FiberCare R resulted in the highest binding to amourphous cellulose, while this endoglucanase achieved a slighlty lower degree of refining value than EndoC. This could be explained by different enzyme mechanisms with EndoC beeing more effcient, while FiberCare R binds strongly and in high amounts to its substrate, but expresses a lower catalytic activity. On the other hand, binding studies using SPR are only possible on regnerated cellulose, as it requires complete dissolution in DMAc/LiCl, hence small differences between the two enzymes could also be related to certain characteristics of the native pulp.

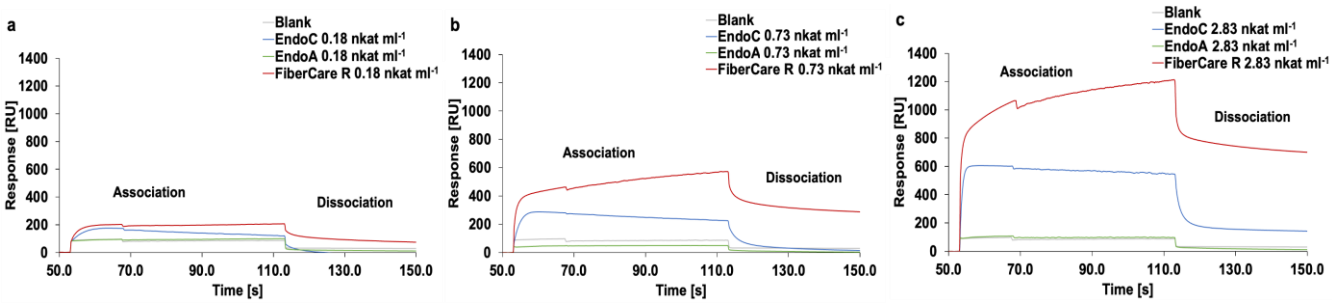

Fig. 38 Sensograms of surface plasmon resonance multi-cycle kinetic analysis of the binding of pure endoglucanases to amorphous regenerated cellulose. Purified endoglucanases EndoA and EndoC as well as the commercial endoglucanase FiberCare R were applied to a gold chip coated with amorphous regenerated cellulose (dissolved in DMAc/LiCl) at three different enzyme activities (based on CellG5 activity assay). Duplicate measurements of two independent sensor chip sections were performed and average response values were calculated. (a) shows the sensogram at 0.18 nkat ml^{-1}, (b) the sensogram at 0.73 nkat ml^{-1} and (c) the sensogram at the highest endoglucanase activity of 2.83 nkat ml^{-1}

5.4.5 Refining trials on hardwood sulfate pulp

To investigate possible synergistic effects of endoglucanases together with β-glucosidases or xylanases in pulp refining, laboratory refining trials on bleached hardwood (eucalyptus) sulfate pulp were conducted. Addition of β-glucosidase from *Aspergillus niger* indeed increased the degree of refining on hardwood pulp. Compared to a reference without enzyme treatment (35.5 °SR at 6000 PFI revolutions of the refiner) the purified endoglucanase EndoC increased the degree of refining to 43.4 °SR. Addition of β-glucosidase induced a further increase to 47.2 °SR **(Fig. 39a).** However, this improvement in the degree of refining was accompanied with some decrease of the length weighted fiber length from 787 µm (EndoC) to 777 µm (EndoC + β-glucosidase), while the reference without enzyme addition showed a fiber length of 804 µm at 6000 PFI revolutions **(Fig. 39b)**.

One important aim of the refining process is the increase in tensile strength; hence the tensile index is another important parameter for the assessment of the refining performance [66], [67]. The tensile index of the reference without enzyme treatment reached a value of 72.1 Nm g^{-1} (at 6000 PFI revolutions) and decreased to 70.6 Nm g^{-1} upon EndoC treatment. Interestingly, treatment of a combination of EndoC and β-Glu led to a synergistic increase of the tensile index to 73.5 Nm g^{-1} **(Fig. 39c),** while treatment with β-Glu alone did not show any effect. Air permeability was determined according to the Bendtsen method and usually decreases during the refining process through formation of the fiber-fiber bonds in the network that increase the density of

paper sheets [68]–[70]. An air permeability Bendtsen value of 404 ml min^{-1} was measured for the reference without enzyme addition at 6000 PFI revolutions, which was decreased to a value of 208 ml min^{-1} by EndoC and further reduced upon addition of β-Glu to 141 ml min^{-1} **(Fig. 39d).** As expected, the use of only β-glucosidases without endoglucanases showed a similar air permeability value (383 ml min^{-1}) as the blank at 6000 PFI revolutions. The results after β-glucosidase addition to endoglucanases indicate that endoglucanases are already inhibited by their products (such as cellobiose), which is avoided by synergistic action of β-glucosidases, that degrade these products to glucose.

