



Mechanistic investigation of the effect of endoglucanases related to pulp refining

Martin Nagl · Oskar Haske-Cornelius · Lukas Skopek · Florian Bausch · Alessandro Pellis · Wolfgang Bauer · Gibson S. Nyanhongo · Georg M. Guebitz

Received: 23 September 2021 / Accepted: 17 December 2021
© The Author(s) 2022

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 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 ¹³C-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

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10570-021-04386-5>.

M. Nagl (✉) · O. Haske-Cornelius · L. Skopek · A. Pellis · G. S. Nyanhongo (✉) · G. M. Guebitz
Institute of Environmental Biotechnology, University of Natural Resources and Life Sciences Vienna, Konrad-Lorenz-Strasse 20, 3430 Tulln an der Donau, Austria
e-mail: martin.nagl@boku.ac.at

G. S. Nyanhongo
e-mail: g.nyanhongo@boku.ac.at

F. Bausch
Institute of Chemistry of Renewable Resources, University of Natural Resources and Life Sciences Vienna, Konrad-Lorenz-Strasse 24, 3430 Tulln an der Donau, Austria

W. Bauer
Institute of Bioproducts and Paper Technology, Graz University of Technology, NAWI Graz, Inffeldgasse 23, 8010 Graz, Austria

G. S. Nyanhongo · G. M. Guebitz
Austrian Centre of Industrial Biotechnology, Konrad-Lorenz-Strasse 20, 3430 Tulln an der Donau, Austria

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

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 (Gharehkhani et al. 2015; Reza et al. 2019; Tripathi 2019). 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 30–50% of the total energy used for paper making and generation of high amounts of fines (Loosvelt 2009; Lecourt et al. 2010; Buzala et al. 2016). Therefore, the use of enzymes during pulp refining emerged as a new measure to reduce energy consumption, resulting in energy savings up to 40% (Fleiter et al. 2012; Torres et al. 2012; Tripathi 2019; Haske-Cornelius et al. 2020).

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 (García et al. 2002; Singh et al. 2015; Nagl et al. 2021). 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; Kampuri

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 (Lecourt et al. 2010). 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 (Haske-Cornelius et al. 2020). 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 (Tripathi et al. 2008; Orłowski et al. 2015).

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 (Evans et al. 1995; Emwas 2015; Haslinger et al. 2019). 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 (Maunu et al. 2000; Rongpipi et al. 2019). Carbohydrate binding modules are non-hydrolytic domains that facilitate the catalytic activity of carbohydrate specific enzymes by binding to their respective substrates (Hashimoto 2006; Karita 2016; Liu et al. 2021). 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 (Hettle et al. 2017; Carbohydrate-Active enZymes Database 2021). 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 (Hägglund et al. 2003; Igarashi et al. 2009).

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 (Boraston et al. 2004; Ribitsch et al. 2013; Weber et al. 2019). 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 (Fox et al. 2013). This characteristic was already used to observe changes in crystallinity when applying an enzyme formulation designed for total hydrolysis on never-dried pulps (Novy et al. 2019). 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 (Ding et al. 2006; Gourlay et al. 2015).

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.

Material & methods

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.

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 (Mangan et al. 2016). As buffer 50 mM citrate buffer, pH 4.8 was used. For this purpose, a volume of 100 μ l of diluted enzyme solution was mixed with 100 μ l of CellG5 substrate in 10 ml glass tubes and incubated at 45 °C for 10 min. 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 μ l of 50 mM citrate buffer (pH 4.8) was used as a blank. Afterwards, 2 μ l 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 [$M^{-1} \cdot cm^{-1}$].

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 (Nagl et al. 2021). 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 to 90% B. EndoB was purified using a 50% B step, followed by a linear gradient ranging from 50 to 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).

Confocal laser scanning microscopy (CLSM)

Labelling of carbohydrate binding modules

The buffer of purchased CBM 28A and CBM 2D was exchanged to 1×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-h 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 min at 1000 g.

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 h on a thermomixer (Eppendorf, Austria). For removal of bound enzymes, a similar washing protocol as in a previous study was applied (Novy et al. 2019). Therefore, pulps were washed 3× with 250 µl fresh ultrapure water and incubated with 250 µl 1% SDS solution at 99 °C, 500 rpm for 10 min to remove residual enzymes from the cellulose fibers. Pulps were then washed 1× in 250 µl absolute ethanol, 1× 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 min 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 10×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 (Haske-Cornelius et al. 2020). 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. S2 and S3).

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 h 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 min 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 min at 12,500 rpm. The supernatant was then filtered 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 min. Data analysis was performed using the Agilent OpenLab software. Cellobiose, celotriose, cellotetraose, glucose and xylose standards ranging between 0 and 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.

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 min). 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 min 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 and 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.

¹³C-NMR analysis of enzyme treated pulps

For the analysis of enzyme treated pulps with ¹³C-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 min at 300 rpm. Solid-state NMR experiments were performed on a Bruker Avance III HD 400 spectrometer (resonance frequency of ¹H of 400.13 MHz, and ¹³C

of 100.61 MHz, respectively), equipped with a 4 mm 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 ^1H 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 $\delta = 176.03$ 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 (Massiot et al. 2002). Assignment of crystalline or amorphous fractions was based on Wickholm et al. (1998). Peaks between 87.8 ppm and 89.3 ppm were assigned to the crystalline (cellulose I α : 89.3 ppm, cellulose I β : 88.8 ppm, cellulose I $\alpha\beta$: 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).

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 h 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. S4. of the Online Resource.

Results and discussion

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. S4) 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 (Nagl et al. 2021). 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 U. 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. 1). 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} (Nagl et al. 2021).

