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Enhancement of enzymatic saccharification of cellulose by cellulose dissolution pretreatments

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ABSTRACT

Attempts were made to enhance cellulose saccharification by cellulase using cellulose dissolution as a pretreatment step. Four cellulose dissolution agents, NaOH/Urea solution, *N*-methylmorpholine-*N*-oxide (NMMO), ionic liquid (1-butyl-3-methylimidazolium chloride; [BMIM]Cl) and 85% phosphoric acid were employed to dissolve cotton cellulose. In comparison with conventional cellulose pretreatment processes, the dissolution pretreatments were operated under a milder condition with temperature <130 °C and ambient pressure. The dissolved cellulose was easily regenerated in water. The regenerated celluloses exhibited a significant improvement (about 2.7- to 4.6-fold enhancement) on saccharification rate during 1st h reaction. After 72 h, the saccharification yield ranged from 87% to 96% for the regenerated celluloses while only around 23% could be achieved for the untreated cellulose. Even with high crystallinity, cellulose regenerated from phosphoric acid dissolution achieved the highest saccharification rates and yield probably due to its highest specific surface area and lowest degree of polymerization (DP).

1. Introduction

Cellulose is an abundant renewable polysaccharide consisting of a linear chain of (1→4) linked β-D-glucopyranose units aggregated to form a highly ordered structure due to its chemical constitution and spatial conformation. The individual cellulose chains are joined by a network of inter- and intra-molecular hydrogen bonding and van der Waals forces. The high order structure and crystallinity of cellulose makes it recalcitrant to hydrolysis. Since cellulose hydrolysis in the context of conversion renewable biomass to fuels and chemicals via fermentation is a very important step, its study has already attracted a substantial research effort. It is generally accepted that the highly ordered structure of cellulose has to be disrupted in a pretreatment step in order to hydrolyze cellulose efficiently into fermentable sugars for the biofuels or other bio-products fermentation. Cellulase is most often employed to hydrolyze cellulose to glucose because of its mild reaction condition and specific action in hydrolysis, producing virtually no glucose degradation products. Cellulose hydrolysis by cellulase involves three steps: cellulase adsorption onto cellulose surface, subsequent breakdown of cellulose through the synergistic action of the endo- and exo-cellulase, and cellulase desorption from cellulose residue into supernatant. The accessibility of cellulase to the limited adsorption sites on crystalline cellulose structure is generally believed to play an important role in determining the cellulose hydrolysis rate. To increase the surface area accessible to cellulases adsorption, cellulose substrate is often subjected to physical or chemical pretreatments that disrupt the tight packing arrangement of cellulose fibrils in the crystalline domains.

Dissolution of cellulose by a cellulose solvent is by no means considered as a most facile method to disrupt the crystalline structure of cellulose. To date, many derivative and non-derivative solvents for cellulose dissolution have been found (Heinze & Koschella, 2005). In order to be recognized and hydrolyzed by cellulase, only non-derivative solvents can be used to prepare regenerated cellulose for enzymatic hydrolysis. Recently, non-derivative reagents such as ionic liquids (Dadi, Varanasi, & Schall, 2006; Liu & Chen, 2006; Rayne & Mazza, 2007), *N*-methyl-morpholine-*N*-oxide (NMMO) (Kuo & Lee, 2009), and concentrated phosphoric acid (Wei, Kumar, & Banker, 1996; Zhang, Cui, Lynd, & Kuang, 2006; Zhang et al., 2007) have been employed for cellulose dissolution. The celluloses regenerated by rapid precipitation of the dissolved cellulose dopes with an anti-solvent such as water have demonstrated a great improvement on enzymatic hydrolysis kinetic. In addition to the costly cellulose dissolution reagent such as ionic liquids and NMMO, recently, the inexpensive NaOH/urea aqueous solutions was found that can also dissolve cellulose directly and quickly at subzero temperature (Cai et al., 2004; Ruan, Zhang, Zhou, Jin, & Chen, 2004). Based on its cellulose dissolution capability, lignocellulosic material has been pretreated with NaOH/urea aqueous solution at low temperature to enhance enzymatic hydro-

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lysis performance (Zhao, Wang, Zhu, Ragauskas, & Deng, 2008). Compared with the conventional pretreatments such as dilute acid, steam explosion, organosolv, and etc. (Mosier et al., 2005; Pan et al., 2005), the mild dissolution condition with temperature <130 °C and ambient pressure is the main feature of cellulose dissolution pretreatment. Due to its mild operation condition, cellulose dissolution has the potential to become an important pretreatment method for enhancing enzymatic hydrolysis of cellulose. To date, however, comparison between the performances of enzymatic hydrolysis of cellulose regenerated from these dissolution processes has not been reported. Information on the structural features of these regenerated celluloses has been limited. The relationship between regenerated cellulose structure and its enzymatic hydrolysis kinetic also remains unclear.