As a next step purified xylanase XylC was added to EndoC, which increased the degree of refining value as well when compared to refining with endoglucanase only. With EndoC a degree of refining of 43.4 °SR was achieved at 6000 PFI revolutions while a further increase to 46.1 °SR was seen upon addition of XylC **(Fig. 39a)**. This increase was, however, accompanied with a small decrease in fiber length from 787 µm (EndoC) to 781 µm **(Fig. 39b)**. Furthermore, improvements in the tensile index were observed to a value of 73.0 Nm g^{-1} at 6000 PFI revolutions for the combination of EndoC and XylC compared to 70.6 Nm g^{-1} with only EndoC **(Fig. 39c)**. Air permeability was reduced as well by the addition of xylanase, reaching a value of 196 ml min^{-1} compared to 208 ml min^{-1} for EndoC at 6000 PFI revolutions **(Fig. 39d)**. This clearly shows that xylanases are beneficial for pulp refining through a synergistic effect, which is in accordance with previous studies [71], [72]. However, the decrease in air permeability was much more pronounced after addition of β-glucosidase (141 min^{-1}), suggesting that the addition of xylanase increased the accessibility to cellulose for endoglucanases through degradation of xylan, but prevention of product inhibition by β-glucosidase was more effective.

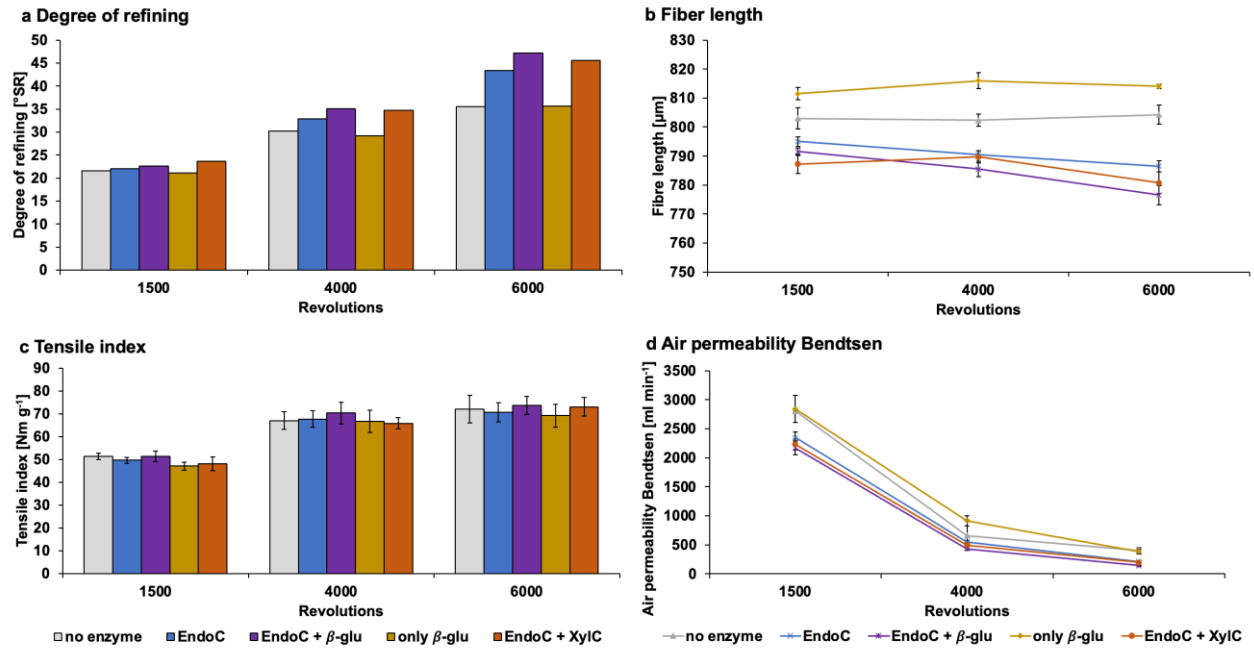

Fig. 39 Enzyme assisted refining of bleached hardwood sulfate pulp. The reference without enzyme addition as well as purified endoglucanase EndoC was refined at 1500, 4000 and 6000 PFI revolutions. Furthermore, β-glucosidase from *Aspergillus niger* (β-Glu) or xylanase (XylC, purified from EnzC formulation) was tested in combination with EndoC to investigate possible synergistic effects on degree of refining and pulp and paper properties such as fiber length, tensile index, and air permeability. (a-d) show the degree of refining values, determined fiber length, tensile index, and air permeability values for combinations of EndoC with β-Glucosidase and with XylC xylanase