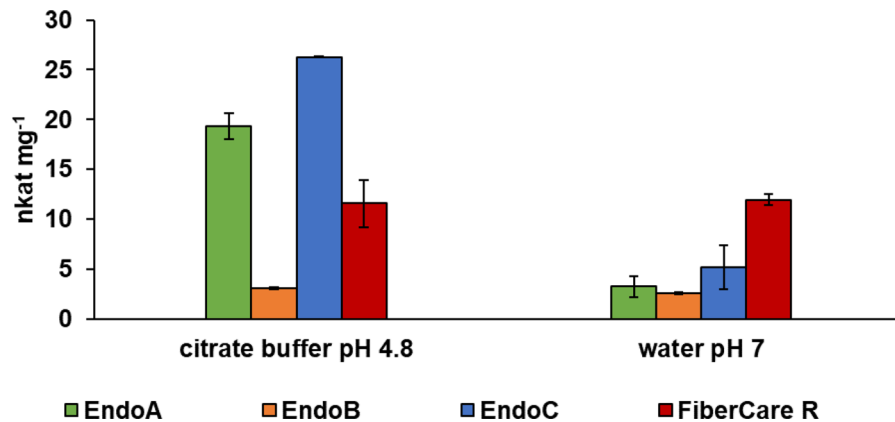


Fig. 1 Activity of purified endoglucanases and of the commercial endoglucanase formulation FiberCare R on derivatized cellopentaose (CellG5). Endoglucanase activity was measured using 50 mM citrate buffer (pH 4.8) or 50% tap water + 50%

deionized water (adjusted to pH 7) simulating refining conditions. Measurements were conducted in duplicates and error bars indicate the standard deviation

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. 2). 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. 2a). 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. 2b). 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 (Vlasenko et al. 2010; Hua et al. 2018). 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 (Song et al. 2016; Zhang et al. 2017; Ghio et al. 2020).

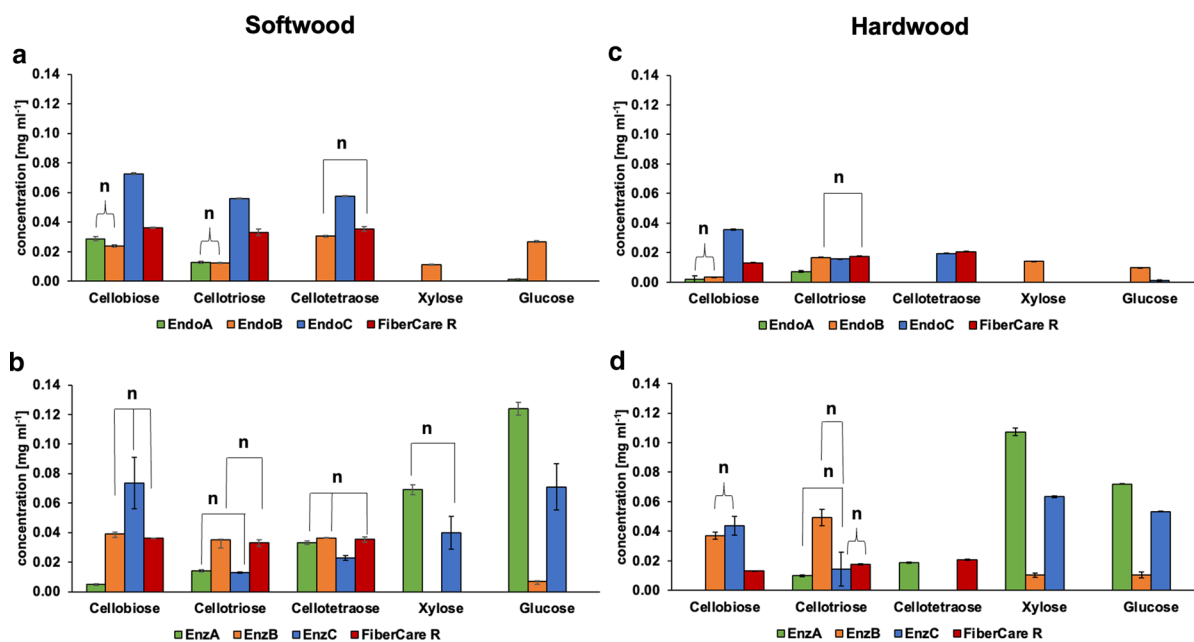


Fig. 2 Quantification (HPLC) of oligo- and monosaccharides released from softwood (**a**, **b**) and hardwood pulp by endoglucanases (**a**, **c**) and enzyme cocktails (**b**, **d**). Long fiber softwood or short fiber hardwood pulp were treated using 50% tap water + 50% deionized water at pH 7 (simulating refining conditions) at a dosage of 0.83 nkat at 45 °C for 4 h and sub-

sequently inactivated at 99 °C for 5 min. 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

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. 2c). 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 (Nagl et al. 2021). 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 cello-oligosaccharides (Chir et al. 2011; Rawat et al. 2015; Cano-Ramírez et al. 2016). 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 (Karlsson et al. 2002). 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. 2d). 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 (Parry et al. 2001; Ahmad Khairudin and Mazlan 2013). 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 celooligosaccharides 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 (Nagl et al. 2021).

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 (Singh et al. 2015; Buzala et al. 2016). 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 (Miller 1959). 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 (Jalak et al. 2012). 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 (Öhgren et al. 2007; Siqueira et al. 2017; Haske-Cornelius et al. 2020). 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 (Nagl et al. 2021). 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. S1 of the Online Resource.

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 (Wang et al. 2013;

Hidayat et al. 2015). On the other hand, labelled isolated carbohydrate binding modules can also be used to identify changes in crystallinity upon enzyme treatment (Li et al. 2018; Novy et al. 2019). 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 (Orłowski et al. 2015). 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 (Velikodvorskaya et al. 2013). 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 (Mclean et al. 2000; Moser et al. 2008; Vuong and Wilson 2010). 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 (Li et al. 2013; Sun et al. 2015; Ázar et al. 2019). 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 (Rabinovich et al. 2002; Iakiviak et al. 2016). 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. (Horikawa and Sugiyama 2009; Zhang et al. 2017).