In this paper, NaOH/urea, NMMO, ionic liquid [BMIM]Cl and concentrated phosphoric acid (85% w/v) were employed as cellulose dissolution agents for the pretreatment of cotton cellulose. The effects of these cellulose dissolution pretreatments on enhancing enzymatic saccharification were compared. The crystalline structure of the celluloses regenerated from dissolution preparations were investigated by using Fourier transformed infrared spectrophotometry (FTIR) and X-ray diffraction (XRD). Furthermore, the influence of particle size, surface area, degree of polymerization (DP) of the regenerated celluloses on enhancing enzymatic hydrolysis was also studied.

2. Material and methods

2.1. Materials

Defatted cotton was obtained from Tong Ho Ltd (Taiwan). *N*-Methylmorpholine- *N*-oxide (NMMO) was obtained from Sigma (St. Louis, MO). Ionic liquid 1-butyl-3-methylimidazolium chloride ([BMIM]Cl) and ortho-phosphoric acid were obtained from Acros (NJ, USA). Cellulase AP3 (8.5 FPU/g) from *Aspergillus niger* strain was supplied by Amano Enzyme Inc. (Nagoya, Japan). All other reagents and chemicals, unless otherwise noted, were analytic grade.

2.2. Cellulose dissolution pretreatment and regeneration

2.2.1. NaOH/urea

Cotton cellulose was cut into small pieces ($0.5 \times 0.5\ cm^2$) and 0.5 g (5% w/w) of cellulose was soaked in a 10 ml solution of 12% w/v urea and 7% w/v NaOH stirred at 100 rpm for 10 min. The slurry was then cooled to −20 °C overnight. After the frozen cellulose slurry was thawed, 60 ml deionized water was added to precipitate the dissolved cellulose. After well-mixed for 30 min, the mixture was filtered with filter paper to collect the precipitated cellulose and washed thoroughly with 150 ml deionized water.

2.2.2. NMMO and [BMIM]Cl

Cellulose solution of 5% w/w was prepared by mixing 0.5 g cotton with 9.5 g NMMO monohydrate or 9.5 g [BMIM]Cl in a 130 °C oil bath at 100 rpm for 20 min. At the end of dissolution, deionized water of 60 ml was rapidly added to the stirred cellulose solution. The stirring continued until the temperature cooled down to the room temperature. The resulting mixture was filtered with filter paper to collect the precipitated cellulose and washed thoroughly with 150 ml deionized water.

2.2.3. Phosphoric acid

Cellulose solution of 5% w/w was prepared by mixing 0.5 g cotton with 5.6 ml (9.5 g) of 85% phosphoric acid in a 50 °C water bath at 100 rpm for 60 min. At the end of dissolution, deionized water of 60 ml was rapidly added to the stirred cellulose solution. The stirring continued until the temperature cooled down to the room temperature. The resulting mixture was filtered with filter paper to collect the precipitated cellulose and washed thoroughly with 150 ml deionized water. All of the washed precipitates were considered as regenerated celluloses and subjected to cellulase hydrolysis.

2.3. Enzymatic hydrolysis

The regenerated cellulose of 0.5 g (based on initial loading in dissolution pretreatment) was suspended in 25 ml of pH 5, 100 mM phosphate buffer supplemented with 0.02% sodium azide. Cellulase AP3 concentration of 10 FPU/g cellulose was used in the hydrolysis reaction. The hydrolysis was carried out at 50 °C under magnetic stirring and the release of soluble reducing sugars was periodically measured by the DNS assay. The reducing sugars content in Cellulase AP3 preparation was also measured as the background concentration and subtracted from that measured during enzymatic hydrolysis reaction. A conversion factor of 1.11 was used to calculate the amount of glucose released from the amount of cellulose consumed.

