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ORIGINAL PAPER

Production of cellulose nanofibrils from bleached eucalyptus fibers by hyperthermostable endoglucanase treatment and subsequent microfluidization

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Abstract A GH5 hyperthermostable endoglucanase from the archaeon *Pyrococcus honkoshii* (ph-GH5) and a commercial endoglucanase FR were used to treat bleached eucalyptus pulp (BEP) fibers to produce cellulose nanofibrils (CNFs) through subsequent microfluidization Enzymatic treatments facilitated CNF production due to the reduced degree of polymerization (DP) of the fibers SEM imaging indicated that FR reduced fiber DP drastically and resulted in much shorter fibers than with Ph-GH5, even at very low dosages (1 mg protein/g fiber) of FR treatment compared with a high dosage (10 mg protein/g fiber) of Ph-GH5. The fibers treated with FR were much more uniform in length perhaps due to the presence of exoglucanase and beta-glucosidase saccharifying short microfibers into glucose. TEM imaging indicated that Ph-GH5 produced longer and entangled CNFs than FR with the same number of microfluidization passes. However, the CNF diameters were approximately the same for all CNFs from enzyme-treated fibers using both endoglucanases at two dosages (1 or 10 mg protein/g fiber). CNFs produced from BEP fibers without enzymatic treatment showed larger diameters than those with enzymatic treatment.

Keywords Hyperthermophilic endoglucanase · Pretreatment · Microfluidization · Nanofibrillated cellulose

This work was conducted on official government time of Zhu, Kersten, Mozuch, and Sabo while Wang was a visiting student at the US Forest Service, Forest Products Lab

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Introduction

Sustainable, green and environmentally friendly mattrials have gained much attention in recent years (Abdul Khalil et al. 2012; Eichhorn et al. 2010). Cellulose is the most abundant renewable natural biopolymer on earth, and is present in a wide variety of living species including plants, animals, and some bacteria (de Souza Lima and Borsali 2004). The development of nanocomposites derived from renewable sources with cellulose nanomaterial as reinforcement is currently an important research topic.

Promising results were obtained in nanocellulose applications to hybrid composite materials, films, dispersions, foams, and in many other areas (Abdul Khalil et al. 2014). Cellulose nanomaterials can be produced by different methods from various lignocelluloses. Recently, considerable interest has been directed to cellulose nanofibrils (CNFs), because of its low thermal expansion (Fukuzumi et al. 2009; Nogi et al. 2009), high aspect ratio (Moon et al. 2011), good mechanical and optical properties which may find many applications in nanocomposites, paper making, coating additives, security papers, food packaging, and gas barriers (Belbekhouche et al. 2011).

Energy consumption is a major concern for mechanical CNF production. Enzymatic pretreatment is able to facilitate nanofibrillation by reducing energy input (Henriksson et al. 2007; López-Rubio et al. 2007; Svagan et al. 2007; Tanpichai et al. 2012; Zhu et al. 2011). By combining mild enzymatic pretreatment with high-shear refining or fibrillation, homogenization, or other mechanical processes, CNFs can be produced with a greater aspect ratio than by acid hydrolysis (Pääkko et al. 2007). Enzyme pretreatment also reduces instrument blockage during high pressure homogenization, thus facilitating processing of cellulosic fibers (Siddiqui et al. 2010). Most reported studies use commercial cellulase enzymes that are not pure and shortened the length of the resultant CNFs (Henriksson et al. 2007; Qing et al. 2013).

To understand the issue of fibril length shortening, we evaluate a purified recombinant GH5 endoglucanase, in comparison with a commercial endoglucanase, for potential in producing strong networked CNFs with lower energy input. Using a purified and well characterized endoglucanase can eliminate unknown constituents in commercial endoglucanase that may affect fibril length shortening. The GH5 endoglucanase from the hyperthermophilic archaeon *Pyrococcus horikoshii* was chosen because cellulose is its best substrate, the enzyme is hyperthermostable (thus providing a higher temperature experiment range), and it is selective (no activity with xylan and xyloglucan, and low activity with soluble cellooligosaccharides) (Ando et al. 2002; Kashima et al. 2005). Also, the crystal structure is known and the active site well-characterized (Kang and Ishikawa 2007; Kim et al. 2008). Therefore, this work differentiates from a published work (Henriksson et al. 2007) that compared a commercial endoglucanase with acid for CNF production. Characteristics of fibers or fibrils after endoglucanase treatment and microfludization were investigated by scanning electron microscopy (SEM), transmission electron microscopy (TEM), light transmittance, and degree of polymerization (DP) as indicators for potential CNF application.

Materials and methods

Materials

Bleached kraft eucalyptus pulp (BEP) from Fibria (Aracruz, Brazil) was used as the feedstock for CNF production. The major chemical components of the pulp were analyzed (Wang et al. 2012): 78.1 ± 1.0 % glucan, 15.3 ± 0.6 % xylan, 0.7 ± 0.1 % Klason lignin. BEP was first soaked in distilled water for 24 h, and then disintegrated by a lab disintegrator (TMI Ronkonkoma, NY). The disintegrated pulp was screened with a Bauer McNett Classifier (Testing machines Inc., Amityville, NY). The fraction between 28 and 48 mesh was collected after vacuum filtration and stored in a freezer until use. Commercial endoglucanase FR was from a commercial source.

