Biorefining: the role of endoglucanases in refining of cellulose fibers

Martin Nagl · Oskar Haske-Cornelius · Lukas Skopek · Alessandro Pellis · Wolfgang Bauer · Gibson Stephen Nyanhongo · Georg Guebitz

Received: 11 April 2021 / Accepted: 14 June 2021 / Published online: 24 June 2021 © The Author(s) 2021

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\(^{-1}\)-13.7 nkat mg\(^{-1}\)), \(\beta\)-glucosidase (0.57 nkat mg\(^{-1}\)-1.34 nkat mg\(^{-1}\)) and xylanase activities (1.78 nkat ml\(^{-1}\)-62.1 nkat mg\(^{-1}\)) on model substrates. Additionally, distinct amounts of reducing sugars from hardwood sulfate pulp were released. Endoglucanases were purified from each formulation by using hydrophobic interaction and anion exchange chromatography and showed molecular weights from 20 to 55 kDa and specific activities ranging between 3.11 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 \[^{\circ}\text{SR}\] 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

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 Iα and Iβ (Zhao et al., 2007). 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 (Lecourt et al. 2010; K. Przybysz et al. 2016). Enzymes are emerging as ideal tools for cellulose fiber refining leading to a reduction of energy consumption of up to 40% (Fleiter et al. 2012; Torres et al., 2012). However, many studies have shown that different commercial formulations perform differently and effects are difficult to predict (Haske-Cornelius et al. 2020).

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 (Dimarogona et al. 2012). 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 (Jalak et al. 2012; Singhania et al. 2013). 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 (Delgado-Aguilar et al. 2015). Moreover, endoglucanases lead to the reduction of cellulose chain length, but the lateral hierarchical structure of the wood fibers is unaffected (Qin et al. 2016).

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 (Oksanen et al. 2000; S. Tripathi 2019). Today, a variety of enzymes preparations have been assessed for refining containing (besides cellulolytic enzymes) biocatalysts having other activities such as xylanase or laccases (Pelletier et al. 2013; Singh 2016). 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 hemicellulloses (Przybysz Buzala et al. 2018). Laccases aid the refining process though degradation of lignin and increasing the amount of carboxyl groups, thus leading to increased bonding of fibres (Lin et al., 2018). 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 (Confederation of European Paper Industries (CEPI), 2019), 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).
Materials and methods

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.

Enzyme activity assays

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 (Mangan et al. 2016). Dilutions were performed in 50 mM citrate buffer (pH 4.8) or 50% tap water + 50% ddH2O (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 min 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.

Endoglucanase activity on carboxymethylcellulose

Endoglucanase activity was also measured on carboxymethylcellulose (CMC) similar to measurements performed by Liu (G. Liu et al. 2006). 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% ddH2O (pH 7). The assay is based on the reducing sugar determination with colour formation by dinitrosalicylic acid (Miller 1958). 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 min 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 min 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 to 20 mM was measured. Activities were measured in duplicates and expressed as nkat ml⁻¹.

β-Glucosidase activity

β-Glucosidase activity was measured according to Parry (Parry et al., 2001) 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% ddH2O (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 min 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 NaPO₄ 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 to 0.2 mM. The β-glucosidase activity was calculated in nkat ml\(^{-1}\). Measurements were performed in duplicates and error bars indicate standard deviation.

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 (Mangan et al., 2017). 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 min 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\(_{2}\)O (pH 7). Xylanase activity was calculated in nkat ml\(^{-1}\). Measurements were performed in duplicates and error bars indicate standard deviation.

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 (Ghose T.K, 1987). Stripes of 0.75 × 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) or 50% tap water + 50% ddH\(_{2}\)O (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 min 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 min. 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 to 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\(^{-1}\).

Cellobiohydrolase I activity

Cellobiohydrolase I cleaves cellulose from reducing ends of the cellulose chain and its activity was determined using 4-nitrophenyl cellulobioside as substrate. The measurement principle was described by Percival Zhang (Percival Zhang et al., 2006). A solution containing 2 mM 4-nitrophenyl cellulobioside in 50 mM citrate buffer (pH 4.8) was prepared. Additionally, 100 mM D-glucono-1,5-lactone to suppress 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 min 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 to 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.

