Enzyme-Assisted Mechanical Fibrillation of Bleached Spruce Kraft Pulp to Produce Well-Dispersed and Uniform-Sized Cellulose Nanofibrils

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Cellulose nanofibrils (CNFs) are bionanomaterials with many promising properties and great potential in composite applications. Herein, well-dispersed and uniform-sized cellulose nanofibrils were successfully obtained from commercially bleached softwood kraft pulp, resulting in yields of 79.15% *via* enzyme-assisted hydrolysis and subsequent homogenization. Field emission scanning electron microscopy (FE-SEM) confirmed the fiber morphology. The water retention value (WRV) was increased from 107% of original pulp to 1384% of the resulting CNFs, while crystallinity had no significant changes. Fourier transform infrared (FTIR) spectroscopy indicated that enzymes degraded a portion of hemicelluloses and residual lignin. CNFs subjected to enzymatic treatment and homogenization had a less entangled network, larger aspect ratio, and higher transmittance than those from pure mechanical treatment. They were well dispersed in an aqueous solution and uniformly sized in morphology, resolving the present challenge of nanomaterials tendency for agglomeration. These results revealed that the enzyme-assisted process had a remarkable effect on the production of CNFs and made CNFs more appealing for nanotechnology applications and nanomaterial.

Keywords: Cellulose nanofibrils; Mechanical pretreatment; Enzyme hydrolysis; Homogenization; Dispersion

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INTRODUCTION

Cellulose nanomaterials, such as cellulose nanocrystals (CNC) and cellulose nanofibrils (CNF), are produced from renewable lignocellulose, are biodegradable and have unique physicochemical properties suitable for developing a range of bioproducts (Moon *et al*. 2011; Satyamurthy and Vigneshwaran 2013). CNFs with widths of 10 to 30 nm and lengths of several microns show potential applications in the area of transparent films, strong materials, foods, cosmetics, flexible displays, pharmaceutical application, and electrochemistry due to their excellent properties of large surface area, light weight, high Young's modulus, low coefficient of thermal expansion, biodegradability, and biocompatibility (Nishino *et al*. 2004; Nishiyama 2009; Nogi and Yano 2009; Nyholm *et al*. 2011; Li *et al*. 2012; Lin and Dufresne 2014). However, efficient production of CNF is still facing the challenges of high energy consumption and a need to increase capacity.

Various types of mechanical methods have been applied to produce CNFs, including homogenization, micro-grinding, microfluidization, and cryocrushing (Nair *et al*. 2014; Wang *et al*. 2015a; Tian *et al*. 2016). These techniques are the most efficient for

delamination of fiber cell walls and CNF production. The first successful isolation of CNF was reported in 1983 using a homogenizer (Osong *et al*. 2016). As a result, individualized nanofibrils were obtained with diameters less than 100 nm. The microfluidizer, an alternative for a homogenizer, also produces nanofibrils of 20 to 100 nm in diameter and several tens of micrometers in length (Zimmermann *et al*. 2010). Due to different shear mechanisms and intensities, the energy demand and size distribution of obtained nanofibers are widely varied (Qing *et al*. 2013). The main barrier for commercial production of CNF is the high-energy consumption. For example, approximately 12000 to 70000 kWh/t of energy is necessary to make gel-like microfibrillated cellulose from a kraft hardwood pulp suspension (Spence *et al*. 2011; Wang *et al*. 2012). Another disadvantage of fibrillation is the occasional clogging of the reaction chamber when using long fibers (Henriksson *et al*. 2007).

To reduce energy requirements and obtain high-value products, various pretreatments have been employed to facilitate the disintegration of cellulose into nanofibrils (Zhu *et al*. 2011). Alkali pretreatments (such as KOH, NaOH, $Ca(OH)_2$ and ammonia, *etc*.) are beneficial to fiber swelling and cellulose accessibility for chemical reagents (Lavoine *et al*. 2012; Wang *et al*. 2014). Mechanical pretreatments, such as refining (*e.g.* PFI-milling and Valley beating) are generally performed to deconstruct the compact structure of cellulose and expose more organized fibrils for further processing (Zhu *et al*. 2009, 2011). A common chemical method for producing CNF with a uniform width of 2 to 5 nm has also been prepared using 2,2,6,6-tetramethylpiperidine-1-oxy (TEMPO)-mediated oxidation followed by mechanical disintegration, even though TEMPO-oxidized cellulose fibers were actually cellulose derivatives (Saito *et al*. 2006). Enzymatic hydrolysis is not commercialized yet, but it has the potential to reduce operating costs and improve efficiency (Wang *et al*. 2016).