Gene construction of Ph-GH5 endoglucanase

Design of Ph-GH5 was based on EGPh sequence of *P. horikoshii* without signal peptide and with deletion of the C-terminal for improved enzyme production (Ando et al. 2002; Kashima et al. 2005). A C-terminal histidine tag was included to aid enzyme purification. The gene for Ph-GH5 was commercially synthesized with codon bias for optimized *E. coli* expression in plasmid pJexpress414 (DNA2.0, Menlo Park, CA, USA). The sequence of Ph-GH5 is listed in Table S1.

Production and purification of recombinant Ph-GH5 endoglucanase

Ph-GH5 was produced with *E. coli* BL21 (DE3) as the expression host using MDG non-inducing medium and auto-induction medium with lactose inducer as described (Studier 2005). MDG cultures were grown overnight at 37 °C and used to inoculate auto-induction medium at 20 °C for approximately 30 h. Cells were harvested by centrifugation, suspended in

50 mM Tris–HCl(pH 8.0), 50 mM NaCl, 5 % glycerol, and lysed by sonication. Supernatant after centrifugation was heat-treated at 70 °C for 30 min and clarified by centrifugation. Supernatant was made 20 mM in imidazole, applied to HisTrap column (GE Healthcare, Uppsala, Sweden) and then washed with 20 mM imidazole in 20 mM sodium phosphate, 0.5 M NaCl, pH 7.4 buffer. The bound protein was eluted with 500 mM imidazole in the phosphate/NaCl buffer, concentrated by ultrafiltration (Amicon Ultra-15 centrifugal filters), and buffer exchanged (50 mM bis–Tris, pH 7.0, 0.15 M NaCl) with a HiPrep 26/10 desalting column (GE Healthcare, Uppsala, Sweden). Enzyme solution was made 20 % in glycerol for storage. Proteins were examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with Coomassie brilliant blue G-250.

Enzyme assays

The specific activities of the Ph-GH5 and FR were determined using *p*-nitrophenyl-ß-D-cellobioside (*p*-NPC; Sigma-Aldrich, St. Louis, MO) and carboxymethyl cellulose (CMC, sodium salt, low viscosity; Sigma-Aldrich, St. Louis, MO) as substrates. Hydrolytic activity toward *p*-NPC (Kang et al. 2007) was measured at 400 nm from the absorbance of *p*-nitrophenol liberated from *p*-NPC at 50 or 70 °C in 50 mM acetate buffer (pH 5.0) using *p*-nitrophenol as standard. The hydrolytic activities of the enzymes towards CMC were determined from the amounts of reducing sugars released (Ghose 1987) at 50 or 70 °C in 50 mM acetate buffer of pH 5.0 using glucose as standard. One unit of activity is defined as 1 µmol of glucose equivalent released per min.

Enzymatic treatment of fibers

The 28-48 mesh BEP fiber fraction was enzymatically treated by Ph-GH5 or FR before mechanical fibrillation. The enzyme loadings were 0.1, 1 and 10 mg protein/g fiber (abbreviated as mg/g in the following discussion). Protein concentration was measured by Bio-Rad protein assay (Bradford 1976) using bovine serum albumin (BSA) as standard. Fibers were mixed with endoglucanase in 50 mM acetate buffer at pH 5.0 with a solid concentration of 5 % (w/v), incubated at 70 °C for Ph-GH5 or 50 °C for FR in a shaker (MaxQ™ 4450, Thermo, Waltham, MA) at 200 rpm for 48 h. The resultant solids after enzymatic treatment were washed with acetate buffer and then several times with water with the aid of centrifugation. The washed solids were used for analysis and mechanical fibrillation.

Glucose determination

The glucose content of hydrolysates were determined as, (1) monomeric glucose, and (2) the glucose in the enzymatic hydrolysate after 4 % sulfuric acid hydrolysis at 121 °C for 60 min (Sluiter et al. 2011). Together, these measurements indicate glucose as free monomer and glucose in soluble oligomeric saccharide. Glucose concentration was determined with a YSI 2700 Biochemistry Analyzer (2700S, YSI Inc., Yellow Springs, OH, USA).

Mechanical fibrillation by microfluidization

BEP fibers with or without enzymatic treatment were fibrillated using a microfluidizer (Microfluidizer M-110EH, Microfluidics Corp., Westwood, MA). The fiber slurry of 0.3 % (w/v) was passed through two different sized chambers, first through a 200 µm chamber up to 20 times, and then up to an additional 20 times through a 87 µm chamber with operation pressure of 275.9 and 1,241 bar, respectively. Samples were taken during microfluidization every five passes for analyses.

Light transmittance

The visible light transmittance of enzymatic and mechanically treated fiber suspension (0.1 %, w/v) was measured at 600 nm with a spectrophotometer (Model 8453, Agilent Technologies, Inc., USA).

Determination of degree of polymerization

The DP of the cellulase treated fibers was measured according to TAPPI Standard Test Method T230 om-99 (TAPPI 1999). Vacuum dried cellulosic solids of 0.1 g was first dispersed with 10 mL distilled water, and then added 10 mL of 1 M cupriethylenediamine solution. The viscosity of the resultant solution was determined with a capillary viscometer. The DP of the cellulose was calculated using the following

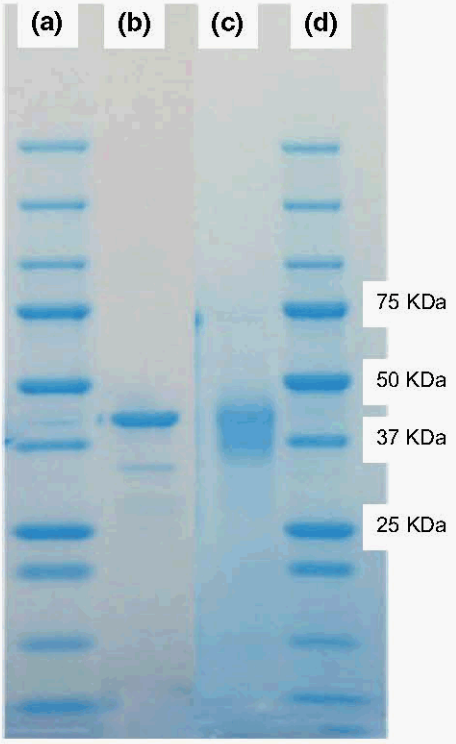


Fig. 1 SDS-PAGE of two endoglucanases: **a** Standard; **b** Ph-GH5; **c** FR; **d** Standard

expression (Mazumder et al. 2000): $DP^{0.905} = 0.75(954 \log X - 325)$, where X is the measured viscosity.