2.4. Analysis

Reducing sugar was measured by the DNS assay (Miller, 1959). Glucose was used as a standard for the reducing sugar measurement. Duplicate were made for each hydrolysate sample. Cellulase activity was determined by the standard filter paper assay and expressed as filter paper units per gram of glucan (FPU) (Ghose, 1987). One FPU is defined as the enzyme that releases 1 μmol of glucose equivalents per minute from Whatman No.1 filter paper. The regenerated cellulose was freeze-dried to maintain the water swelling state and prevent the structure collapse for FTIR, XRD, BET and DP assay. Fourier transformed IR (FTIR) was measured by BIO-RAD FTS-3500 spectrometer. The spectra (4000–400 cm^{-1}) were recorded with a resolution of 4 cm^{-1} and 64 scans per sample. About 2 mg samples were prepared by mixing with 120 mg of spectroscopic grade KBr then pressed in a standard device using a pressure of 6000 Ψ to produce 13 mm diameter pellets. The background spectrum of pure potassium bromide was subtracted from that of the sample spectrum.

Crystallinity was determined by X-ray diffraction using a Rigaku X-ray diffractometer (Rigaku Co., Japan). Samples of 50 mg were pressed in a standard device using a pressure of 6000 Ψ to produce 13 mm diameter pellets and scanned at 2°/min from $2\theta = 10°–30°$. The crystallinity index, C_r, which represents the percentage of crystalline materials was calculated from the XRD patterns by an empirical method based on that of Nelson and Oconner (Nelson & O'Connor, 1964b),

$$C_r = \frac{I_{cr} - I_{am}}{I_{cr}}$$

where I_{cr} is the diffraction intensity at peak position $2\theta° \approx 22.6°$ for cellulose *I*, 21.7° for cellulose II and I_{am} is the intensity at suitable locations for the amorphous background ($2\theta° \approx 19°$ for cellulose *I*, 16° for cellulose (II). The specific surface area was measured by BET method using Autosorb-1 (Quantachrome Co., USA). The samples were degassed for 3 h at 60 °C before analysis. The analyzed relative pressures (P/P_0) were in the range of 0.05–0.985.

DP measurement was based on the insoluble fibers reducing power (IFRP) determined by the Nelson–Somogyi method (Nelson, 1944). It is calculated according to the equation DP = TC × 1.1/IFRP, where TC is the total cellulose weight and 1.1 is the correction factor that expresses cellulose as glucose equivalents (Pala, Mota, & Gama, 2007). Five replicas were made for each sample.

3. Results and discussion

3.1. Cellulose dissolution and hydrolysis

Transparent cellulose dope of 5% (w/w) was readily prepared from cotton cellulose by using dissolution agents NMMO and [BMIM]Cl at 130 °C, and 85% phosphoric acid at 50 °C. However, the cellulose solution prepared by dissolving cellulose in NaOH/Urea aqueous solution at subzero temperature appeared to be turbid. This indicates that unlike previous reports on cellulose dissolution in NaOH/Urea aqueous solution (Jin, Zha, & Gu, 2007), cotton cellulose because of its high DP (~2000) (Wang, Zhao, & Deng, 2008) can not readily be dissolved. After precipitating with excess amount of water, the regenerated cellulose obtained either from transparent cellulose dope or turbid mixture was thoroughly washed and subjected to enzymatic hydrolysis without further drying. As shown in Fig. 1, the cellulose dissolution pretreatment greatly enhances the hydrolysis of cellulose. After 1st h enzymatic hydrolysis, approximately 6.2, 7.2, 8.1 and 10.6 mg/ml of reducing sugars were released from cellulose regenerated from NaOH/Urea, NMMO, [BMIM]Cl and phosphoric acid dissolution pretreatment, respectively. In contrast, only 2.3 mg/ml of reducing sugars were released from the untreated cotton. In other words, at least 2.5-fold enhancement on initial hydrolysis rate can be achieved by applying cellulose dissolution pretreatment. No appreciable reducing sugars accumulation was observed for the untreated cotton after 12 h even though the saccharification conversion was only approximately 20%. In contrast, the significant accumulation of released reducing sugars continued for 7 h, 12 h, 24 h, and 48 h, for cellulose regenerated from phosphoric acid, [BMIM]Cl, NMMO, and NaOH/urea, respectively. The final conversion in the range of 85–95% was achieved after 72 h hydrolysis of these regenerated celluloses. This implies that approximately 4-fold enhancement on cellulose saccharification conversion can be achieved by employing cellulose dissolution pretreatment. The effectiveness of cellulose dissolution pretreatments on enhancing regenerated cellulose saccharification by cellulase followed the order of phosphoric acid > [BMIM]Cl > NMMO > NaOH/urea.