Commercially bleached spruce kraft pulp (BSKP) with a high cellulose content has been widely used for producing CNFs. Cellulose is covered or interlaced with hemicelluloses and lignin in the cell wall, which limits its efficient utilization (Fratzl *et al*. 2004). In addition, water molecules have difficulty in permeating and diffusing into the microfibril layers due to the intermolecular hydrogen bonds between microfibrils and microfibril bundles (Hult *et al*. 2000). Therefore, enzymatic hydrolysis has been combined with mechanical fibrillation to loosen microfibril layers and produce nanofibers. Preparation of cellulose nanocrystals (CNC) using sulfuric acid hydrolysis was optimized to obtain a high yield of CNC from cellulase-pretreated fibers (Beltramino *et al*. 2016). Apart from CNC, microfibrillated cellulose (MFC) and CNF have been prepared using endoglucanase hydrolysis with subsequent high-pressure homogenization or microfluidization (Pääkkö *et al*. 2007; Wang *et al*. 2015b). Moreover, xylanse was also utilized with chemical treatment and homogenization to isolate CNF from banana peels (Tibolla *et al*. 2014). Despite all the studies describing the enzyme-assisted method to produce CNF, the effect of mixed enzyme pretreatment and mechanical disintegration process on the morphology and properties of CNF is still not clear.

This study investigated the effect of enzyme treatment on the mechanical fibrillation of cellulose nanofibrils. To produce two different cellulose nanofibrils, three steps containing mechanical pretreatment, enzyme hydrolysis, and homogenization were designed in this study. The composition and yield of different cellulose fibers were investigated. The morphology of CNFs with and without the enzyme-assisted processes were compared using field emission scanning electron microscopy (FE-SEM). The water retention value (WRV) and crystallinity index (CrI) were determined as indicators of fiber

quality. Functional groups of the original pulp and enzymatic hydrolysis residues were measured using Fourier transform infrared spectroscopy (FTIR). The optical properties of each suspension were also characterized by a UV-vis spectrometer.

EXPERIMENTAL

Materials

Bleached spruce kraft pulp (BSKP) was supplied by the Zellstoff Celgar mill, Canada, in the form of dry sheet. The chemical composition of BSKP was as follows: glucan (74.21%), xylan (13.93%), mannan (4.76%), Klason lignin (2.95%), and ash (2.56%). The fiber length and diameter were 1.7 to 5 mm and 30 to 75 μm, respectively. Sodium hydroxide (NaOH, wt.% ≥ 96 %) was purchased from Nanjing Chemical Reagent Co., Ltd. (Nanjing, China). Xylanase (powder, ≥ 2500 units/g) was supplied from Sigma-Aldrich (St. Louis, MO, USA). Laccase (approximately 2.5 to 3 units/g) was provided by the Institute of Chemical Engineering at Nanjing Forestry University. Both were used as received without further purification.

Methods

Determination of chemical composition

Samples derived from all fractions were hydrolyzed using sulfuric acid in two steps for carbohydrates and lignin analyses by the Analytical Chemistry and Microscopy Laboratory (ACML) of the USDA Forest Service.

Cutting and crushing

Fibers of approximately 1 wt.% were cut for 45 min using a Valley beater (ZQS2, Shaanxi University of Science and Technology Machinery Plant, Shaanxi, China). The average fiber size was measured by a fiber quality analyzer (LDA02, Optest Equipment Inc., Hawkesbury, Ontario, Canada). The cutting process was stopped when the average length was 0.4 mm. The short-fibers were screened through a 40-mesh sieve using a Bauer-McNett screening instrument, and fibers were collected as the feedstock. The short-fibers were immersed in a 6 wt.% NaOH solution at 0 °C for 24 h. A Valley beater was used again for crushing. Compared with cutting, the fiber concentration was controlled at 3 wt.%. The lever arm load was adjusted to 38 N and the rotate speed of the knife drum was 498 ± 12 rpm. The fiber morphological changes were observed on site. The fibers were filtered through a 200-mesh filter cloth, neutralized with 1 wt.% acetic acid, and washed with deionized water three times.