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 min. 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 min.
Absorbance was measured at 540 nm using an Infinite 200 Pro Tecan Reader (Zurich, Switzerland). A glucose standard curve ranging from 0 to 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$^{-1}$.

**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$_4$)$_2$SO$_4$, 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 min. 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.

**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.

**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$^{-1}$]).

**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 (Laemmli 1970). A volume of 5 μl of 5 × 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 min 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 min. Afterwards the gel was stained in Coomassie staining solution (ddH$_2$O, acetic acid, ethanol in the ratio 6:3:1 with 0.125% Coomassie G-250) for 1 h and 3 times in destaining solution (ddH$_2$O, acetic acid, ethanol in the ratio 6:3:1) for 1 h. Image acquisition of the stained gel was performed using the Biorad ChemiDoc (Hercules, California).

**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 disintegrated for 10 min 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 min and inactivated using 30% H$_2$O$_2$. 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 16,065–2 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) und air permeance Gurley (ISO 5636–5:2013) were determined after conditioning the samples for at least 24 h 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 10,500–15,000 fibers were analyzed. Error bars indicate the standard deviation.

**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 1000 × magnification and are available in Fig. S3, Fig. S4, Fig. S5 and Fig. S6 of the Online Resource.

**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 (Lee et al. 2007) 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 min. 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.

Results and discussion

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 (Pere et al. 1997). 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. 1). 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 (McCleary et al. 2014) when compared to CMC which can also be cleaved by cellobiohydrolases or even xylanases (Gilkes et al. 1997; Wang et al. 2019). 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 monoxygenases (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 (Kolev et al. 1991; Mboowa et al. 2020).

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 (Deshpande et al. 1984) 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 (Kou et al. 2014), 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 (Pastor et al. 2003). 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 (Suchy et al. 2009). The activity of β-glucosidase differed greatly between the investigated enzyme formulations (0.57–1.3 nkat mg⁻¹). β-Glucosidase activity may be necessary to avoid product inhibition of endoglucanases by accumulation of cellobiose and cellotriose (Teugjas and Väljamäe, 2013). 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 negligible (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 (Tao et al. 2019). 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 (Miletzky et al. 2015; K. Przybysz et al. 2016; Roberts et al. 1990).

Determined volumetric activities in nkat mL\(^{-1}\) of the enzyme formulations are listed in Table S1 of the Online Resource.

**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 \(\beta\)-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 (Lecourt et al. 2010). 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 (Karnchanatat et al. 2008). 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 (Claeyssens and Henrissat 1992). 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 (Tsumoto et al. 2007). The purified enzymes had a similar protein content ranging between 7.1 and 10.9 mg ml\(^{-1}\) (Fig. 2).

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. 2). 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 (Dave et al. 2015; Messner et al. 1988; Miotto et al. 2014). The SDS-PAGE pictures of the enzyme formulations are shown in Fig. S1 of the Online Resource.

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. 3). The difference in activities was more pronounced on CMC as substrate (Fig. 3), 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 (Chang et al. 2011; Tan et al. 2018). 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 (Coward-kelly et al., n.d.). 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 S1 of the Online Resource.

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 s. 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 fastest 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 (Søren Hvidt 2016). 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 (J. Liu and Hu 2012).

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 (Fig. 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 S2 of the Online Resource.

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. 5). 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 (Mboowa 2021). Lignin and hemicelluloses can affect cellulase accessibility (Baig 2016). For example, one study showed an increase of cellulase accessibility on sugarcane bagasse after lignin removal (Siqueira et al. 2017). Haske-Cornelius already showed varying content of hemicelluloses between different pulps using NMR, although a different short fibre pulp was used in our study (Haske-Cornelius et al. 2020). 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.