5.5 Conclusions

Due to the high variety of available enzyme formulations and their included enzymes, prediction of enzyme performance for pulp refining is still challenging. Endoglucanases resulting in the highest reduction in molecular weight during SEC-MALLS of CMC, also showed the highest efficiency in pulp refining experiments, while analysis of the molecular size distribution indicates the formation of narrow size distributions is also advantageous for pulp refining. However, SEC-MALLS analysis of enzyme treated hardwood pulp suggests that the decrease in molecular weight cannot be used for prediction of enzyme behaviour on the pulps themselves, the most efficient enzymes in pulp refining rather differ in increased formation of low molecular weight fractions. Enzyme formulations containing additional enzymes like β-glucosidases or xylanases expressed the highest reduction in molecular weight on both CMC and hardwood pulp, however, no additional increase of refining performance could be observed, which complicates enzyme prediction. In addition, excessive degradation of cellulose could also affect the properties of paper sheets negatively, which could explain this behaviour. FTIR analysis revealed that endoglucanases that are most efficient in the

degradation of amorphous parts of cellulose are also the best performing enzymes during pulp refining, thereby this method can indeed be used for the identification of the most efficient enzyme in pulp refining if the endoglucanase specific CellG5 activity assay is applied. Prediction of enzyme behaviour was only possible for pure endoglucanases using SEC-MALLS, however, this method could provide a valuable insight into the enzyme mechanisms though observation of the resulting size distributions. This is especially the case for SEC-MALLS of enzyme treated pulp, which revealed changes to the insoluble part of cellulose, a crucial part in pulp refining, as it is transformed to the final paper product. Evaluation of β-glucosidase or xylanase addition in refining also highlighted the benefits of these enzymes in complex enzyme formulations, but high amounts of these enzymes may affect pulp refining negatively through successive degradation of cellulose layers from pulp. Furthermore, enzymes like β-glucosidases, also increase the chemical oxygen demand, thereby a sensitive balance point between β-glucosidase addition for endoglucanase activity enhancement in pulp refining and resulting wastewater treatment requirements must be found. Analogous, xylanase addition increases accessibility for endoglucanase enzymes through degradation of xylan, while excessive degradation thereof may affect paper properties like the tensile index negatively. The use of SPR enabled the prediction of the most effective endoglucanases in pulp refining by analysis of the binding behaviour to regenerated amorphous cellulose, therefore this method can be used as tool for quick screening of suitable enzymes.

CRediT authorship contribution statement

Martin Nagl: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing – original draft, Visualization, Funding acquisition

Oskar Haske-Cornelius: Conceptualization, Methodology, Project administration. Funding acquisition **Georg M. Guebitz and Wolfgang Bauer:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Supervision

Florian Csarman and Roland Ludwig: Conceptualization, Methodology, Writing – review & editing

Gibson Stephen Nyanhongo: Project administration, Funding acquisition, Supervision, Writing – review & editing

Declarations of interest

None.

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5.7 Supplementary material

Refractive index value of SEC-MALLS after enzymatic treatment of CMC

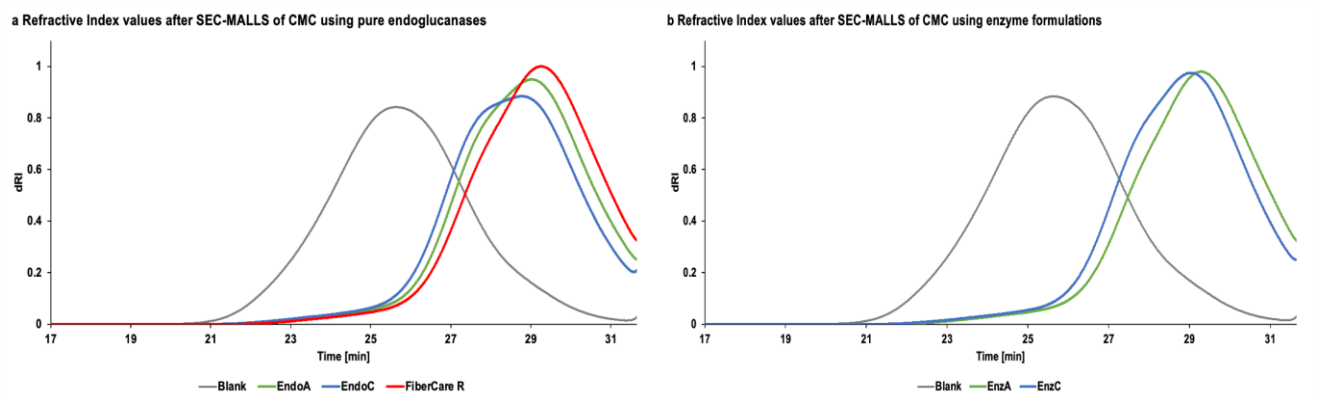

Fig. S40 Recorded refractive index values after size exclusion chromatography of enzyme hydrolyzed carboxymethylcellulose (degree of substitution: 0.9, average molecular weight 700.000 g mol^{-1}). CMC was hydrolyzed at identical CellG5 endoglucanase activity with pure endoglucanases or complex enzyme formulations. Chromatograms of average values of duplicate measurements highlight changes in refractive index after 2-hour enzyme hydrolysis. (a) shows the chromatograms for the purified endoglucanases EndoA and EndoC as well as the commercial endoglucanase FiberCare R, (b) for the corresponding complex enzyme formulations EnzA and EnzC.