Refining

Refining was performed according to TAPPI T248 sp-08 (2008) using a PFI mill (ZQS7-PFI, Shaanxi University of Science and Technology Machinery Plant, Shaanxi, China). After cutting and crushing pretreatment, 30 g o.d. pulp was refined at 10% consistency.

Enzymatic treatment

Enzymatic treatments were performed in a 250 mL flask at 3% pulp consistency with enzymes and a citric acid buffer solution (pH 4.5 to 5) at 50 °C for 12 h. The loading of laccase and xylanase was 30 IU/g pulp OD and 100 IU/g pulp OD, respectively. The

incubation was carried out in a thermostatic bath to preserve heat. The pulps were mixed with water at 90 °C for 30 min to denature and inactivate the enzymes. The resulting pulp was washed with deionized water and separated by centrifugation (8000 rpm) at 5 °C for 15 min. As a control, the pulps were treated under identical conditions without enzymes.

Homogenization

Fibrillation treatments of cellulose fibers were performed using a high pressure homogenizer (FB-110Q, LiTu Mechanical Equipment Engineering Co., Ltd, Shanghai, China). Fiber suspensions of 0.5% (w/v) were passed through a homogenizer 20 times with an operation pressure of 500 bar.

The entire experimental process is shown schematically in Fig. 1. For ease of discussion, all the samples were given a label designation of C, CR, CRE, CEH, and CREH in which C stood for cutting and crushing, R corresponded to PFI refining, and E and H were enzymatic hydrolysis and homogenization, respectively. The detailed information for different processing methods is shown in Table 1.

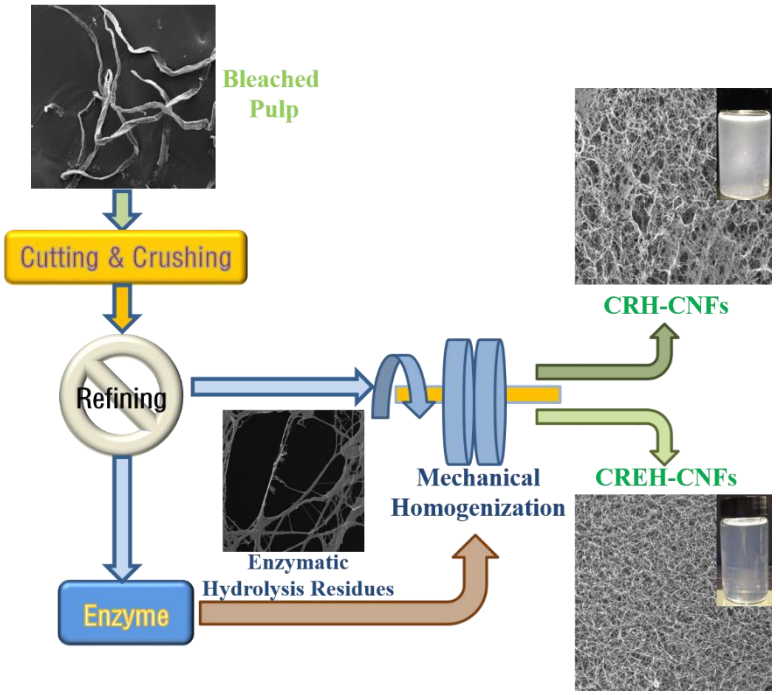

Fig. 1. Schematic process flow diagram of experiments

Table 1. Preparation Approaches for Cellulose Nanofibrils (CNF)

Materials	Preparation Approach			
	Cutting and Crushing	Refining	Enzyme	Homogenizing
BSKP	No	No	No	No
C	Yes	No	No	No
CR	Yes	Yes	No	No
CRE	Yes	Yes	Yes	No
CRH	Yes	Yes	No	Yes
CREH	Yes	Yes	Yes	Yes

Characterization

Morphology observation

Specimens for SEM analysis were freeze-dried and sputter-coated with gold to provide adequate conductivity. Images were observed with a field emission scanning electron microscope (FE-SEM, JSM-7600F, JEOL, Tokyo, Japan) at a voltage of 20 kV. Fiber diameter was measured using imaging software (Image-Pro Plus, Media Cybernetics, Rockville, USA).