Electron microscopy and imaging analysis

Specimens for SEM were prepared by drying drops of the aqueous slurry of 0.2 g/L on aluminum mounts. All SEM specimens were sputter-coated with gold to provide adequate conductivity for examination in a Zeiss EVO 40 SEM (Carl Zeiss NTS, Peabody, MA) under ultrahigh vacuum conditions. For TEM, aqueous CNF suspensions (0.2 g/L) were further diluted and sonicated to disperse the particles. TEM grids (ultrathin carbon films supported by fenestrated carbon films) were floated on drops of approximately 5 µl sample for 1-2 min. They were then swished through two consecutive 250 µl drops of 2 % aqueous uranyl acetate. Excess stain was removed by capillary action and gentle blotting resulting in negatively stained particles. The samples were imaged using a Philips CM100 transmission electron microscope (FEI Company, Portland OR) with an accelerating potential of 100 keV. Images were captured on Kodak SO-163 electron image film and later scanned into digital images using 600 dpi resolution.

The diameters of the CNFs from TEM images were quantified using an imaging software (Nano Measure, Fudan University, China). Fibrils were randomly and manually picked. The fibril diameter sizing was accomplished by manually drawing the width of selected fibrils based on image gray level. Total of 50-100 fibril diameters were measured for each sample. The diameter distribution probabilities were reported. The reported ranges of fibril length were estimated based on visual observation of the SEM and TEM images.

Results and discussion

Characterization of Ph-GH5 and FR

Purified Ph-GH5 appeared much purer than FR (Fig. 1), possibly due to glycosylation of FR as a result of production in the eukaryotic host. The yield of purified Ph-GH5 was 442 mg/L culture and the theoretical molecular weight of 45,417 Da is in good agreement with empirical results (Fig. 1).

Ph-GH5 and FR have very different activities with *p*-NPC and CMC (Table 1). Ph-GH5 has a high *p*-NPC activity of 0.67 and 1.55 IU/mg at 50 and 70 °C, respectively, in agreement with literature (Ando et al. 2002; Kang et al. 2007). Ph-GH5 as a hyperthermophilic endoglucanase has higher activity at 70 °C, more than 2-3 times the values at 50 °C by both the *p*-NPC and CMC measures. In contrast, FR has almost no activity with *p*-NPC, while having high CMC activity of 21 IU/mg at 50 °C.

Endoglucanase treatment of fibers

Endoglucanase cleaves p-1,4 linkages of cellulose which creates new chain ends and reduces the cellulose chain length. Solid yield and DP of fibers after Ph-GH5 and FR treatment at different cellulase loadings are listed in Table 2. Approximately 2-6 % of fibers were lost during enzymatic treatment due to degradation to sugars and loss of fines through centrifugal washing. DP of fibers was decreased to different degrees, with greater DP reduction achieved at higher enzyme loadings. As a hyperthermostable

Table 1 Characteristics of Ph-GH5 and FR

Eudoglucanase	Molecular weight (g/mol)	Protein concentration (mg/mL)	*P*-NPC activity (IU/mg)		CMC activity (IU/mg)	
			50 °C	70 °C	50 °C	70 °C
Ph-GH5	45,417	7.58	0.67	1.55	5.50	14.00
FR	46,000	7.53	0.01	ND	21.04	ND

ND not detectable

Table 2 Solid yields and degrees of polymerization (DP) of endoglucanase pretreated fibers

Endoglucanase	Solid yield (%)			DP		
	Enzyme loading (mg/g)			Enzyme loading (mg/g)		
	10	1	0.1	10	1	0.1
Ph-GH5-50 °C	96.5 ± 0.4	97.3 ± 0.3	97.9 ± 0.3	984 ± 6	1,071 ± 12	1,122 ± 10
Ph-GH5-70 °C	95.1 ± 0.6	96.9 ± 0.5	97.7 ± 0.3	857 ± 21	941 ± 4	1,021 ± 14
FR-50 °C	94.1 ± 0.7	95.2 ± 0.5	96.5 ± 1.0	424 ± 9	542 ± 10	639 ± 21

Table 3 Glucose (wt% of g/g fiber) released from endoglucanase treatments

Endoglucanase loading	Ph-GH5-70 °C		FR-50 °C	
	10 mg/g	1 mg/g	10 mg/g	1 mg/g
Glucose 1 (wt%)[a]	1.7 ± 0.4	0.2 ± 0.1	6.5 ± 0.6	3.8 ± 0.9
Glucose 2 (wt%)[b]	5.2 ± 0.5	2.2 ± 0.3	6.7 ± 0.8	5.7 ± 0.8

[a] Released from endoglucanase pretreatments

[b] Measured from the endoglucanase hydrolysate after 4 % sulfuric acid hydrolysis at 121 °C for 60 min

cellulase, Ph-GH5 facilitated reduction in cellulose DP at an elevated incubation temperature, in agreement with the activity results. FR reduced DP from 1,160 to 542 (Table 2) or by approximately 50 % with only 1 mg/g enzyme loading.