Activity assay results

β-glucosidase activity using 50% tap water + 50% deionized water (adjusted to pH 7.0):

Table S9 β-glucosidase activity of Megazyme β-glucosidase E-BGLUC using 50% tap water + 50% deionized water (adjusted to pH 7):

Enzyme	Activity [nkat ml^{-1}]	Standard deviation
Megazyme β-glucosidase E-BGLUC	51.31	1.71

Xylanase activity using 50% tap water + 50% deionized water (adjusted to pH 7.0):

Table S10 XylX6 xylanase activity of XylC xylanase (purified from EnzC formulation) using 50% tap water + 50% deionized water (adjusted to pH 7):

Enzyme	Activity [nkat ml^{-1}]	Standard deviation
XylC xylanase (purified from EnzC formulation)	6.731	0.0063

Scanning electron microscopy pictures of refined paper sheets at 6000 U

Refined paper sheets of hardwood pulp were analysed using the scanning electron microscope at 1000× magnification. Pictures of paper sheets refined at 1500 PFI revolutions (**Fig. S41**), 4000 revolutions (**Fig. S42**) and 6000 revolutions (**Fig. S43**) revealed increased fibrillation of all enzyme treated paper sheets when compared to sheets without enzyme addition. Highest fibrillation levels could be observed with addition of β-glucosidase or XylC xylanase to EndoC endoglucanase.