Evaluation of water retention value (WRV)

The WRV was defined as the amount of water retained by the fibers in the pores over the oven dry mass of the fibers after centrifugation of a fiber pad under standard conditions. WRV was used to characterize for fiber swelling. WRV was measured according to a modified Scandinavian test method SCAN-C 62:00 (Luo and Zhu 2011). The weight of the sediment was recorded as m_1. The sample was dried at 105 °C to a constant mass and reweighed (m_2). WRV was calculated using Eq. 1. Each sample was measured three times, and the average value was recorded.

$$\mathrm{WRV}(\%) = (m_1 - m_2)/m_2 \times 100\% \quad (1)$$

X-ray diffraction (XRD)

X-ray diffraction analysis was carried out on a Rigaku-D/MAX instrument (Ultima IV, Rigaku Corp., Tokyo, Japan). Ni-filtered Cu-Kα radiation generated at a voltage of 40 kV and a current of 30 mA with a scan speed of 2 °/min from 5° to 40° was used. The degree of crystallinity was calculated according to the Segal method (without base line subtraction) (Segal *et al*. 1959). The crystallinity index (CrI) of the cellulose samples was calculated according to Eq. 2, where I_{002} is the counter reading at peak intensity ($2\theta = 22°$) representing crystallized regions, and I_{am} is the counter reading at peak intensity ($2\theta = 18°$), representing amorphous regions in cellulosic fibers.

$$\mathrm{CrI}(\%) = (\mathrm{I}_{002} - \mathrm{I}_{am})/\mathrm{I}_{002} \times 100\% \quad (2)$$

Fourier transform infrared spectroscopy (FTIR)

Functional groups present in the original pulp and enzymatic hydrolysis residues were carried out using a Fourier transform infrared spectrometer (FTIR-650, GANGDONG Sci & Tech Development Co., Ltd., Tianjin, China) with an infrared region of 1800 to 800 cm^{-1}.

Light transmittance

The visible light transmittance of CRE, CRH-CNFs, and CREH-CNFs suspensions (0.2%, w/v) was measured at 400 to 800 nm wavelength using a UV-vis spectrometer (UV757CRT, Precision & Scientific Instrument Co., Ltd., Shanghai, China).

RESULTS AND DISCUSSION

Chemical Composition and Yield

The chemical composition along with yields of different cellulose fibers are listed in Table 2. The hemicellulose (xylan and mannan) and lignin content were around 17% and

2.5%, respectively, for all mechanically treated fibers, suggesting that mechanical fibrillation had no influence on the chemical composition of fibers. There was only a 11% yield loss of cellulose fiber (CRH = 88.72%) without the enzymatic treatment. As expected, most of the hemicellulose and lignin lost in the enzyme-assisted process resulted in a CREH yield of 79.15%. According to the glucan content and the yield of BSKP and CREH, cellulose was essentially conserved when enzyme-assisted mechanical fibrillation was employed for producing CNFs.

Table 2. Chemical Composition and Yield of Different Cellulose Fibers

Materials	Glucan (%)	Xylan (%)	Mannan (%)	Klason Lignin (%)	Yield (%)
BSKP	74.21	13.93	4.76	2.95	100
C	73.88	13.24	4.17	2.45	96.65
CR (5000 revolution)	74.56	13.89	3.71	2.59	91.36
CR (15000 revolution)	74.07	13.05	4.01	2.68	91.02
CRE	85.84	6.05	2.76	0.53	81.39
CRH	76.16	12.38	4.24	2.40	88.72
CREH	87.11	5.52	2.87	0.52	79.15

Morphological Characteristics

Figure 2 shows the fiber morphologies under various conditions. Untreated BSKP fiber had an average width of about 30 μm (Fig. 2a). Figure 2b shows that the P and S_1 layer were peeled off, and the S_2 layer was exposed to the surface. The water molecules and hydrated-sodium ions mainly permeated into the interior of the C fibers. As shown in Fig. 2c, refining at 15000 revolutions was not sufficient to completely convert the cellulose fibers to nanofibrils.

However, refining led to more fiber fines and fragments, and imparted the fiber with a smaller diameter and higher accessibility to enzyme. Notably, a small portion of the fibers was converted to nano-scaled fibers. Compared with the sample CR, enzymatically-treated fibers had a shorter diameter of approximately 1 μm due to the removal of hemicelluloses and lignin (Fig. 2d). The morphological differences between CR and CRE indicated that the enzymes hindered the aggregation of microfibrils and resulted in more uniform structures.