Glucose release from endoglucanase treatments (Table 3) indicates that monomeric glucose concentration is much lower with Ph-GH5 treatment than with FR treatment. However, total soluble glucose (monomeric and soluble oligomeric glucose) between Ph-GH5 and FR treatment was comparable, especially at the high cellulase loading of 10 mg/g. The results indicate that less than one-third of the soluble sugars from Ph-GH5 treatment was glucose in comparison with more than two-thirds by FR. This suggests FR contains some exoglucanase and/or ß-glucosidase activities. Soluble sugars (such as cellobiose) released during enzymatic treatment can inhibit cellulase activity (Teugjas and Valjamäe 2013), which might negatively affect DP reduction by Ph-GH5.

SEM was used to reveal the morphologies of BEP fibers with and without enzymatic treatment. Fractionation by Bauer McNett Classifier produced fibers with relatively uniform size (Fig. 2a). Endoglucanase cut cellulose chains to result in shorter fibers (Fig. 2b–f) than those shown in Fig. 2a. A lot of fines, a term referred to fibers shorter than 200 µm, were found along with fibers from approximately 0.5–1 mm in the Ph-GH5 treated sample at 10 mg/g dosage (Fig. 2b). Fines were not present in FR treated sample (Fig. 2d, f). Compared with Ph-GH5, FR produced relatively uniform and shorter fibers of approximately 0.2–0.3 mm by visual observation of the SEM images at enzyme loading of 10 mg/g (Fig. 2d). This uniformity with FR is evidently a result of the hydrolysis of fine cellulosic particles all the way to monomeric glucose, consistent with the lack of oligomeric sugars in the FR treated hydrolysate compared to Ph-GH5 (Table 3). The average fiber length is longer (between 0.3 and 1.0 mm) at a

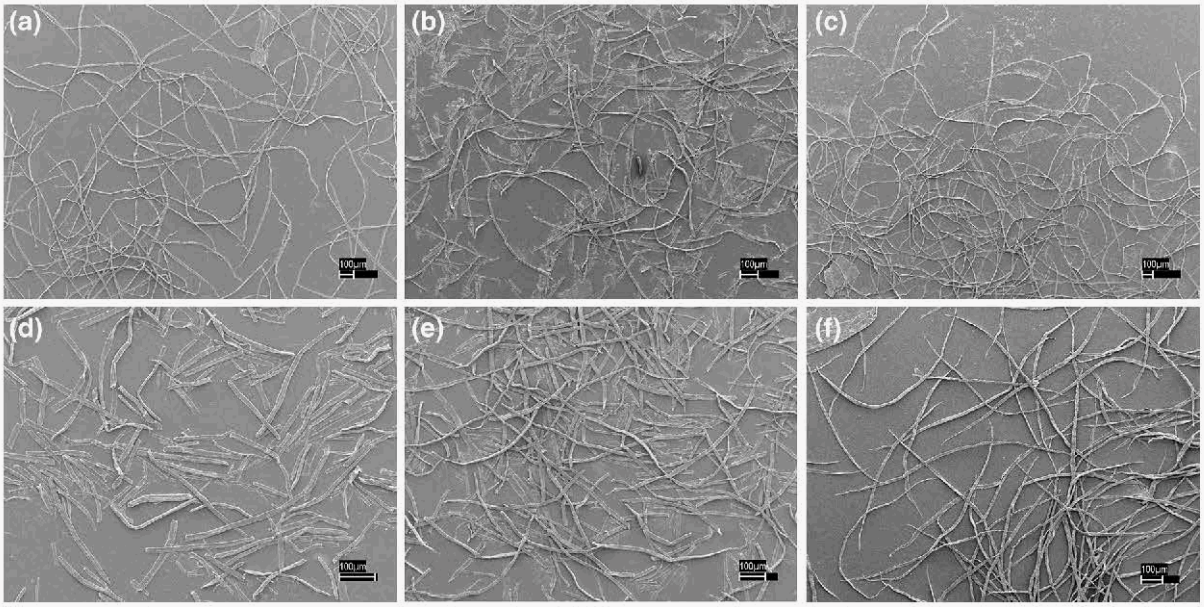


Fig. 2 SEM images of original untreated and endoglucanase treated BEP fibers (all scales = 100 μm): **a** untreated fibers: **b** Ph-GHS at 10 mg/g; **c** Ph-GH5 at 1 mg/g; **d** FR at 10 mg/g; **e** FR at 1 mg/g: **f** FR at 0.1 mg/g

lower FR loading of 1 mg/g by visual observation of the SEM image (Fig. 2e). The differences between the two endoglucanases indicate that they may work differently on fibers, with FR providing extensive hydrolysis to glucose, while Ph-GH5 is more selective with limited hydrolysis between hydrolytic nicks of the cellulose fibers. FR may contain exoglucanase and other enzyme activities that may also have aggressively attacked crystalline cellulose in addition to hydrolysis of disordered cellulose regions, while Ph-GH5 may have primarily worked on non-crystalline regions of cellulose. Even at the lowest FR loading of 0.1 mg/g, the resultant fibers appeared shorter than those from Ph-GH5 treatment at 1 mg/g loading (comparing Fig. 2f with c).