CLSM images of the softwood pulp (Fig. 3a) 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. 3b). To confirm this assessment, a semi-quantitative analysis of the colour intensities was performed with ImageJ, the results are highlighted in Fig. 5. 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 (Nagl et al. 2021). 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 (Aksenov et al. 2020). Another factor for

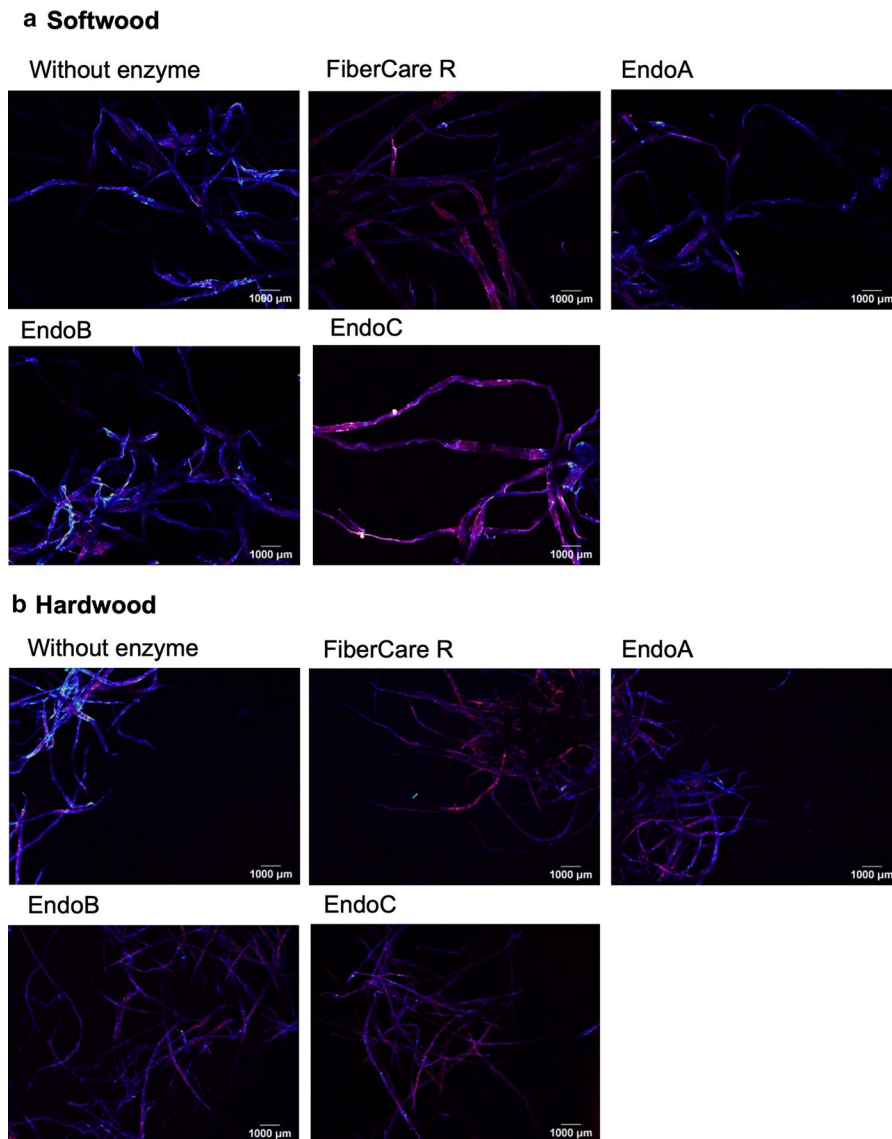


Fig. 3 CLSM images (10×magnification) on pulps incubated before and after endoglucanase treatment with two different carbohydrate binding modules for probing crystallinity changes. Duplicates of each combination of enzyme and pulp were measured. Specific binding of CBM 28 from *Caldicellulosiruptor bescii* 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 4 h in 50 mM citrate buffer (pH 4.8)

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 (Mussatto et al. 2008; Qing et al. 2010; Siqueira et al. 2017).

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. 4a). 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 (Nagl et al. 2021).

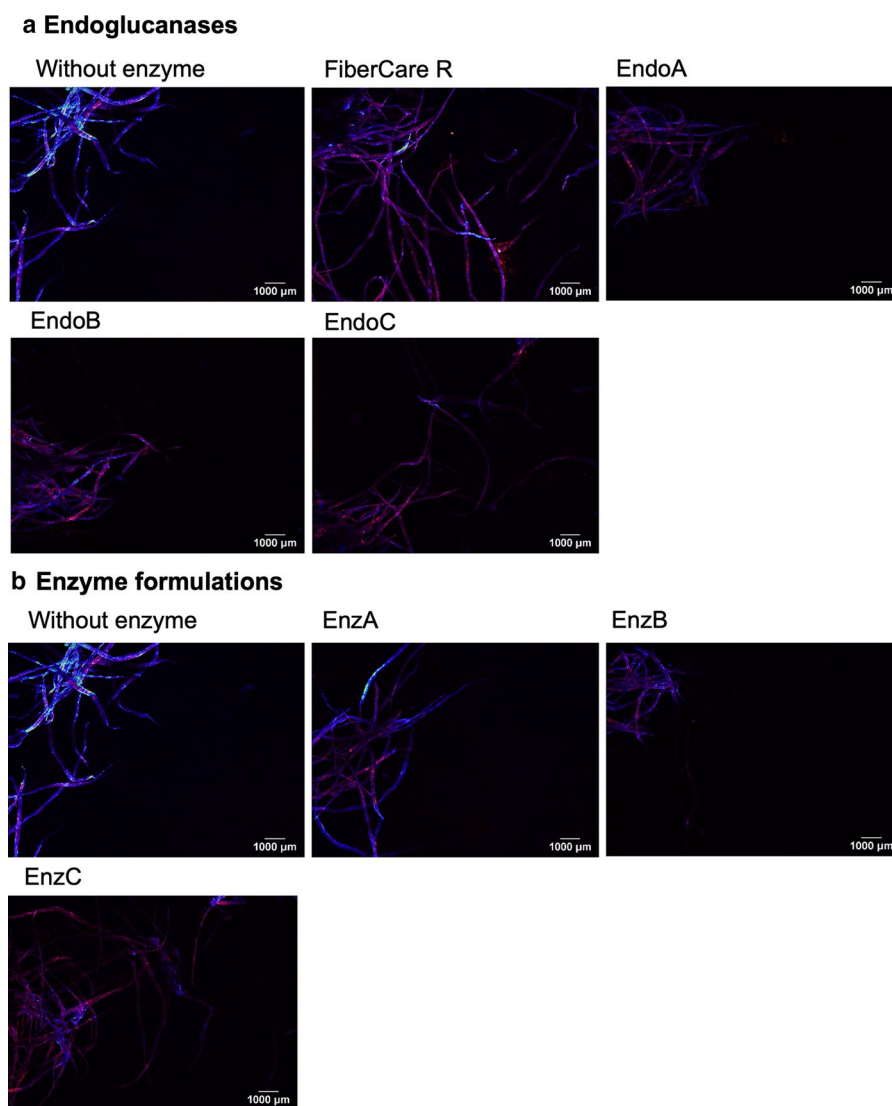


Fig. 4 CLSM images (10×magnification) on short fiber hardwood pulp incubated before and after endoglucanase treatment at refining conditions (50% tap water+50% deionized water at pH 7) with two different carbohydrate binding modules for probing crystallinity changes. Duplicates of each combination of 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

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. 4b). 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 (Nagl et al. 2021). 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. 5.