Figures 2e and 2f show two different CNFs prepared by homogenization using CR and CRE. Fibers without enzymatic treatment clogged the reaction chamber at the beginning of homogenization, causing the device to be emptied and cleaned. However, no clog was detected when using enzymatically treated fibers. High-pressure homogenization effectively liberated cellulose bundles into more uniform fibrils, such as the gel-like CNF that was formed after 20 passes. Compared with CREH-CNFs, CRH-CNFs were entangled fibril networks with incomplete separation (Fig. 2e). CREH-CNFs had relatively smaller diameters of approximately 20 nm and a higher aspect ratio (length/diameter), which demonstrated that the enzyme-assisted process played an important role in producing uniform-sized, well-dispersed cellulose nanofibers as part of an integrated biorefinery approach.

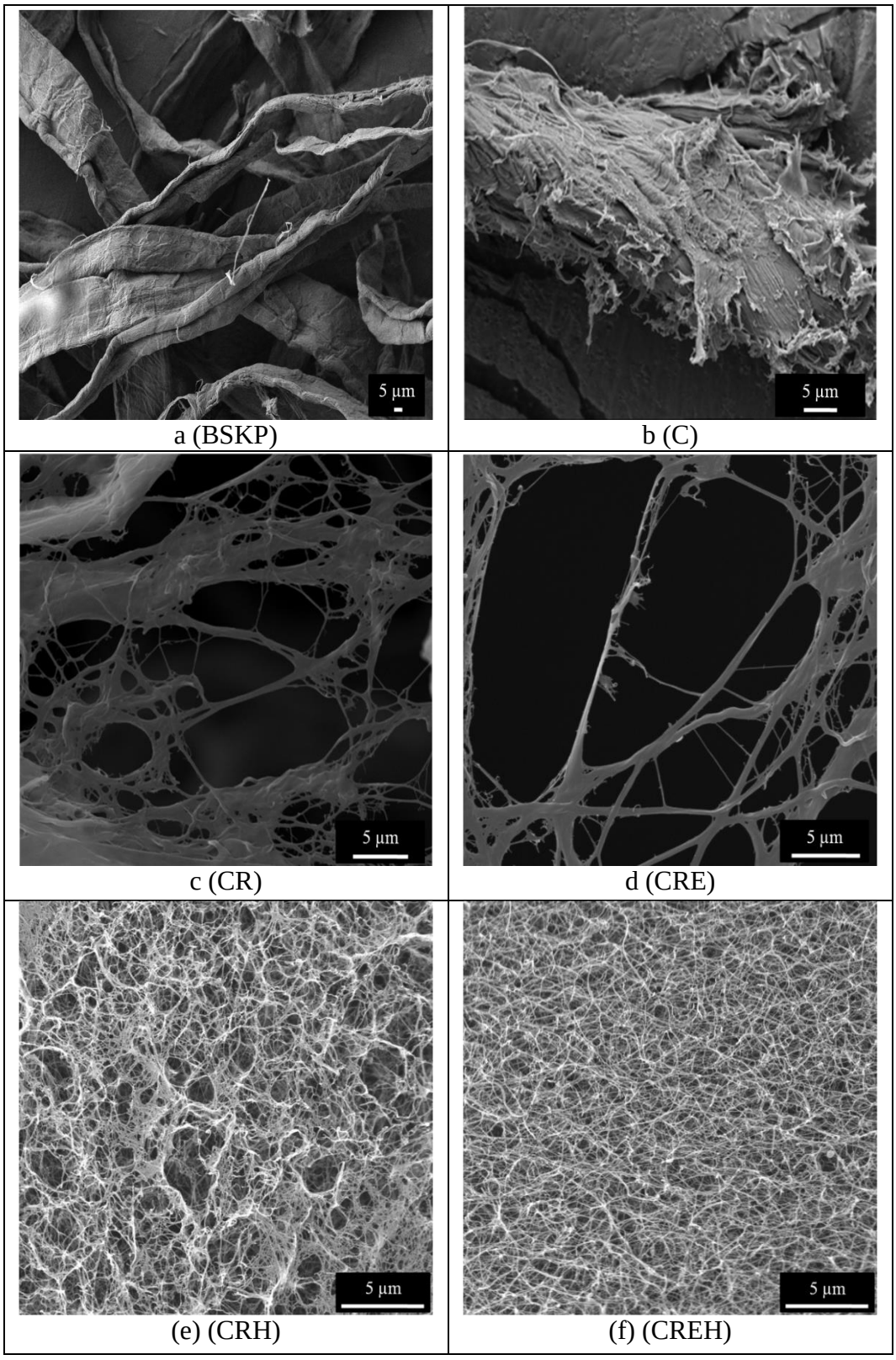

Fig. 2. SEM images of BSKP fibers under different conditions. (a) - (f) represent BSKP, C, CR, CRE, CRH, and CREH, respectively. The scale bar is 5 µm.

Effect of Different Treatment on Fiber Swelling

The water retention value (WRV), an indicator of the interaction between the water and cellulose fibers, was used to identify the degree of cellulose fibrillation and fiber swelling. WRV also correlates to total internal pores, fibril surface areas, and enzyme accessibility to substrates (Luo and Zhu 2011).