CNF properties

Photos of CNF suspensions (0.1 % w/v) from microfluidization of fibers with and without enzymatic treatment are shown in Fig. 3a–e. The CNF suspensions were contained in glass vials of 10 mm in diameter. A piece of white paper with parallel black inked lines was used as background to visually demonstrate the opacity of the CNF suspensions. For each initial fiber sample, different degrees of fibrillation produced CNFs with different levels of dispersion in water. The fibers before microfluidization settled to the bottom of the vials. Fibrillation through microfluidization increased dispersity of the resultant fibrils as can be seen from the increased opacities of the suspensions. After 20 passes through the 200 μm chamber and 5 passes through the 87 μm chamber, all samples were well dispersed in water, and became more and more transparent with increased numbers of passes of microfluidization. Treatments using both endoglucanases improved fibril dispersity and the transparency of fibril suspension; however, FR treatment had a more pronounced effect (compare Fig. 3b–d with a). At FR loading of 10 mg protein/g fiber, only five passes through 200 μm chamber were sufficient to produce well dispersed fibrils (Fig. 3d).

Light transmittances of suspensions of fibrils produced using different enzymatic treated fibers after only five passes of microfluidization all decreased to 0 % (Fig. 4), suggesting fibrillation of the initial fibers to result in good dispersion of the cellulosic material in water that increased the opacity of the suspensions. It took 25 passes to result in 0 % transmittance using fibers without enzymatic treatment. The nonzero transmittance at zero pass of microfluidization was due to settling of fibrous material (or lack of dispersion). Further fibrillation up to total of 20 passes did not change the transmittance for all suspensions

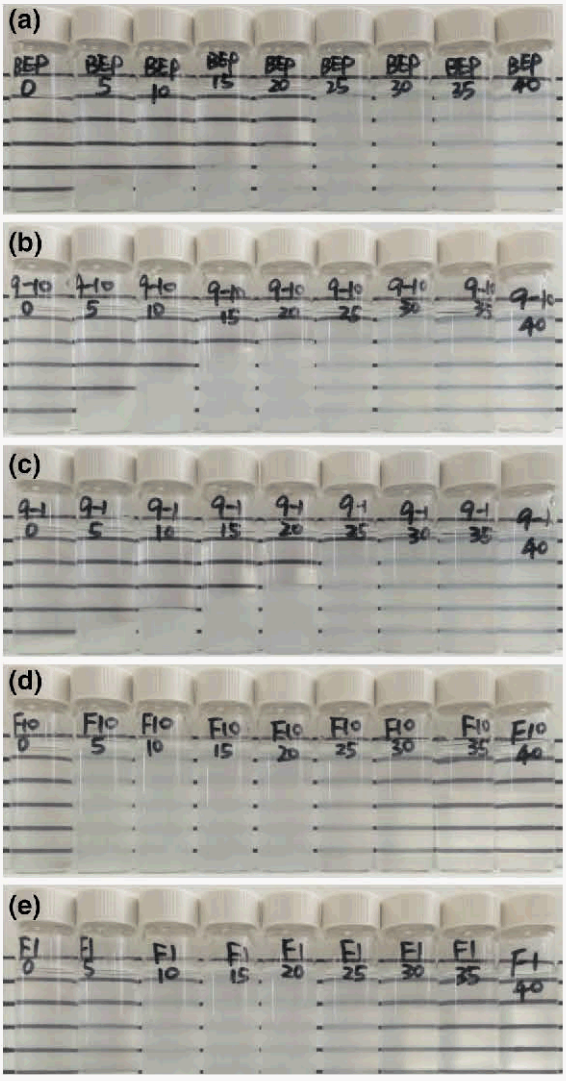


Fig. 3 Photos of suspensions of cellulosic BEP fibrils (0.1 w/v %) from microfluidization with or without enzymatic treatment. Numbers on the vials indicate passes of microfluidization. 0–20 passes were through the 200 μm chamber with additional passes through the 87 μm chamber. **a** untreated fibers; **b** Ph-GH5 at 10 mg/g; **c** Ph-GH5 at 1 mg/g; **d** FR at 10 mg/g; **e** FR at 1 mg/g

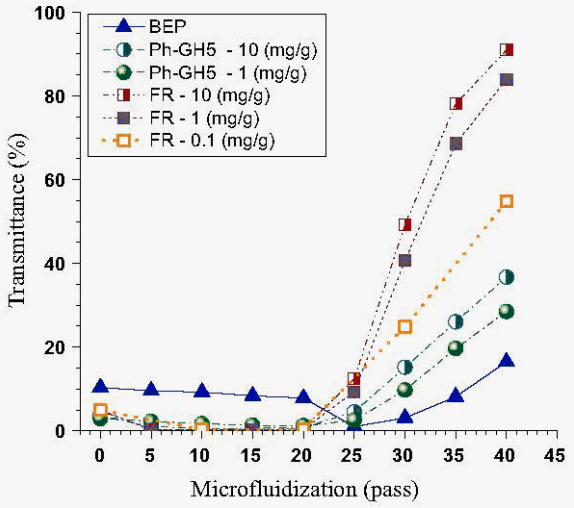


Fig. 4 Optical transmittances of suspensions of cellulosic BEP fibrils shown in Fig. 3

(Figs. 3a–e, 4). Transmittance was decreased for the fibers without enzymatic treatment with additional fibrillation using a 87 μm chamber. With additional ten passes (all refer to 87 μm chamber in below discussion), the fibril suspension of FR treated fibers became semi-transparent (Fig. 3d, e) with transmittance of 40–50 % (Fig. 4), suggesting increased hydrogen bonding between water and accessible hydroxyl groups resulting from fibrillation (Pääkko et al. 2007). It took additional 20 passes for the suspensions of fibrils from Ph-GH5 treated fibers at 10 and 1 mg/g dosages to achieve transmittance of 36, and 16 % (Fig. 4), respectively. The differences between enzyme loading of 10 and 1 mg protein/g fiber were not substantial (compare Figs. 3b–e, 4) for both endoglucanases. With the same number of microfluidization passes (presumably approximately the same energy consumption), endoglucanase pretreatment dramatically increased the suspension transmittance, especially when FR was used; a transmittance of 84 % was achieved even at a low enzyme loading of 1 mg/g.