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. 5. 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

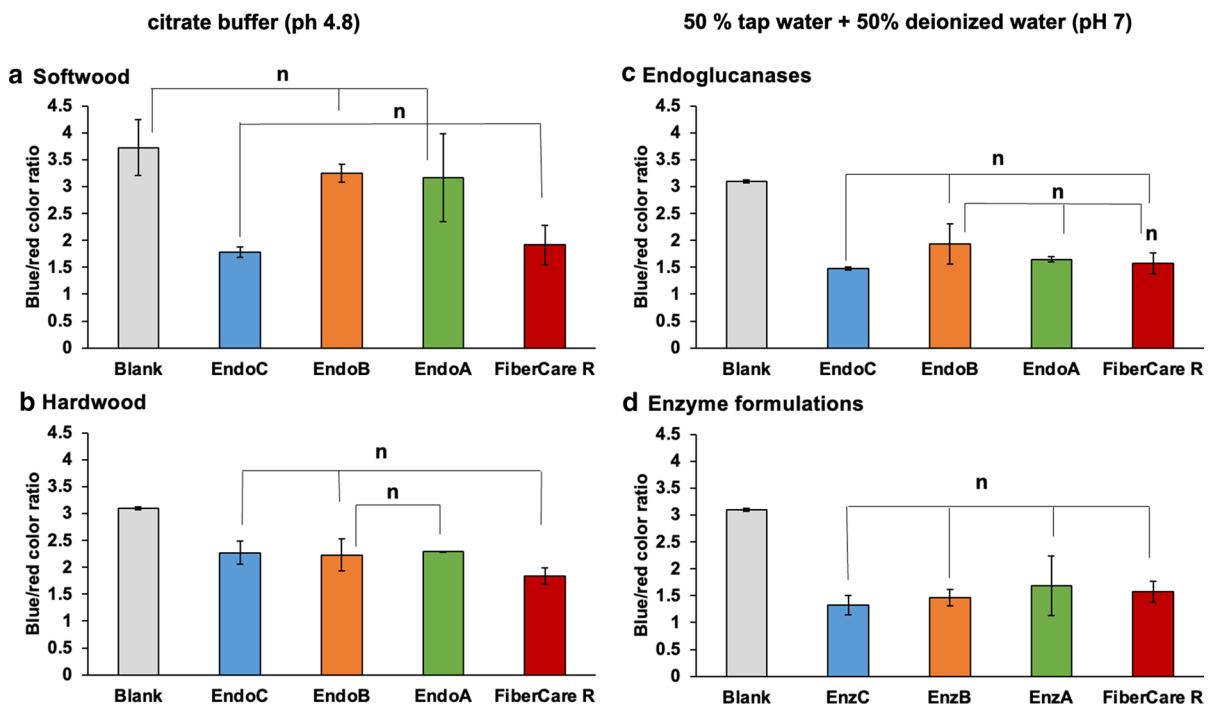


Fig. 5 Calculated blue/red colour ratios of the CLSM images after quantification of the blue and red colour intensities using ImageJ. The average ratios of the duplicate images were calculated, error bars indicate the standard deviation. **a** and **b** show the ratios of the purified endoglucanases (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

softwood pulp (Fig. 5a), 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. 5b) and 50% tap water+50% deionized water (Fig. 5c), 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. 5b), 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.

¹³C-NMR of enzyme treated pulps

Changes of the pulps properties upon enzyme treatment were investigated using ¹³C-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. 6a). 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 (Ioelovich et al. 2010; Sun et al. 2014). 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 monoxygenases 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 (Villares et al. 2017). 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