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. 6a). 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. 6. Additionally, fibril area and air resistance according to Gurley was determined (shown in Fig. S2 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 (Reza et al. 2019). 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 (Ghosh et al. 2018). 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 \(\mu\)m for the control and decreased to values between 794 and 766 \(\mu\)m in the presence of enzymes (Fig. 6b). Fibre shortening can have negative impacts on the tensile strength and on tear index (P. Przybysz et al. 2020). 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. 6d). 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 a decrease at higher refining stages (Garcia et al. 2002). 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 and 0.835 mmol s\(^{-1}\) (Fig. 6c) and even more for the purified endoglucanases with values between 7.51\(\times\)10\(^{-3}\) and 0.450 mmol s\(^{-1}\) (Fig. 6h). Moreover, the enzyme causing the lowest release of reducing sugars lead to the highest degree of refining (Fig. 6e). 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 (Pere et al. 1997). 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 (Gil et al. 2009; Wu et al. 2020).

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. 6f). 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. 6h). 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 (Edgar et al. 1998). Regarding fibre length, the reduction was expectedly less pronounced with the pure enzyme when compared to the enzyme formulations (Fig. 6g). 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 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 mNm² g⁻¹, EnzC: 7.41 mNm² g⁻¹), suggesting additional enzyme components in EnzC affecting the tear index negatively.

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.

Author contributions MN and LS performed the experiments. MN, OH, AP, GG and WB planned the experiments and analysed the data. GG and WB supervised the work. MN wrote the manuscript. OH, AP, WB, GSN and GG 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 and the Government of Lower Austria. The authors thank the participating companies of the pulp and paper industry of Austria for their scientific contribution.

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

Declarations

Conflict of interest The authors have no conflicts 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

Baig KS (2016) Adsorption of cellulase enzymes on lignocel-lulosic materials and influencing factors: a review. Int J Waste Resource 6(3):27–31. https://doi.org/10.4172/2252-
5211.1000239

Chang L, Ding M, Bao L, Chen Y, Zhou J, Lu H (2011) Char-acterization of a bifunctional xylanase/endoglucanase from yak rumen microorganisms. Appl Microbiol Biotechnol 90(6):1933–1942. https://doi.org/10.1007/s00253-011-3182-x

Claeyssens M, Henrisat B (1992) Specificity mapping of cel-lulolytic enzymes: classification into families of structurally related proteins confirmed by biochemical analysis. Protein Sci 1(10):1293–1297. https://doi.org/10.1002/pro.5560011008

Confederation of European Paper Industries (CEPI). (2019). Key Statistics 2019 European pulp & paper industry

Coward-kelly G, Aiello-mazzari C, Kim S, Granda C, & Claeyssens M, Henrissat B (1992) Specificity mapping of cel-lulolytic enzymes: classification into families of structurally related proteins confirmed by biochemical analysis. Protein Sci 1(10):1293–1297. https://doi.org/10.1002/pro.5560011008

Confederation of European Paper Industries (CEPI). (2019). Key Statistics 2019 European pulp & paper industry

Coward-kelly G, Aiello-mazzari C, Kim S, Granda C, & Holtzapple M (n.d.). Suggested Improvements to the Standard Filter Paper Assay Used to Measure Cellulase Activity. https://doi.org/10.1002/bit.10620

Dave BR, Sudhir AP, Subramanian RB (2015) Purification and properties of an endoglucanase from Thermoascus aurantiacus. Biotechnol Reports 6:85–90. https://doi.org/10.1016/j.btre.2014.11.004

Delgado-Aguilar M, Tarres Q, Puig J, Blanco A, Mutjé P (2015) Enzymatic refining and cellulose nanofiber addition in papermaking processes from recycled and deinked slurries. BioResources 10(3):5730–5743

Deshpande MV, Eriksson KE, Göran Pettersson L (1984) An enzymatic assay for endo-1,4-β-glucanase in a mixture of cellulolytic enzymes. Anal Biochem 138(2):481–487. https://doi.org/10.1016/0003-2697(84)90843-1

Dimarogona M, Topakas E, Christakopoulos P (2012) Cellulose degradation by oxidative enzymes. Comput Struct Biotechnol J 2(3):e201209015. https://doi.org/10.5936/csbj.201209015