Figure 3 presents the water retention value of different cellulose fibers. WRV increased with enzymatic treatment and mechanical fibrillation. The WRV of BSKP was 107.32%, and increased to 249.08% after refining, suggesting that fibers were easily crushed overall in the radial direction and fibrillated into microfibrils with the peeling of P and S_1 layers in cell wall, as shown in Fig. 2c.

Compared with the WRV of CR fiber, enzymatically treated fiber increased to 313.29%. This was due to the removal of hemicellulose and lignin with the assistance of enzymes, resulting in more hydroxyl groups exposed on the fiber surface. Subsequent homogenization resulted in CNFs with smaller diameters and more water-accessible surfaces. Therefore, WRV of CRH and CREH were increased to 852.41% and 1383.92%, respectively.

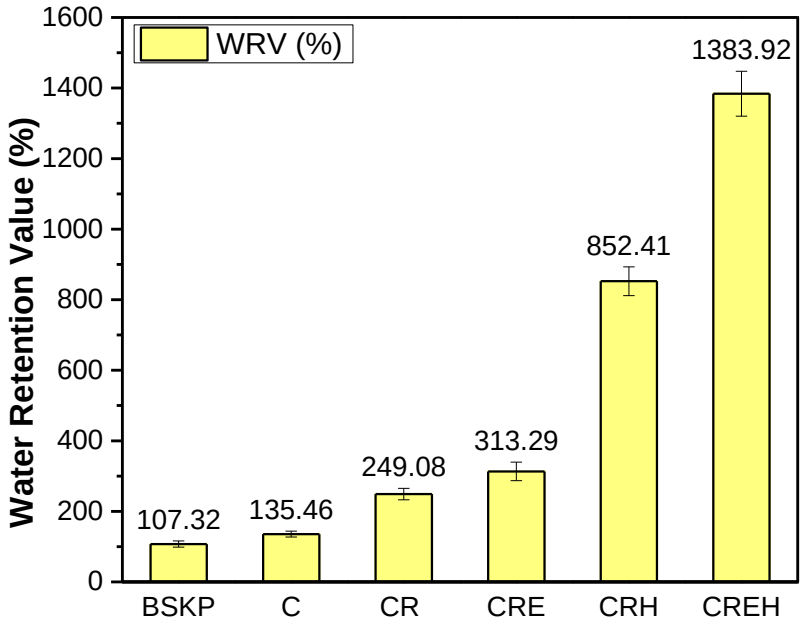

Fig. 3. Water retention value (WRV) of cellulose fiber from different conditions

Crystallinity

The influence of mechanical and enzymatic treatments on the crystallinity of the resulting CNFs was investigated. The XRD patterns for original BSKP and fibers prepared by different approaches are shown in Fig. 4. All samples exhibited a sharp peak at 2θ = 22.3°, suggesting that the fibers contained a significant portion of native cellulose. Therefore, these methods for producing CNFs did not alter the crystal structure. Although the crystal structure was not changed, the intensity of diffraction peaks varied among the samples. To quantitatively analyze the differences in crystallinity of the samples, the CrI was calculated as shown in Table 3.