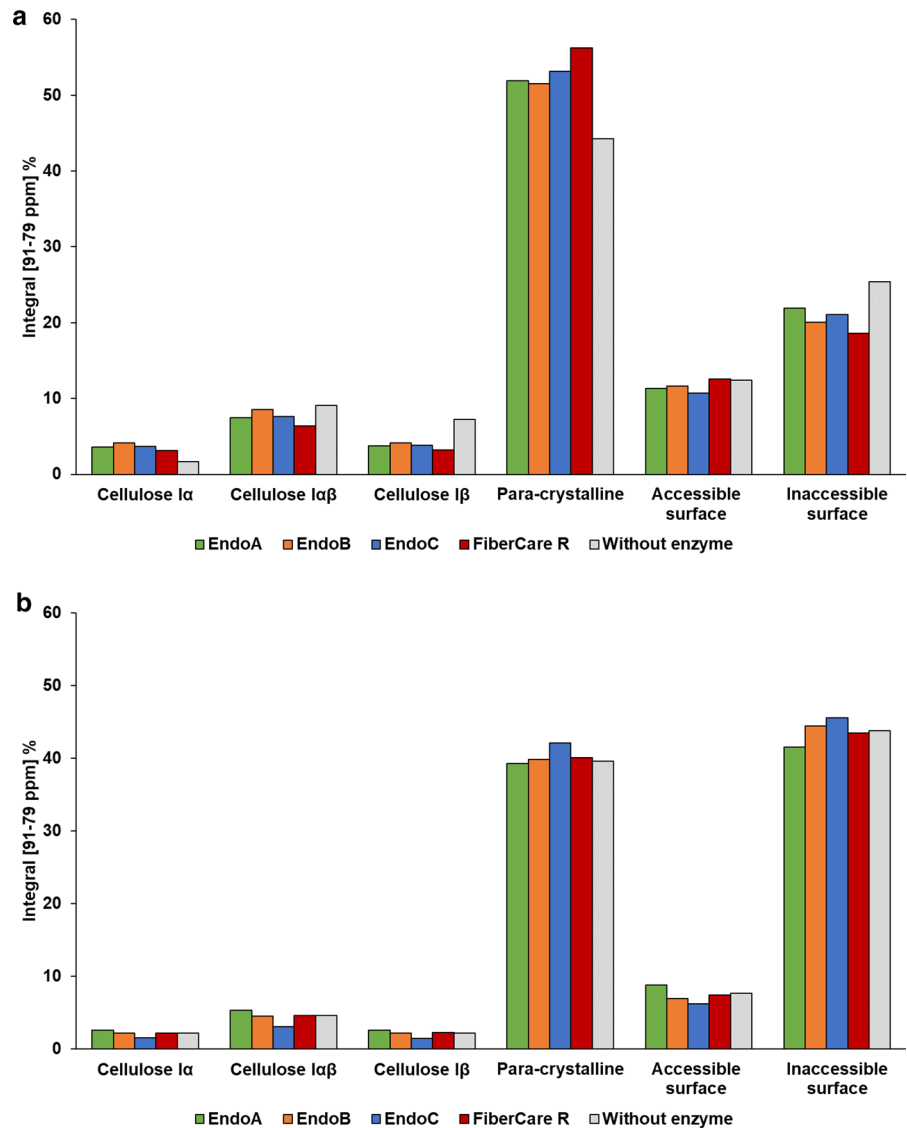


Fig. 6 ^{13}C -NMR analysis of long fiber softwood or short fiber hardwood pulp after treatment with purified endoglucanases for 4 h in 50 mM citrate buffer (pH 4.8). NMR measurements were performed without replicates. Differences in the amorphous and crystalline fractions caused by the purified endo-

glucanases 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

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 (Foston 2014; Gourlay et al. 2015; Rongpipi et al. 2019). 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 (Bali et al. 2016). 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 (Reyes-Ortiz et al. 2013; Bernardes et al. 2019). The theory that the packing of cellulose is weakened is also supported by the amount of cellulose I $\alpha\beta$, as this fraction was decreasing slightly for all endoglucanases, the highest decrease was caused by FiberCare R (–29.5%). Interestingly, the fraction of I α cellulose increased while the I β cellulose fraction decreased, although being the thermodynamically more stable form (Pu et al. 2006). 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 (Shoseyov et al. 2006; Szijártó et al. 2008; Contreras et al. 2020). 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 (Orłowski et al. 2015). A similar observation was made with endoglucanase III from *Trichoderma reesei*, disrupting the structure of crystalline cellulose through the breakage of hydrogen bonds (Xiao et al. 2001). 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. 6b). 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 (Arantes et al. 2014; Aksenov et al. 2020). 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 (Park et al. 2010; Novy et al. 2019). 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 (Nagl et al. 2021). This suggests a different mode of action of the endoglucanases, which might also be attributed to differences in the catalytic domain, as for example *Trichoderma reesei* exhibits at least 5 different endoglucanases, each showing distinct catalytic domains (Miettinen-Oinonen and Suominen 2002; Nakazawa et al. 2008). 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 $\alpha\beta$ 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 I α and I β 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.

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. ¹³C-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 endoglucanases correlate to a different extent of crystallinity changes and oligomers liberated from softwood and hardwood pulps.

Author contributions MN and LS performed the experiments and analysed the data. MN, OHC, AP, GMG and WB planned the experiments and participated in the conceptualization of the experiments. FB performed the formal analysis of the ¹³C-NMR data. GG and WB supervised the methodology. MN wrote the manuscript. AP, WB, GSN and GMG corrected the manuscript. The presented data was discussed by all authors prior to submission and all authors agreed to submit.

Funding Open access funding provided by University of Natural Resources and Life Sciences Vienna (BOKU). This work has been supported by the Austrian Research Promotion Agency (FFG) within the scope of the FibreZyme Project, the Government of Lower Austria and the BioMatInt doctoral school. The authors thank the participating companies of the Austrian pulp and paper industry for their scientific contribution.

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

Declarations

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

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

References

- Ahmad Khairudin N, Mazlan N (2013) Molecular docking study of beta-glucosidase with cellobiose, cellotetraose and cellotriose. *Bioinformation* 9(16):813–817. <https://doi.org/10.6026/97320630009813>
- Aksenov AS et al (2020) Biocatalysis of industrial kraft pulps: similarities and differences between hardwood and softwood pulps in hydrolysis by enzyme complex of penicillium verrucosum. *Catalysts*. <https://doi.org/10.3390/catal10050536>
- Arantes V, Gourelay K, Saddler JN (2014) The enzymatic hydrolysis of pretreated pulp fibers predominantly involves 'peeling/erosion' modes of action. *Biotechnol Biofuels* 7(1):1–10. <https://doi.org/10.1186/1754-6834-7-87>
- Ázar RISL et al (2019) Impact of protein blocking on enzymatic saccharification of bagasse from sugarcane clones. *Biotechnol Bioeng* 116(7):1584–1593. <https://doi.org/10.1002/bit.26962>
- Bali G et al (2016) Characterization of cellulose structure of Populus plants modified in candidate cellulose biosynthesis genes. *Biomass Bioenerg* 94:146–154. <https://doi.org/10.1016/j.biombioe.2016.08.013>