Edgar CD, Mansfield SD, Gübitz GM, Saddler JN (1998) The synergistic effects of endoglucanase and xylanase in modifying Douglas fir kraft pulp. ACS Symp Ser 687:75–87. https://doi.org/10.1021/bk-1998-0687.ch007

Fleiter T, Fehrenbach D, Worrell E, Eichhammer W (2012) Energy efficiency in the German pulp and paper industry e A model-based assessment of saving potentials. Energy 37(4):146–155. https://doi.org/10.1016/j.energy.2012.02.025

Garab S, Offermanns B, Streithoffer B, & Heitzmann G (2007) Enzymatic refining of different pulps. J Biotechnol 114(1):359–365. https://doi.org/10.1016/j.jbiotec.2010.06.009

Ghose TK (1987) Measurement of cellulase activities. Pure App Chem 59(2):257–268

Ghosh A, Thornton B, & Hart PW (2018) Effect of pH and enzymes on strength of recycled fibers during refining, TAPPI JOURNAL July 2018. July. https://doi.org/10.32964/TJ17.07.047

Gilkes NR, Kwan E, Kilburn DG, Warren RAJ, Miller C (1997) Attack of carboxymethylcellulose at opposite ends by two cellulbiohydrolases from celhomonas fimii. J Biotechnol 57:83–90

Gil N, Gil C, Amaral ME, Costa AP, Duarte AP (2009) Use of enzymes to improve the refining of a bleached Eucalyptus globulus Kraft pulp. Biochem Eng J 46(2):89–95. https://doi.org/10.1016/j.bej.2009.04.011

Haske-Cornelius O, Hartmann A, Brunner F, Pellis A, Bauer W, Nyanhongo GS, Guebitz GM (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

Jalak J, Kuraśin M, Teuggas H, Väljamä P (2012) Endo-exo synergism in cellulose hydrolysis revisited. J Biol Chem 287(34):28802–28815. https://doi.org/10.1074/jbc.M112.381624

Karnchanatat A, Petsom A, Sangvanich P, Piapukiew J, Whalley AJ, Reynolds CD, Gadd GM, & Sihanonth P (2008) A novel thermostable endoglucanase from the wood-decaying fungus Daldinia eschscholzii (Ehrenb: Fr ) Rehm. 42, 404–413. https://doi.org/10.1016/j.enzmitec.2007.11.009

Kolev D, Witte K, Wartenberg A (1991) Synergistic cooperation of cellulosases of Trichoderma reesei as distinguished by a new filter-paper destruction assay. Acta Biotechnol 11(4):359–365. https://doi.org/10.1007/90.0710114

Kou Y, Xu J, Cao Y, Lv X, Zhao G, Chen G, Zhang W, Liu W (2014) Gluconolactone induces cellulase gene expression in cellulolytic filamentous fungus Trichoderma reesei. RSC Adv 4(68):36057–36063. https://doi.org/10.1039/c4ra06731b

Laemmli UK (1970) Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature 227:680–685

Lee JM, Heitmann JA, Pawlak JJ (2007) Rheology of carboxymethyl cellulose solutions treated with cellulases. BioResources 2(1):20–33

Lin X, Wu Z, Zhang C, Liu S, Nie S (2018) Enzymatic pulping of lignocellulosic biomass. Ind Crops Prod 120(April):16–24. https://doi.org/10.1016/j.indcrop.2018.04.033

Liu J, & Hu H (2012) The role of cellulose binding domains in the adsorption of cellulases onto fibers and its effect on the enzymatic beating of bleached Kraft pulp. BioResources, 7(1), 878–892. https://doi.org/10.1537/biores.7.1.0878-0892

Liu G, Tang AX, Deng X, & Xing AM (2006) Improvement of the cellulolytic activity of Trichoderma reesei endoglucanase IV with an additional catalytic domain. 1301–1305. https://doi.org/10.1016/s11274-006-9176-7