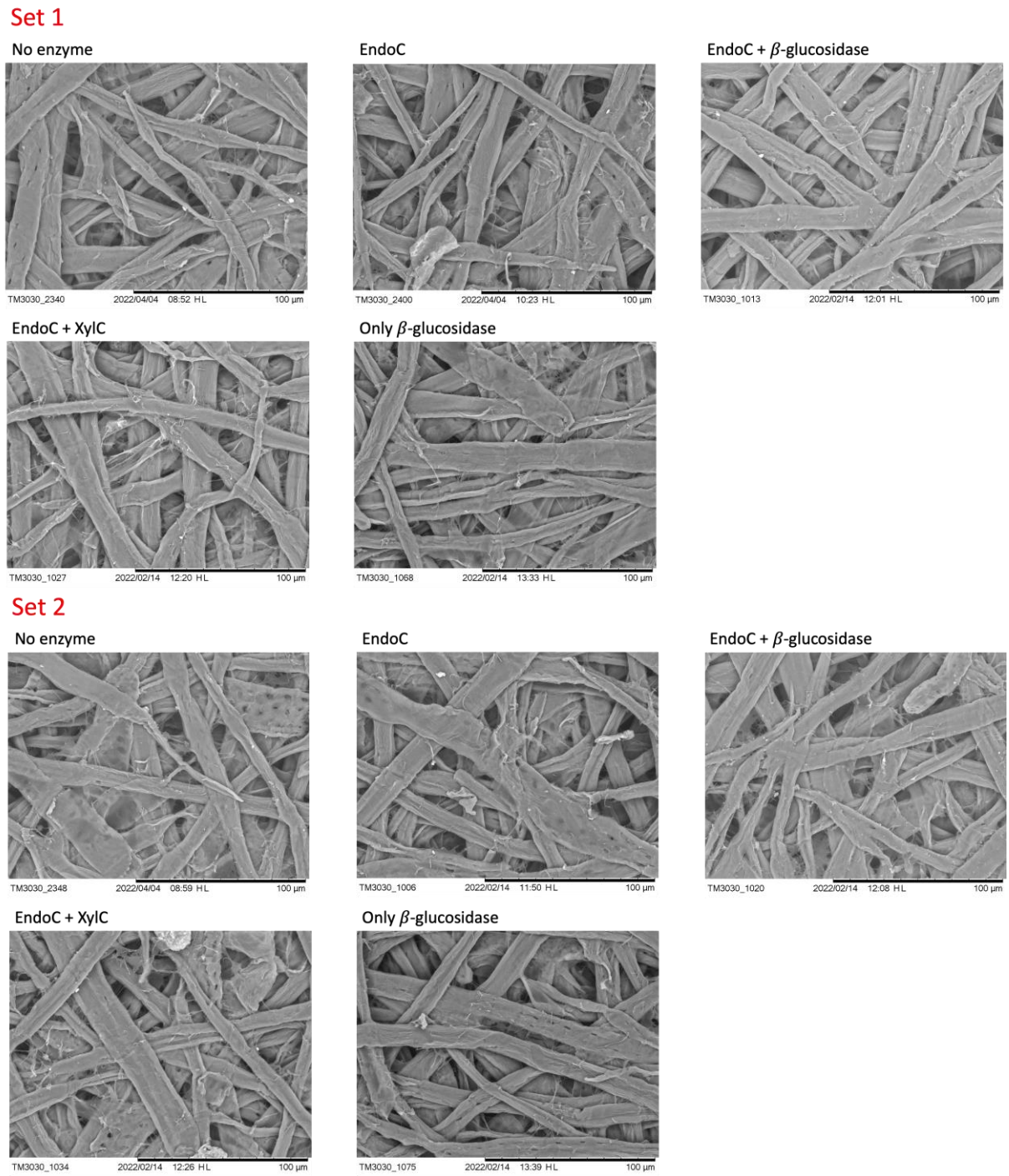

Fig. S41 Scanning electron microscopy pictures of the duplicate measurements of refined paper sheets at 1500 revolutions. Paper sheets were formed after refining on hardwood pulp using a reference without enzymes, EndoC, EndoC with β-glucosidase addition, EndoC with XylC addition and with only β-glucosidase and subsequently analyzed using the scanning electron microscope at 1000× magnification.