Mechanical properties of CNFs are strongly dependent on fibril length (Henriksson et al. 2008). DP is a measure of the length and branching of cellulose chains, therefore, DP can be an intrinsic parameter of the mechanical properties of CNFs. The average DP reduction in the first 20 passes (through the 200 μm chamber) was approximately 16–19 per pass for fibers pretreated by FR at cellulase loadings of 10, 1, and 0.1 mg/g and by Ph-GH5 at 10 mg/g (Fig. 5). DP reduction was approximately 10 per pass at a low Ph-GH5 loading of 1 mg/g compared with 6 per pass for the untreated fibers. A rapid reduction in DP was observed after the fibrils were sent through the 87 μm chamber (Fig. 5). This is especially true for fibers without enzymatic pretreatment. DPs of fibrils from

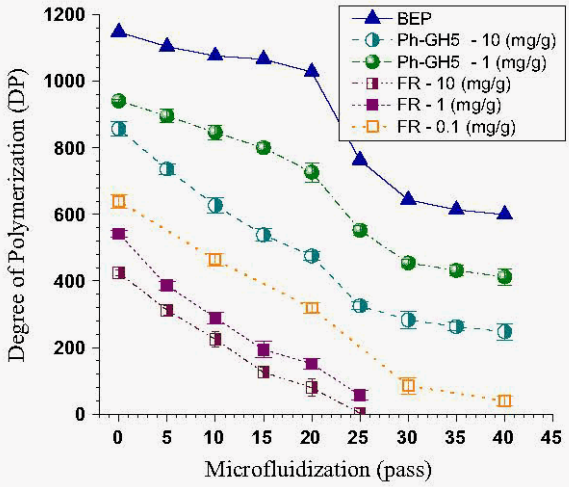


Fig. 5 Degrees of polymerization of BEP fibrils after different passes of microfluidization

FR treated fibers were so low after just an additional 5–10 passes through the 87 μm chamber that they could not be measured accurately with the method employed (Fig. 5).

CNF morphologies

Scanning electron microscopy (SEM) imaging can provide morphological information about the mechanically fluidized fibrils. After 20 passes through the 200 μm chamber, the fibrils were still large enough to be seen by SEM with diameters greater than 1 μm (Fig. 6a1–f1). Microfluidization also decreased the average length of the fibers. The length of fibrils from untreated fibers were approximately 500–1,000 μm (Fig. 6a1) and 10–100 μm from Ph-GH5 (10 mg/g) pretreated fibers based on visual observation of the SEM images (Fig. 6b1). Reducing Ph-GH5 loading to 1 mg/g substantially increased fibril length to approximately 50–500 μm (Fig. 6c1). The lengths of fibrils from FR (10 mg/g) pretreated fibers were more uniform between 5 and 30 μm (Fig. 6d1). The small amount exoglucanase and beta-glucosidase activities in FR may act as scavengers to saccharify the very short fibrils resulted from enzymatic treatment into glucose resulting in very uniform fibril morphology. Reducing FR loading to 1 mg/g only increased fibril length slightly, approximately to 10–40 μm (Fig. 6e1). The fibril lengths were approximately 10 – 200 μm at a very low loading of FR of 0.1 mg/g

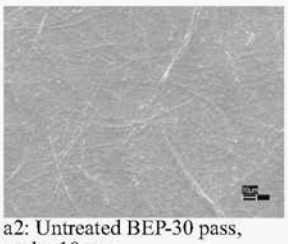
Fig. 6 SEM images of BEP fibrils from different passes of microfluidization using BEP fibers with or without enzymatic treatment: **a** untreated fibers; **b** Ph-GH5 at 10 mg/g; **c** Ph-GH5 at 1 mg/g; **d** FR at 10 mg/g; **e** FR at 1 mg/g; **f** FR at 0.1 mg/g

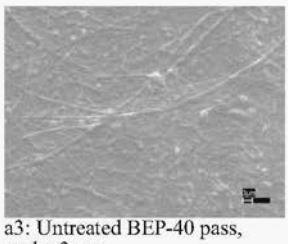
(Fig. 6f1). The reduction in cellulose chain length by FR was much more drastic than by Ph-GH5 even when comparing the fibrils from the low dosage (0.1 mg/g) of FR (Fig. 6f1) with those from the high dosage (10 mg/g) of Ph-GH5 (Fig. 6b1). This is in agreement with the measured fibril DP values reported in the previous section (Fig. 5).

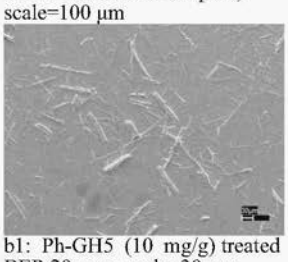
With additional ten passes through the 87 μm chamber (total 30 passes), substantial reduction in fibril diameter were observed (Fig. 6a2–f2). The lengths of fibrils from Ph-GH5 treated fibers were much longer and were not able to be determined by SEM (Fig. 6b2, c2). The lengths of fibrils from FR treated fibers with loadings of 1 and 10 mg/g were approximately 10 μm (Fig. 6d2, e2). The lowest FR loading of 0.1 mg/g resulted in fibrils <100 μm (Fig. 6f2). Additional processing of 20 passes through the 87 μm chamber further reduced the physical dimensions of the fibrils (Fig. 6a3, b3, c3, d3, e3, f3). Although some large, micrometer-scale fibers were still present for all samples even after 40 passes, the fibrils were not able to be seen using SEM; however, the reductions in fibril diameters were obvious comparing with those with less processing.