Mangan D, Cornaggia C, Mckie VA, Kargelis T, Mccleary BV, Mccormack N, Ivory R, Mccleary BV, & Ivory R (2017) Novel suberin-degrading endo-1,4-β-glucanase. Bioanal Chem. https://doi.org/10.1007/s00216-016-9507-y

Mangan D, Cornaggia C, Liadova A, Mccormack N, Ivory R, Mckie VA, Ormerod A, Mccleary BV (2017) Novel sub-strates for the automated and manual assay of endo-1, 4-β-glucanase . Anal Bioanal Chem. 57:83–90. https://doi.org/10.1016/j.carres.2017.02.009

Mangas D, Cornaggia C, Liadova A, Mccormack N, Ivory R, Mckie VA, Ormerod A, Mccleary BV (2017) Novel sub-
Mboowa, D. (2021) A review of the traditional pulping methods and the recent improvements in the pulping processes.

Mboowa, D., Chandra, R.P., Hu, J., Saddler, J.N. (2020) Substrate characteristics that influence the filter paper assay’s ability to predict the hydrolytic potential of cellulose mixtures. ACS Sustain Chem Eng 8(28):10521–10528. https://doi.org/10.1021/acssuschemeng.0c02883

McCleary, B.V., Mangan, D., Daly, R., Fort, S., Ivory, R., McCormack N. (2014) Novel substrates for the measurement of endo-1,4-β-glucanase (endo-cellulase). Carbohydr Res 385:9–17. https://doi.org/10.1016/j.carres.2013.12.001

Messner, R., Gruber, F., Kubicke, C.P. (1988) Differential Regulation of Synthesis of Multiple Forms of Specific Endoglucanases by Trichoderma reesei QM9414. 170(8):3689–3693

Miletzky, A., Fischer, W.J., Czibula, C., Teichert, C., Bauer, W. (1999) Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. III. 426–428. doi.org/10.1021/ac60147a030

Miettinen, L.S., De Rezende, C.A., Bernardes, A., Serpa, V.I., Tsang, A., & Polikarpov, I. (2014) The characterization of the endoglucanase Cel12A from Gloeoplylum trabeum reveals an enzyme highly active on β-glucan. PLoS ONE, 9(9). https://doi.org/10.1371/journal.pone.0108393

Oksanen, T., Pere, J., Paavilainen, L., Buchert, J., & Viikari, L. (2000) Treatment of recycled kraft pulps with Trichoderma reesei hemicellulases and cellulases. 78, 39–48

Parry, N.J., Beever, D.E., Owen, E., Vandenberghe, I., Becu- men, J. V. A. N., & Bhat, M. K. (2001) β-glucosidase purified from Thermosascus aurantiacus. 127, 117–127

Pastor, F.I., Diaz, P., & Sa MM. (2003) Exo-mode of action of cellulbiohydrolase Cel48C from Paenibacillus sp. BP-23. 2919, 2913–2919. https://doi.org/10.1046/j.1432-1033.2003.03673.x

Pelletier, A., Zhao, Yu., Xiao Chun, Lei KL. (2013) Improved fiber separation and energy reduction in thermomechanical pulping refining using enzyme-pretreated wood. BioResource 8: 3385–3398

Percival, Zhang Y.H., Himmel ME., Mielenz JR. (2006) Outlook for cellulase improvement: screening and selection strate- gies. Biotechnol Adv 24(5):452–481. https://doi.org/10.1016/j.biotechadv.2006.03.003

Perez, J., Buchert, J., & Viikari, L. (1997) The effect of Tricho- derma reesei cellulases and hemicellulases on the paper technical properties of never-dried bleached kraft pulp

Przybysh, Buzkała K., Kalinowska H., Borkowski J., Przybysh P. (2018) Effect of xylanases on refining process and kraft pulp properties. Cellulose 25(2):1319–1328. https://doi.org/10.1007/s10570-017-1607-y

Przybysh P., Dubowski M., Malachowska E., Kucner M. (2020) The effect of the refining intensity on the progress of internal fibrillation and shortening of cellulose fibers. BioResources 15(1):1482–1499