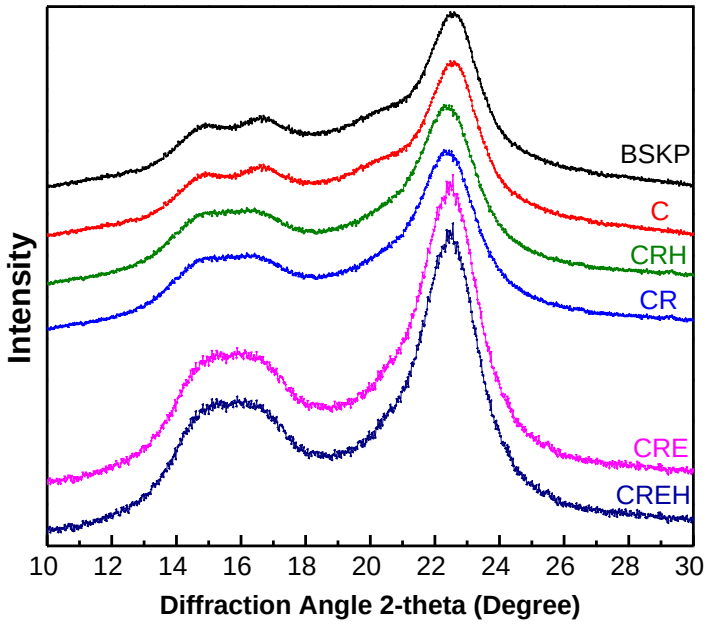

Fig. 4. XRD patterns for BSKP and different cellulose fibers

Table 3. Crystallinity Indices of Different Cellulose Fibers

Materials	CrI (%)
BSKP	63.95 ± 0.53
C	66.56 ± 1.33
CR (5000 revolutions)	Not detected
CR (15000 revolutions)	58.47 ± 0.34
CRE	67.33 ± 2.07
CRH	54.75 ± 1.61
CREH	63.81 ± 0.81

The CrI of fibers initially increased slightly and then significantly decreased to 58.47% (CR) and 54.75% (CRH). This tendency indicated that mechanical fibrillation by refining and homogenization broke the crystalline region of cellulose apart, resulting in a decrease in crystallinity for CR and CRH fibers. Interestingly, the enzymatically-treated samples showed a remarkable increase in crystallinity. These results stemmed from partial hemicellulose and lignin elimination from the fiber during the enzymatic hydrolysis, which was consistent with a previous report (Tibolla *et al*. 2014). Compared with untreated pulp, CREH-CNFs ultimately had the same CrI due to the combined effects of different treatment conditions.

FTIR Analysis of Enzymatic Hydrolysis Residues

FTIR analysis identified the functional groups present in each sample and revealed how the structure of bleached softwood fibers changed. Figure 5 contains several absorption peaks of the BSKP fiber and enzymatic hydrolysis residues. The band at 896 cm^{-1} was assigned to the β-glycosidic linkages between glucose unites in the cellulose (Oh *et al*. 2005). The peak that emerged in the region of 1034 cm^{-1} in all the spectra was ascribed to the xyloglucans fractions associated with non-hydrolyzed hemicelluloses strongly bound within the cellulose microfibrils (Viikari *et al*. 1994). Furthermore, the spectrum of the untreated pulp differed from the enzymatic hydrolysis residues by having prominent

absorption bands at 1663 to 1665 cm^{-1}, which were ascribed to a C=O stretch in aryl ketones. These results proved that the feedstock contains not only cellulose, but also other two biopolymers, namely hemicellulose and lignin (Adel *et al*. 2011). In addition, FTIR spectra demonstrated reduced or disappeared characteristic absorption peaks at 811 cm^{-1}, 1056 cm^{-1}, 1161 cm^{-1} and 1460 cm^{-1}, which were attributed to galactoglucomannan, benzene stretching vibrations (lignin), C-O-C asymmetric stretching vibrations, and -CH_2 deformation vibrations, respectively (Wong *et al*. 1996). Thus, the addition of enzymes weakened or removed the absorption peaks related to lignin and hemicellulose and decreased amorphous components in the pulp.