- Bernardes A et al (2019) Carbohydrate binding modules enhance cellulose enzymatic hydrolysis by increasing access of cellulases to the substrate. *Carbohydr Polym* 211:57–68. <https://doi.org/10.1016/j.carbpol.2019.01.108>
- Boraston AB et al (2004) Carbohydrate-binding modules: fine-tuning polysaccharide recognition. *Biochem J*. <https://doi.org/10.1042/BJ20040892>
- Buzala KP et al (2016) Effect of cellulases and xylanases on refining process and kraft pulp properties. *PLoS ONE* 11(8):1–14. <https://doi.org/10.1371/journal.pone.0161575>
- Cano-Ramírez C et al (2016) Expression, purification and characterization of an endoglucanase from *Serratia proteamaculans* CDBB-1961, isolated from the gut of *Dendroctonus adjunctus* (Coleoptera: Scolytinae). *AMB Express*. <https://doi.org/10.1186/s13568-016-0233-9>
- Carbohydrate-Active enZymes Database (2021) *Carbohydrate-Binding Module family classification*. Available at: <http://www.cazy.org/Carbohydrate-Binding-Modules.html> (Accessed: October 20, 2021)
- Chir JL et al (2011) Hydrolysis of cellulose in synergistic mixtures of β -glucosidase and endo/exocellulase Cel9A from *Thermobifida fusca*. *Biotech Lett* 33(4):777–782. <https://doi.org/10.1007/s10529-010-0500-9>
- Contreras F et al (2020) Engineering robust cellulases for tailored lignocellulosic degradation cocktails. *Int J Mol Sci*. <https://doi.org/10.3390/ijms21051589>
- Ding SY et al (2006) Versatile derivatives of carbohydrate-binding modules for imaging of complex carbohydrates approaching the molecular level of resolution. *Biotechniques* 41(4):435–443. <https://doi.org/10.2144/000112244>
- Emwas AHM (2015) The strengths and weaknesses of NMR spectroscopy and mass spectrometry with particular focus on metabolomics research. *Methods Mol Biol*. https://doi.org/10.1007/978-1-4939-2377-9_13
- Evans R et al (1995) Changes in cellulose crystallinity during kraft pulping: comparison of infrared, X-ray diffraction and solid state NMR results. *Holzforschung* 49(6):498–504. <https://doi.org/10.1515/hfsg.1995.49.6.498>
- Fleiter T et al (2012) Energy efficiency in the German pulp and paper industry—a model-based assessment of saving potentials. *Energy* 40(1):84–99. <https://doi.org/10.1016/j.energy.2012.02.025>
- Foston M (2014) Advances in solid-state NMR of cellulose. *Curr Opin Biotechnol*. <https://doi.org/10.1016/j.copbio.2014.02.002>
- Fox JM et al (2013) A single-molecule analysis reveals morphological targets for cellulase synergy. *Nat Chem Biol*. <https://doi.org/10.1038/nchembio.1227>
- García O et al (2002) Effect of cellulase-assisted refining on the properties of dried and never-dried eucalyptus pulp. *Cellulose* 9(2):115–125. <https://doi.org/10.1023/A:1020191622764>
- Gharehkhani S et al (2015) Basic effects of pulp refining on fiber properties—a review. *Carbohydr Polym* 115:785–803. <https://doi.org/10.1016/j.carbpol.2014.08.047>
- Ghio S et al (2020) Synergic activity of Cel8Pa β -1,4 endoglucanase and Bgl1Pa β -glucosidase from *Paenibacillus xylanivorans* A59 in beta-glucan conversion. *Biotechnol Rep* 28:1–9. <https://doi.org/10.1016/j.btre.2020.e00526>
- Gourlay K et al (2015) The use of carbohydrate binding modules (CBMs) to monitor changes in fragmentation and cellulose fiber surface morphology during cellulase- and swollenin-induced deconstruction of lignocellulosic substrates. *J Biol Chem* 290(5):2938–2945. <https://doi.org/10.1074/jbc.M114.627604>
- Häglund P et al (2003) A cellulose-binding module of the *Trichoderma reesei* β -mannanase Man5A increases the mannan-hydrolysis of complex substrates. *J Biotechnol* 101:37–48. [https://doi.org/10.1016/s0168-1656\(02\)00290-0](https://doi.org/10.1016/s0168-1656(02)00290-0)
- Hashimoto H (2006) Recent structural studies of carbohydrate-binding modules. *Cell Mol Life Sci*. <https://doi.org/10.1007/s00018-006-6195-3>
- Haske-Cornelius O et al (2020) Effects of enzymes on the refining of different pulps. *J Biotechnol* 320(June):1–10. <https://doi.org/10.1016/j.jbiotec.2020.06.006>
- Haslinger S et al (2019) Solid-state NMR method for the quantification of cellulose and polyester in textile blends. *Carbohydr Polym* 207:11–16. <https://doi.org/10.1016/j.carbpol.2018.11.052>
- Hettle A et al (2017) Properties of a family 56 carbohydrate-binding module and its role in the recognition and hydrolysis of β -1,3-glucan. *J Biol Chem* 292(41):16955–16968. <https://doi.org/10.1074/jbc.M117.806711>
- Hidayat BJ et al (2015) The binding of cellulase variants to dislocations: a semi-quantitative analysis based on CLSM (confocal laser scanning microscopy) images. *AMB Express* 5(1):1–14. <https://doi.org/10.1186/s13568-015-0165-9>
- Horikawa Y, Sugiyama J (2009) Localization of crystalline allomorphs in cellulose microfibril. *Biomacromol* 10(8):2235–2239. <https://doi.org/10.1021/bm900413k>
- Hua C et al (2018) Characterization of a novel thermostable GH7 endoglucanase from *Chaetomium thermophilum* capable of xylan hydrolysis. *Int J Biol Macromol* 117:342–349. <https://doi.org/10.1016/j.ijbiomac.2018.05.189>
- Iakiviak M et al (2016) Functional and modular analyses of diverse endoglucanases from *Ruminococcus albus* 8, a specialist plant cell wall degrading bacterium. *Sci Rep* 6(March):1–13. <https://doi.org/10.1038/srep29979>
- Igarashi K et al (2009) High speed atomic force microscopy visualizes processive movement of *Trichoderma reesei* cellobiohydrolase I on crystalline cellulose. *J Biol Chem* 284(52):36186–36190. <https://doi.org/10.1074/jbc.M109.034611>
- Ioelovich M, Leykin A, Figovsky O (2010) Study of cellulose paracrystallinity. *BioResources* 5(3):1393–1407. <https://doi.org/10.15376/biores.5.3.1393-1407>
- Jalak J et al (2012) Endo-exo synergism in cellulose hydrolysis revisited. *J Biol Chem* 287(34):28802–28815. <https://doi.org/10.1074/jbc.M112.381624>
- Kampuri T et al (2016) Characterization of endoglucanase rich *Trichoderma reesei* cellulase mixtures and their effect on alkaline solubility of dissolving pulp. *Cellulose* 23(6):3901–3911. <https://doi.org/10.1007/s10570-016-1055-2>
- Karita S (2016) Carbohydrate-binding modules in plant cell wall-degrading enzymes. *Trends Glycosci Glycotechnol*. <https://doi.org/10.4052/tigg.1403.1E>
- Karlsson J et al (2002) Enzymatic properties of the low molecular mass endoglucanases Cel12A (EG III) and Cel45A