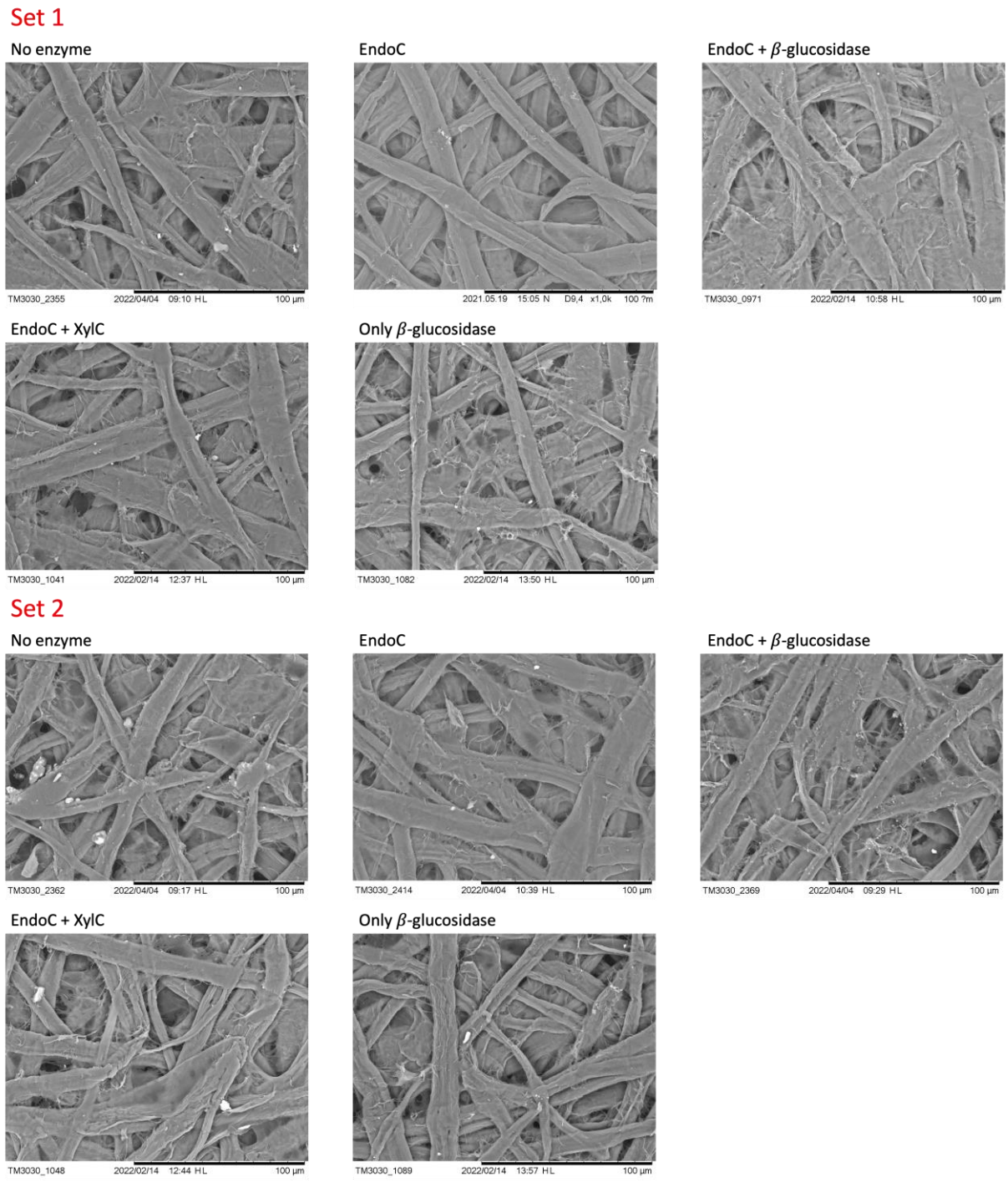

Fig. S42 Scanning electron microscopy pictures of the duplicate measurements of refined paper sheets at 4000 revolutions. Paper sheets were formed after refining on hardwood pulp using a reference without enzymes, EndoC, EndoC with β-glucosidase addition, EndoC with XylC addition and with only β-glucosidase and subsequently analyzed using the scanning electron microscope at 1000× magnification.

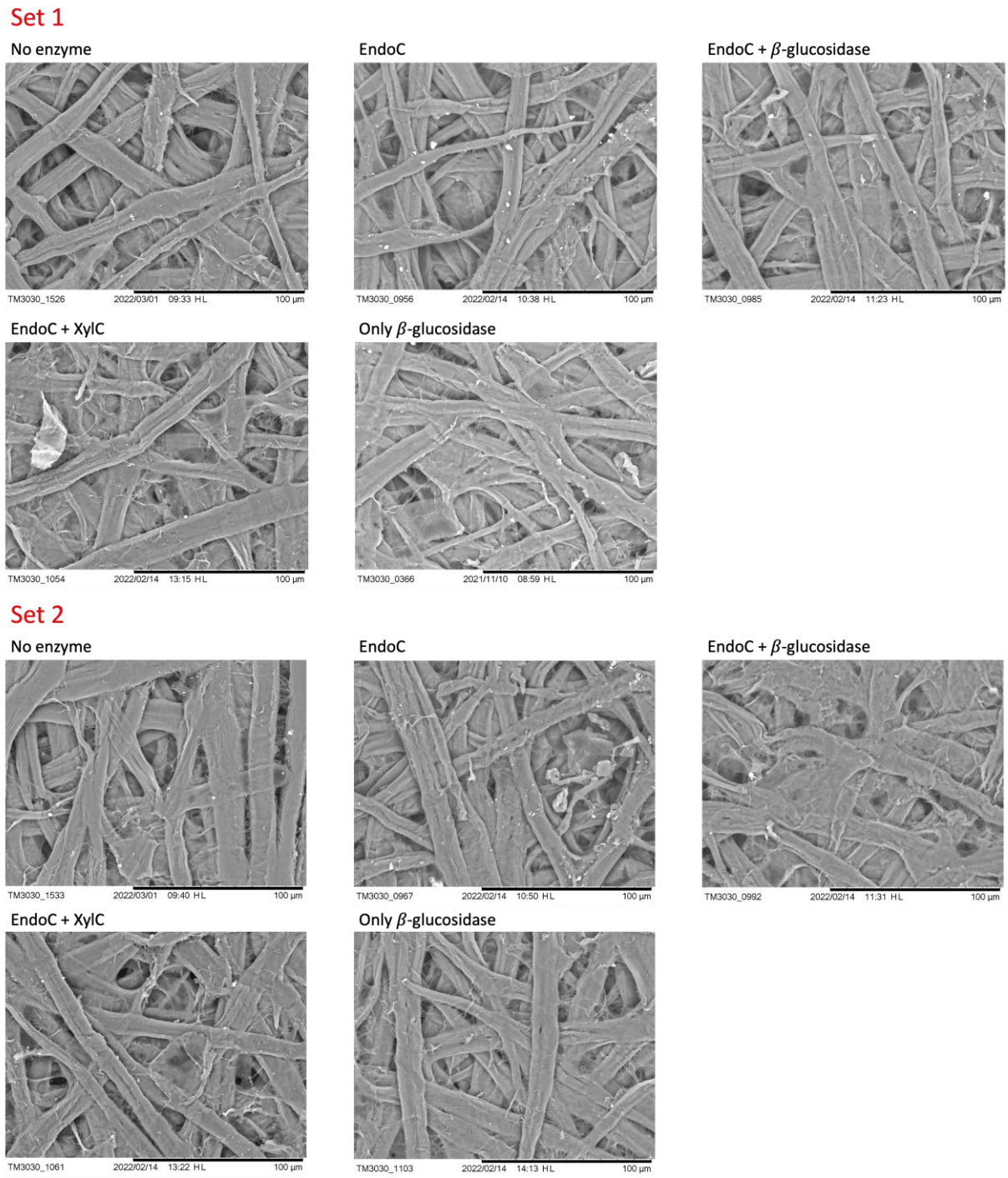

Fig. S43 Scanning electron microscopy pictures of the duplicate measurements of refined paper sheets at 6000 revolutions. Paper sheets were formed after refining on hardwood pulp using a reference without enzymes, EndoC, EndoC with β-glucosidase addition, EndoC with XylC addition and with only β-glucosidase and subsequently analyzed using the scanning electron microscope at 1000× magnification.