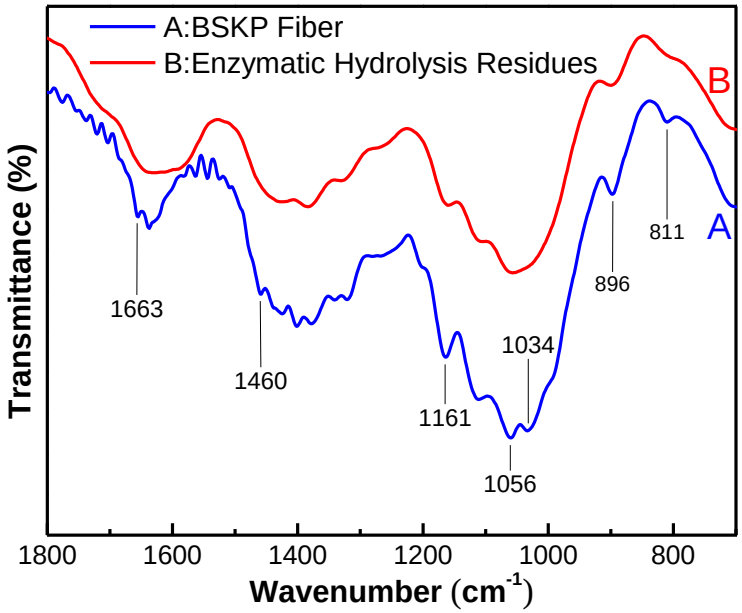

Fig. 5. FTIR spectra patterns of the untreated pulp and enzymatic hydrolysis residues

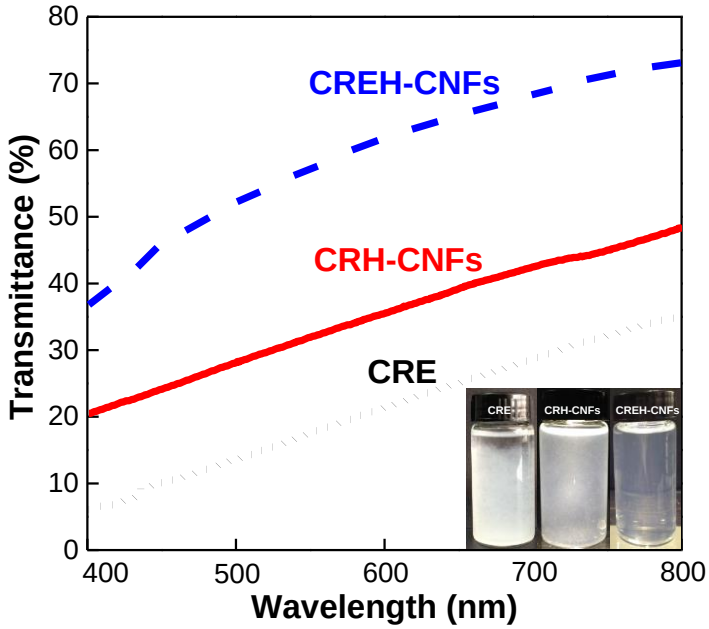

Fig. 6. UV-vis light transmittance of suspension from CRE, CRH-CNFs, and CREH-CNFs

UV-vis Light Transmittance

The visual appearance and light transmittance at 400 to 800 nm wavelength for different suspensions using CRE, CRH, and CREH treatment is shown in Fig. 6. Compared with CRE, samples subjected to homogenization possessed higher transmittance and were well dispersed in aqueous solution. Thus, the effect of homogenization on transmittance was obvious. The most transparent suspension was obtained from CREH-CNFs, which was consistent with the SEM image (Fig. 2f). This was due to the removal of hemicellulose and lignin and more accessible hydroxyl groups resulting from a microfibril liberation action (Pääkkö *et al*. 2007). As a consequence, transmittance strongly depended on the degree of fibrillation and chemical composition.

CONCLUSIONS

1. Enzyme-assisted mechanical fibrillation was successfully used to produce well-dispersed and uniform-sized CNFs with a yield of 79.15% from BSKP fibers.
2. Mechanical pretreatment before enzyme hydrolysis exposed disordered regions of the fiber and made them more accessible to enzymatic attack.
3. Enzymes removed most of the hemicelluloses and lignin present in the pulp, reducing the amount of clogging that occurred in subsequent homogenization.
4. Enzymatic treatment and mechanical fibrillation had dramatic effects on the morphological properties and water retention values of each sample. However, these differences had limited influence on the degree of crystallinity.
5. Compared with CNFs without enzymatic treatment, CNFs obtained from mild enzyme hydrolysis and mechanical fibrillation appeared to be thinner or less entangled networks and more individualized in SEM images and had higher transmittance in aqueous solutions.

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