Przybysh K., Przybysh P., Kalinowska H. (2016) Effect of cellulases and xylanases on refining process and kraft pulp properties. PLoS ONE. https://doi.org/10.1371/journal.pone.0161575

Qin, Y., Qiu, X., Zhu, J.Y. (2016) Understanding longitudinal wood fiber ultra-structure for producing cellulose nanofibrils using disk milling with diluted acid prehydrolysis. Sci Rep 6(May):1–12. https://doi.org/10.1038/srep35602

Reza, H., Armim, M., Artem, E.H., Fines, S.A., Fiber, A.D.A. (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

Roberts J.C., McCarthy, A.J., Flynn, N.J., Broda P. (1990) Modification of paper properties by the pretreatment of pulp with Saccharomonospora viridis xylanase. Enzyme Microbiol Technol 12(3):210–213. https://doi.org/10.1016/0141-0229(90)90040-W

Singh G. (2016) Enzymes: applications in pulp and paper industry. Agro-Industrial Wastes as Feedstock for Enzyme Product. https://doi.org/10.1016/B978-0-12-802392-1.00007-1

Singhaniya RR, Patel AK, Sukumaran RK, Larroche C, Pandey A. (2013) Role and significance of beta-glucosidases in the hydrolysis of cellulose for bioethanol production. Biorec. Technol 127:500–507. https://doi.org/10.1016/j.biortech.2012.09.012

Siqueira, G., Arantes, V., Saddler J.N., Ferraz A., Milagres AMF. (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

Søren, Hvidt. (2016). Rapid Discrimination of Endo- and Exo-cellulases. Transactions of the Nordic Rheology Society, 24

Suchy, M., Hakala T., Kangas H., Tammelin T., & Pursula T. (2009) Effects of commercial cellobiohydrolase treatment on fiber. 63(1999), 731–736. https://doi.org/10.1515/HF.2009.104

Tan, H., Miao, R., Liu, T., Yang, L., Yang, Y., Chen, C., Lei, J., Li, Y., He, J., Sun, Q., Peng, W., Gan, B., Huang, Z. (2018) A bifunctional cellulase–xylanase of a new Chryseobacterium strain isolated from the dung of a straw-fed cattle. Microb Biotechnol 11(2):381–398. https://doi.org/10.1111/1751-7915.13034

Tao, W., Guo, L., Meng, A., Wang, L., Ren, H., Zhai, H. (2019) Effects of xylanase pretreatment on the quality of refiner mechanical mulberry branch fibers. Adv Polym Technol 2019:1–10. https://doi.org/10.1155/2019/6252013

Teugjas, H., Väljamäe P. (2013) Product inhibition of cellulases studied with C-labeled cellulose substrates. Biotechnol Biofuels. https://doi.org/10.1186/1754-6834-6-104

Torres CE, Negro C, Fuente E, Blanco A (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 SPV 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

Tsumoto K., Ejima D., Senczuk AM, Kita Y., Arakawa T. (2007) The effect of Tricho- derma reesei cellulases and hemicellulases on the paper properties. Cellulose 25(2):1319–1328. https://doi.org/10.1007/s10570-017-1607-y

Wang, K., Cao, R., Wang, M., Lin, Q., Zhan, R., Xu, H., Wang, S. (2019) A novel thermostable GH10 xylanase with activities on a...
wide variety of cellulosic substrates from a xylanolytic Bacillus strain exhibiting significant synergy with commercial Celluclast 1.5 L in pretreated corn stover hydrolysis. Biotechnol Biofuels 12:1–13. https://doi.org/10.1186/s13068-019-1389-8

Wu S, Ma X, Cao S, Chen L, Huang L, Huang F (2020) Application of enzymes for the reduction of PFI revolutions in the secondary pulping process and characteristics of thermomechanical pulp. BioResources 15(4):7487–7502

Zhao H, Kwak JH, Zhang ZC, Brown HM, Arey BW, Holladay JE (2007) Studying cellulose fiber structure by SEM XRD, NMR and acid hydrolysis. Carbohydr Polym 68:235–241. https://doi.org/10.1016/j.carbpol.2006.12.013

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