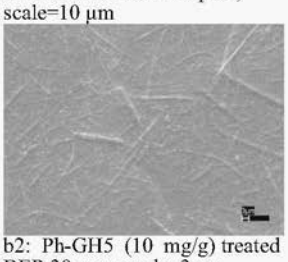
Transmission electron microscopy (TEM) reveals that most cellulose fibrils were nano sized fibrils (Fig. 7a1–e1). CNF from BEP fibers without enzymatic treatment after 40 passes microfluidization had a length between 1 and 2 μm (Fig. 7a1) and diameter between 5 and 14 nm (Fig. 7a2). With the application of Ph-GH5 at just 1 mg/g, similar lengths of 1–2 μm CNFs (Fig. 7b1) but slightly finer and more uniform in diameter of 5–10 nm (Fig. 7b2) were obtained with only 30 pass microfluidization, suggesting that application of enzymatic treatment can save mechanical energy input by at least 30 % for nanofbrillation of cellulosic fibers. The application of FR even at 1 mg/g cut the cellulose chain to result in much shorter fibril length as discussed previously using SEM imaging and DP data and further verified by TEM (Fig. 7c1). The fibril lengths were approximately 0.2–0.8 μm. Increasing the number of passes to 40 did not affect fibril morphology for sample treated by Ph-GH5 (Fig. 7d1 vs b1). High cellulase dosage also cut the

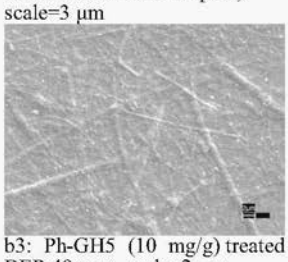

a1: Untreated BEP-20 pass, scale=100 μm

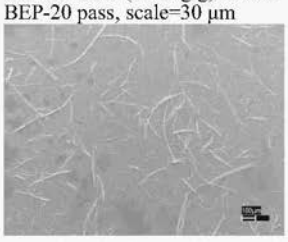

a2: Untreated BEP-30 pass, scale=10 μm

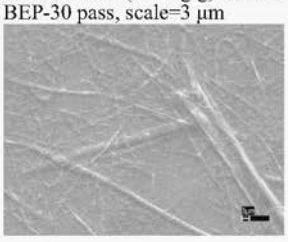

a3: Untreated BEP-40 pass, scale=3 μm


b1: Ph-GH5 (10 mg/g) treated BEP-20 pass, scale=30 μm


b2: Ph-GH5 (10 mg/g) treated BEP-30 pass, scale=3 μm


b3: Ph-GH5 (10 mg/g) treated BEP-40 pass, scale=2 μm


c1: Ph-GH5 (1 mg/g) treated BEP-20 pass, scale=100 μm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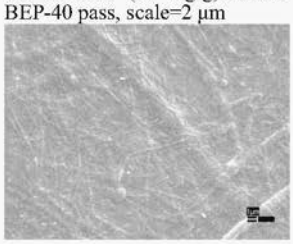
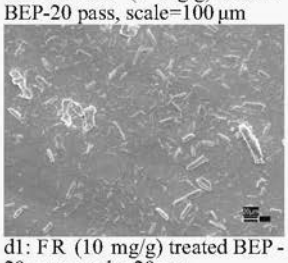
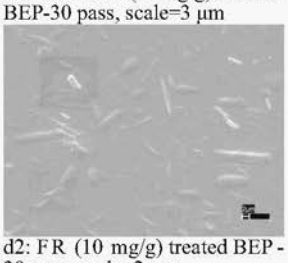
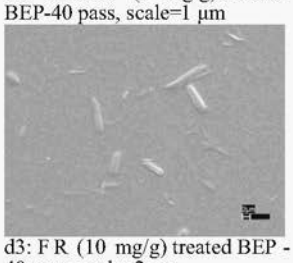
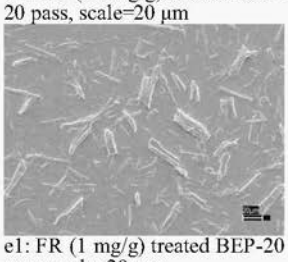
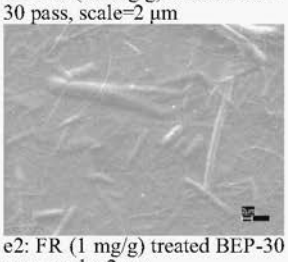