6

General Conclusion

Paper products are an integral part of our lives with versatile applications ranging from tissue papers, to printing papers over to packaging materials. However, paper production consumes a lot of energy, which has negative effects on the environment. In this thesis, the use of enzymes in paper production and recycling was investigated with the aim to provide better means of prediction of enzyme behaviour.

In fact, the investigation of commonly used complex enzyme formulations in the pulp and paper industry showed a high variety of included enzymes (endoglucanases, β-glucosidases, xylanases etc.). Enzyme dosage according to enzyme activity in pulp refining was previously based on reducing sugar assays, however, these assays are not specific, which is a major limitation for formulations containing a high number of different enzymes. Therefore, the new derivatized cellopentaose based substrate (CellG5) was tested in refining trials. This substrate is specific to endoglucanases, the enzymes responsible for energy reduction in pulp refining, which was proven by purification of these enzymes from their respective formulations.

Endoglucanase purification also enabled the identification of beneficial enzyme traits for most efficient refining. Indeed, endoglucanases could increase the degree of refining on hardwood pulp in laboratory refining trials with EndoC and FiberCare R exhibiting the highest effect, while EndoA and EndoB showed a lower increase. Endoglucanases are therefore definitely the enzymes responsible for energy reduction, but differences between the enzymes remained despite the use of the new CellG5 substrate, presumably due to different catalytic domains, with varying substrate affinity. Moreover, complex enzyme formulations, from which the endoglucanases were purified, were studied in laboratory refining trials and the enzyme formulation EnzC containing EndoC endoglucanase, showed a high performance as well. The specificity of the CellG5 substrate for endoglucanases (no degradation by other enzymes such as cellobiohydrolases or β-glucosidases), resulted in strong correlation of CellG5 activity to refining performance for the complex enzyme formulations. However, it was

also shown, that the additional enzymes in the complex enzyme formulations have a positive or negative effect on refining (e.g., lower performance of EnzA formulation compared to its purified endoglucanase EndoA). Therefore, it is essential to study, which individual enzymes are beneficial.

The lack of prediction of enzyme behaviour is the most important obstacle for the use of enzymes in industry. Carbohydrate binding modules have been previously utilized for applications such as monitoring of total hydrolysis of pulp using a hydrolytic enzyme formulation. However, enzyme formulations have a different enzyme composition in pulp refining and especially for mechanistic studies, the investigation of individual enzymes such as endoglucanases is of higher interest, which could also contribute to a better understanding of recycling processes. Therefore, the characteristic trait of CBMs to bind to a high variety of substrates of different crystallinity was successfully adapted in a new method to visualize the effect of endoglucanases on pulp. Indeed, enzyme performance correlated with obtained refining results, which makes CBMs suitable for the selection of the most efficient enzymes. Since CBMs are already available commercially, they can be easily adopted, and it is expected that their usage will expand to other fields as well. Because endoglucanases can only degrade amorphous regions of cellulose, this fraction is expected to decrease upon enzyme treatment, which led in increased binding of the respective CBM. This principle was also applied using Fourier-transform infrared spectroscopy (FTIR), which resulted in the highest decrease of the amorphous region for the most efficient endoglucanases, although it was not that pronounced for complex formulations. As FTIR measurements are conducted in a non-destructive and quick manner, this method can easily be implemented in the pulp and paper industry.

Surface plasmon resonance was previously used for characterization of enzyme kinetics, however, for the direct comparison of different endoglucanases the binding at identical activity is of interest to mimic the set-up of the laboratory refining trials. Therefore, a new method was developed to investigate the binding of endoglucanases to amorphous regenerated cellulose films at identical CellG5 activity.
Indeed, the most effective enzymes in pulp refining were also binding in high amounts. However, differences between FiberCare R and EndoC were still apparent, as FiberCare R bound to a much higher extent, while EndoC exhibited a greater refining

performance. This suggests that remaining variation between enzyme performance is related to the catalytic centre or differences in accessibility to native pulp, as only regenerated cellulose could be used for this method.