- (EG V) of *Trichoderma reesei*. *J Biotechnol* 99:63–78. [https://doi.org/10.1016/s0168-1656\(02\)00156-6](https://doi.org/10.1016/s0168-1656(02)00156-6)
- Lecourt M et al (2010) Energy reduction of refining by cellulases. *Holzforschung* 64(4):441–446. <https://doi.org/10.1515/HF.2010.066>
- Li J et al (2013) A novel cost-effective technology to convert sucrose and homocelluloses in sweet sorghum stalks into ethanol. *Biotechnol Biofuels*. <https://doi.org/10.1186/1754-6834-6-174>
- Li T et al (2018) Visualizing cellulase adsorption and quantitatively determining cellulose accessibility with an updated fungal cellulose-binding module-based fluorescent probe protein. *Biotechnol Biofuels* 11(1):1–14. <https://doi.org/10.1186/s13068-018-1105-0>
- Liu J et al (2021) Carbohydrate-binding modules targeting branched polysaccharides: overcoming side-chain recalcitrance in a non-catalytic approach. *Bioresour Bioprocess*. <https://doi.org/10.1186/s40643-021-00381-7>
- Loosvelt, I. (2009) “Modifying the Quality of Fiber with Enzymes,” *Paper Age*, (September/October 2009), pp. 20–22
- Mangan D et al (2016) A novel automatable enzyme-coupled colorimetric assay for endo-1,4- β -glucanase (cellulase). *Anal Bioanal Chem* 408(15):4159–4168. <https://doi.org/10.1007/s00216-016-9507-y>
- Mansfield SD, Meder R (2003) Cellulose hydrolysis—the role of monocomponent cellulases in crystalline cellulose degradation. *Cellulose* 10:159–169
- Massiot D et al (2002) Modelling one- and two-dimensional solid-state NMR spectra. *Magn Reson Chem* 40(1):70–76. <https://doi.org/10.1002/mrc.984>
- Maunu S et al (2000) 13C CPMAS NMR investigations of cellulose polymorphs in different pulps. *Cellulose Polymers Mater* 7:147–159. <https://doi.org/10.1023/A:1009200609482>
- Mclean BW et al (2000) Analysis of binding of the family 2a carbohydrate-binding module from *Cellulomonas fimi* xylanase 10A to cellulose: specificity and identification of functionally important amino acid residues. *Protein Eng Des Sel* 13(11):801–809
- Miettinen-Oinonen A, Suominen P (2002) Enhanced production of *Trichoderma reesei* endoglucanases and use of the new cellulase preparations in producing the stone-washed effect on denim fabric. *Appl Environ Microbiol* 68(8):3956–3964. <https://doi.org/10.1128/AEM.68.8.3956-3964.2002>
- Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem* 31(3):426–428. <https://doi.org/10.1021/ac60147a030>
- Miotto LS et al (2014) The characterization of the endoglucanase Cel12A from *Gloeophyllum trabeum* reveals an enzyme highly active on β -glucan. *PLoS ONE*. <https://doi.org/10.1371/journal.pone.0108393>
- Moser F et al (2008) Regulation and characterization of thermobifida fusca carbohydrate-binding module proteins E7 and E8. *Biotechnol Bioeng* 100(6):1066–1077. <https://doi.org/10.1002/bit.21856>
- Mussatto SI et al (2008) Effect of hemicellulose and lignin on enzymatic hydrolysis of cellulose from brewer’s spent grain. *Enzyme Microb Technol* 43(2):124–129. <https://doi.org/10.1016/j.enzmictec.2007.11.006>
- Nagl M et al (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>
- Nakazawa H et al (2008) Characterization of the catalytic domains of *Trichoderma reesei* endoglucanase I, II, and III, expressed in *Escherichia coli*. *Appl Microbiol Biotechnol* 81(4):681–689. <https://doi.org/10.1007/s00253-008-1667-z>
- Novy V et al (2019) Quantifying cellulose accessibility during enzyme-mediated deconstruction using 2 fluorescence-tagged carbohydrate-binding modules. *Proc Natl Acad Sci USA* 116(45):22545–22551. <https://doi.org/10.1073/pnas.1912354116>
- Öhgren K et al (2007) Effect of hemicellulose and lignin removal on enzymatic hydrolysis of steam pretreated corn stover. *Biores Technol* 98(13):2503–2510. <https://doi.org/10.1016/j.biortech.2006.09.003>
- Orłowski A et al (2015) How endoglucanase enzymes act on cellulose nanofibrils: role of amorphous regions revealed by atomistic simulations. *Cellulose* 22(5):2911–2925. <https://doi.org/10.1007/s10570-015-0705-0>
- Park S et al (2010) Cellulose crystallinity index: Measurement techniques and their impact on interpreting cellulase performance. *Biotechnol Biofuels* 3(1):1–14. <https://doi.org/10.1186/1754-6834-3-10>
- Parry NJ et al (2001) Biochemical characterization and mechanism of action of a thermostable β -glucosidase purified from *Thermoascus aurantiacus*. *Biochem J* 353(1):117–127. <https://doi.org/10.1042/bj3530117>
- Pu Y, Ziemer C, Ragauskas AJ (2006) CP/MAS 13C NMR analysis of cellulase treated bleached softwood kraft pulp. *Carbohydr Res* 341(5):591–597. <https://doi.org/10.1016/j.carres.2005.12.012>
- Qing Q, Yang B, Wyman CE (2010) Xylooligomers are strong inhibitors of cellulose hydrolysis by enzymes. *Biores Technol* 101(24):9624–9630. <https://doi.org/10.1016/j.biortech.2010.06.137>
- Rabinovich ML, Melnick MS, Bolobova AV (2002) The structure and mechanism of action of cellulolytic enzymes. *Biochem Mosc* 67(8):850–871. <https://doi.org/10.1023/A:1019958419032>
- Rawat R et al (2015) An acidothermophilic functionally active novel GH12 family endoglucanase from *Aspergillus niger* HO: purification, characterization and molecular interaction studies. *Antonie Van Leeuwenhoek, Int J Gen Mol Microbiol* 107(1):103–117. <https://doi.org/10.1007/s10482-014-0308-z>
- Reyes-Ortiz V et al (2013) Addition of a carbohydrate-binding module enhances cellulase penetration into cellulose substrates. *Biotechnol Biofuels*. <https://doi.org/10.1186/1754-6834-6-93>
- Reza H et al (2019) Mechanisms of strength and stiffness improvement of paper after PFI refining with a focus on the effect of fines. *Cellulose* 26(6):4099–4124. <https://doi.org/10.1007/s10570-019-02349-5>
- Ribitsch D et al (2013) Fusion of binding domains to *Thermobifida cellulolytica* cutinase to tune sorption characteristics and enhancing PET hydrolysis. *Biomacromol* 14(6):1769–1776. <https://doi.org/10.1021/bm400140u>
- Rongpipi S et al (2019) Progress and opportunities in the characterization of cellulose—an important regulator