c3: Ph-GH5 (1 mg/g) treated BEP-40 pass, scale=1 μm

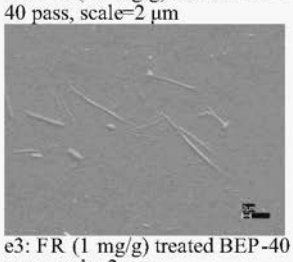

d1: FR (10 mg/g) treated BEP-20 pass, scale=20 μm

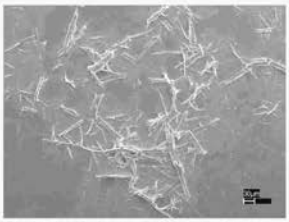

d2: FR (10 mg/g) treated BEP-30 pass, scale=2 μm

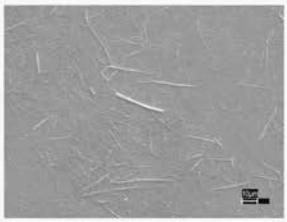

d3: FR (10 mg/g) treated BEP-40 pass, scale=2 μm


e1: FR (1 mg/g) treated BEP-20 pass, scale=20 μm


e2: FR (1 mg/g) treated BEP-30 pass, scale=2 μm


e3: FR (1 mg/g) treated BEP-40 pass, scale=2 μm


f1: FR (0.1 mg/g) treated BEP-20 pass, scale=30 μm


f2: FR (0.1 mg/g) treated BEP-30 pass, scale=10 μm


f3: FR (0.1 mg/g) treated BEP-40 pass, scale=2 μm

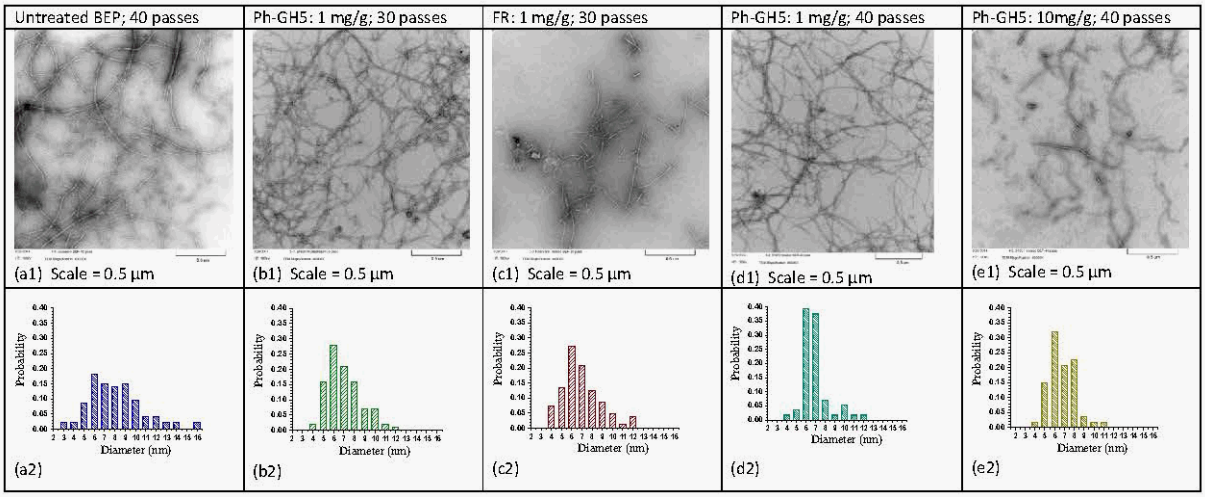


Fig. 7 Comparisons of the morphologies by TEM analysis of cellulose nanofibrils (CNFs) from microfluidization of BEP fibers with or without enzymatic treatment: **a** untreated fibers, **b** Ph-GH5 at 1 mg/g, 30 passes: **c** FR at 1 mg/g; 30 passes: **d** Ph-GH5 at 1 mg/g, 40 passes: **d** Ph-GH5 at 10 mg/g, 40 passes

cellulose chain length as evidenced from the TEM images of fibrils from Ph-GH5 treatment at 10 mg/g (Fig. 7e1). No obvious benefit in reducing CNF diameter was observed (Fig. 7e2).

Imaging analyses indicated that overall cellulase types and dosages did not affect the nominal diameters of the resultant CNFs (Fig. 7a2–e2). Fibril diameters from untreated fibers after 40 passes of microfluidization had a broader distribution between 3 and 16 nm with a relatively uniform distribution between 5 and 10 nm. Cellulase treatment facilitated nanofibrillation and resulted in finer fibrils. Diameters of the CNFs from fibers treated with Ph-GH5 at 1 mg/g with only 30 passes of microfluidization ranged from 4 to 12 nm (Fig. 7b2), similar to the diameters of the fibrils from fibers treated by FR (1 mg/g) after 30 passes (Fig. 7c2). This indicated cellulase treatment can reduce processing by at least 30 % (from 40 passes to 30 passes) to achieve equivalent fibril diameters. Further processing to 40 passes reduced large fibrils as can be seen from the narrower fibril diameter distributions (Fig. 7d2, e2).

Conclusions

Endoglucanase pretreatments combined with microfluidization for production of CNFs were investigated in this study. Ph-GH5 and FR differed in several key parameters during hydrolysis of fibers prior to microfluidization; Ph-GH5 released mostly multimeric soluble sugars whereas FR produced mostly glucose, FR was much more aggressive in lowering DP compared to Ph-GH5, and Ph-GH5 released fines whereas none where observed with FR. These differences most likely reflect inherent catalytic differences of the enzymes as exemplified in the contrasting substrate preferences for *p*-NPC and CMC. The application of Ph-GH5 to treat cellulosic fibers at 1 mg protein/g fiber, with subsequent microfluidization, produced CNFs of longer length compared with those produced using the commercial cellulase at the same loading, and also produced relatively uniform sized CNFs with a mean diameter of approximately 6 nm, suitable as a reinforcement polymer. The Ph-GH5 pretreatment can also reduce mechanical energy input by at least 30 % when compared with using fibers without enzymatic treatment to produce similar quality CNFs in terms of fibril length, diameter, and optical transparency.

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