Analysis of the size profile using size exclusion chromatography resulted in similar molecular weight distribution for the most effective enzymes EndoC and FiberCare R on hardwood pulp. Moreover, there was also a variation of enzyme behaviour on different pulps such as hardwood vs. softwood using NMR, which suggest that a more detailed investigation of the catalytic cavities of the enzymes is beneficial for a deeper understanding of reaction mechanisms. This information could result in a library of structurally similar enzymes, with high effect on a certain pulp type, from which paper manufacturers may choose. However, most of the enzymes intended for the pulp and paper industry are provided by commercial enzyme suppliers, which legitimately want to protect obtained knowledge about the enzymes and the modifications they performed, which complicates the elucidation of enzyme mechanisms. Another factor for efficient enzyme usage is the exploitation of synergistic enzyme effects. Synergistic effects of cellulase enzymes have been previously described, however, the application of the CellG5 assay enabled for the first time their detailed study in enzyme formulations because of the high specificity of this substrate and subsequent comparison with their pure endoglucanases. While this thesis has shown that pure endoglucanases themselves are the main drivers for reduction of energy consumption, their effect was even higher with the addition of enzymes such as β-glucosidases, which prevent product inhibition, or xylanases, which degrade hemicelluloses and provide better accessibility for endoglucanases. On the other hand, the addition of β-glucosidases increases glucose concentration in the process water, thereby requiring wastewater treatment during paper production, while it is ideal for recycling processes that use glucose for new products.

Therefore, a careful selection of enzyme formulations is essential. The potential for energy saving in the pulp and paper industry is huge and the results of this thesis highlight the importance of effective screening techniques for enzymes. The obtained information is also highly relevant for recycling processes, as they also depend on efficient enzymes for the degradation of waste materials, which saves energy as well, if these processes can be accelerated.

7

Appendix

7.1 List of Figures

7.2 List of tables

7.3 Publications

Nagl, Martin, Haske-Cornelius, Oskar, Skopek, Lukas, Pellis, Alessandro, Bauer, Wolfgang, Nyanhongo, Gibson S., & Guebitz, Georg (2021). Biorefining: the role of endoglucanases in refining of cellulose fibers. Cellulose, 28(12), 7633-7650. https://doi.org/10.1007/s10570-021-04022-2

Nagl, Martin, Haske-Cornelius, Oskar, Skopek, Lukas, Bausch, Florian, Pellis, Alessandro, Bauer, Wolfgang, Nyanhongo, Gibson S., & Guebitz, Georg M. (2022). Mechanistic investigation of the effect of endoglucanases related to pulp refining. Cellulose, 29(4), 2579-2598. https://doi.org/10.1007/s10570-021-04386-5

Nagl, Martin, Haske-Cornelius, Oskar, Bauer, Wolfgang, Csarman, Florian, Ludwig, Roland, Nyanhongo, Gibson S., & Guebitz, Georg M. (2022). Towards a better understanding of synergistic enzyme effects during refining of cellulose fibers.

Carbohydrate Polymer Technologies and Applications, 4, 100223. [100223]. https://doi.org/10.1016/j.carpta.2022.100223

7.4 Oral presentations at scientific conferences as presenting author

Nagl, Martin; Haske-Cornelius, Oskar; Skopek, Lukas; Pellis, Alessandro; Bauer, Wolfgang; Nyanhongo, Gibson S.; Guebitz, Georg M. (2022): Refining enzymes – mechanistic study of endoglucanases.
Paper & Biorefinery, 18.05.2022 - 19.05.2022, Graz

Nagl, Martin; Haske-Cornelius Oskar; Skopek Lukas; Pellis Alessandro; Bauer Wolfgang; Nyanhongo Gibson S.; Guebitz Georg M. (2022): A mechanistic study of enzymes used for energy saving in pulp refining. Biocatalysis for the Biological Transformation of Polymer Science, 27.07.2022-29.07.2022, Cologne

7.5 Poster presentations at scientific conferences as presenting author

Nagl, Martin; Haske-Cornelius, Oskar; Skopek, Lukas; Hartmann, Alexandra; Brunner, Florian; Pellis, Alessandro; Bauer, Wolfgang; Nyanhongo, Gibson S.; Guebitz, Georg M. (2021): Enzymes for pulp refining – How do they work? EFB 2021, May 10-14, 2021, Online

Nagl, Martin; Haske-Cornelius, Oskar; Skopek, Lukas; Pellis, Alessandro; Bauer, Wolgang; Nyanhongo, Gibson S.; Guebitz, Georg M. (2021): The role of endoglucanases in pulp refining. [Poster] [Designer Biology 2021, Online, 08.09.2021 - 10.09.2021] The European Federation of Biotechnology, Designer Biology: From proteins and cells to scaffolds & materials in Europe

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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.

Martin Nagl