- of cell wall growth and mechanics. *Front Plant Sci* 9(March):1–28. <https://doi.org/10.3389/fpls.2018.01894>
- Shoseyov O, Shani Z, Levy I (2006) Carbohydrate binding modules: biochemical properties and novel applications. *Microbiol Mol Biol Rev* 70(2):283–295. <https://doi.org/10.1128/MMBR.00028-05>
- Singh R, Bhardwaj NK, Choudhury B (2015) Cellulase-assisted refining optimization for saving electrical energy demand and pulp quality evaluation. *J Sci Ind Res* 74(8):471–475
- Siqueira G, Arantes V, Saddler JN et al (2017) Limitation of cellulose accessibility and unproductive binding of cellulases by pretreated sugarcane bagasse lignin. *Biotechnol Biofuels*. <https://doi.org/10.1186/s13068-017-0860-7>
- Song HT et al (2016) Synergistic effect of cellulase and xylanase during hydrolysis of natural lignocellulosic substrates. *Biores Technol* 219:710–715. <https://doi.org/10.1016/j.biortech.2016.08.035>
- Sun Q et al (2014) Effect of lignin content on changes occurring in poplar cellulose ultrastructure during dilute acid pretreatment. *Biotechnol Biofuels* 7(1):1–14. <https://doi.org/10.1186/s13068-014-0150-6>
- Sun FF et al (2015) Accessory enzymes influence cellulase hydrolysis of the model substrate and the realistic lignocellulosic biomass. *Enzym Microb Technol* 79–80:42–48. <https://doi.org/10.1016/j.enzmictec.2015.06.020>
- Szjártó N et al (2008) Hydrolysis of amorphous and crystalline cellulose by heterologously produced cellulases of *Melanocarpus albomyces*. *J Biotechnol* 136(3–4):140–147. <https://doi.org/10.1016/j.jbiotec.2008.05.010>
- Torres CE et al (2012) Enzymatic approaches in paper industry for pulp refining and biofilm control. *Appl Microbiol Biotechnol* 96(2):327–344. <https://doi.org/10.1007/s00253-012-4345-0>
- Tripathi S et al (2008) Enzymatic refining of chemical pulp. *IPPTA Q J Indian Pulp Pap Tech Assoc* 20(3):129–132
- Tripathi S, Verma P et al (2019) Reduction in refining energy and improvement in pulp freeness through enzymatic treatment—lab and plant scale studies. *J Sci Ind Res (JSIR)* 78(01):50–54
- Velikodvorskaia GA et al (2013) The family 28 carbohydrate-binding module of the thermostable endo-1,4-beta-glucanase CelD *Caldicellulosiruptor bescii* maximizes the enzyme's activity and binds irreversibly to amorphous cellulose. *Molecular Biology*. 47(4):581–586. <https://doi.org/10.7868/s0026898413040150>
- Villares A et al (2017) Lytic polysaccharide monoxygenases disrupt the cellulose fibers structure. *Sci Rep* 7(January):1–9. <https://doi.org/10.1038/srep40262>
- Vlasenko E et al (2010) Substrate specificity of family 5, 6, 7, 9, 12, and 45 endoglucanases. *Biores Technol* 101(7):2405–2411. <https://doi.org/10.1016/j.biortech.2009.11.057>
- Vuong TV, Wilson DB, Master E (2010) Engineering *Thermobifida Fusca* cellulases: Catalytic mechanisms and improved activity, in Mallorie N. Sheehan (ed.) *Protein Engineering: Design, Selection and Applications*. Nova Science Publishers, Inc, pp 29–52
- Wang H et al (2013) Effect of bovine serum albumin (BSA) on enzymatic cellulose hydrolysis. *Appl Biochem Biotechnol* 170(3):541–551. <https://doi.org/10.1007/s12010-013-0208-0>
- Weber J et al (2019) Interaction of carbohydrate-binding modules with poly(ethylene terephthalate). *Appl Microbiol Biotechnol* 103(12):4801–4812. <https://doi.org/10.1007/s00253-019-09760-9>
- Wickholm K, Larsson T, Iversen T (1998) Assignment of non-crystalline forms in cellulose I by CP/MAS 13 C NMR spectroscopy. *Carbohydr Res* 312:123–129. [https://doi.org/10.1016/S0008-6215\(98\)00236-5](https://doi.org/10.1016/S0008-6215(98)00236-5)
- Xiao Z et al (2001) Cellulose-binding domain of endoglucanase III from *trichoderma reesei* disrupting the structure of cellulose. *Biotech Lett* 23(9):711–715. <https://doi.org/10.1023/A:1010325122851>
- Zhang L et al (2017) Discovery of cellulose surface layer conformation by nonlinear vibrational spectroscopy. *Sci Rep* 7:1–7. <https://doi.org/10.1038/srep44319>
- Zhang Z et al (2017) Synergistic effect of thermostable β -glucosidase TN0602 and cellulase on cellulose hydrolysis. *3 Biotech* 7(1):1–7. <https://doi.org/10.1007/s13205-017-0672-2>
- Zuckerstätter G et al (2009) The elucidation of cellulose supramolecular structure by 13C CP-MAS NMR. *Lenzinger Berichte* 87:38–46

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.