3.2. Effect of crystallinity

Since the solid-state structures including crystalline and non-crystalline structures, and others have great influence on cellulose hydrolysis kinetic, the crystallinity and crystal structures of the cellulose regenerated from dissolution preparations were studied. Infrared spectra in the 850–1500 cm^{-1} region have been used to characterize the polymorphs of highly crystalline cellulose (Nelson & O'Connor, 1964a). Several general trends relating cellulose structures and infrared spectra features have been found. The absorption band at 1430 cm^{-1} assigned to the CH_2 scissoring motion is strong in type I crystalline (cellulose I); very weak and shifts to 1420 cm^{-1} in type II crystalline (cellulose II) and amorphous cellulose. The absorption band at 897 cm^{-1} assigned as C–O–C stretching at the β-(1→4)-glycosidic linkage is weak and broad in cellulose I; strong and sharp in cellulose II and amorphous cellulose. Hence, the Fourier transformed IR spectra in the 700–1500 cm^{-1} region was employed to characterize the structure of cotton celluloses regenerated from dissolution pretreatment. As shown in Fig. 2, the absorption band at 1430 cm^{-1} was strong for untreated cotton, but weak for the regenerated celluloses. Besides, the band at 897 cm^{-1} was weak for untreated cotton, but strong for regenerated celluloses. According to the aforementioned findings, the spectra shown in Fig. 2 indicates that the untreated cotton crystalline possesses a cellulose I crystal type which is in accord with the well-known fact that almost all native celluloses in the higher plants have the crystal structure of cellulose I. It also indicates that the cellulose I structure was transformed into amorphous or cellulose II structure after cotton cellulose was regenerated from dissolution pretreatment. Since the absorbance at 1430 cm^{-1} and 897 cm^{-1} are quite sensitive to the crystal structure of cellulose, the absorbance ratio A1430/A897 or lateral order index (LOI) has been used to reflect the cellulose I fraction in the cellulose structure (Oh et al., 2005). As shown in Table 1, the LOI of cotton cellulose decreased significantly from 1.32 to 1.04, 1.11, 1.14 and 1.21after regeneration from NaOH/urea, NMMO, [BMIM]Cl and phosphoric acid dissolution, respectively. The higher LOI of the P-cellulose (cellulose regenerated from phosphoric acid

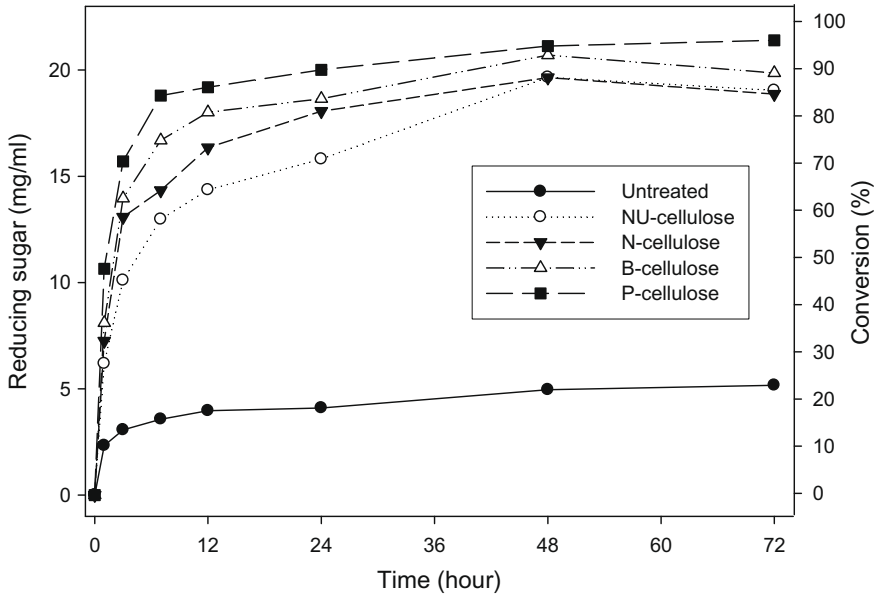

Fig. 1. Reducing sugars released during enzymatic hydrolysis of untreated and dissolution pretreated celluloses. Hydrolysis condition: 20 mg/ml cellulose, Cellulase AP3 10 FPU/g cellulose, and 50 °C. NU-, N-, B-, and P-cellulose are cellulose regenerated from NaOH/Urea, NMMO, [BMIM]Cl, and 85% phosphoric acid dissolution, respectively.

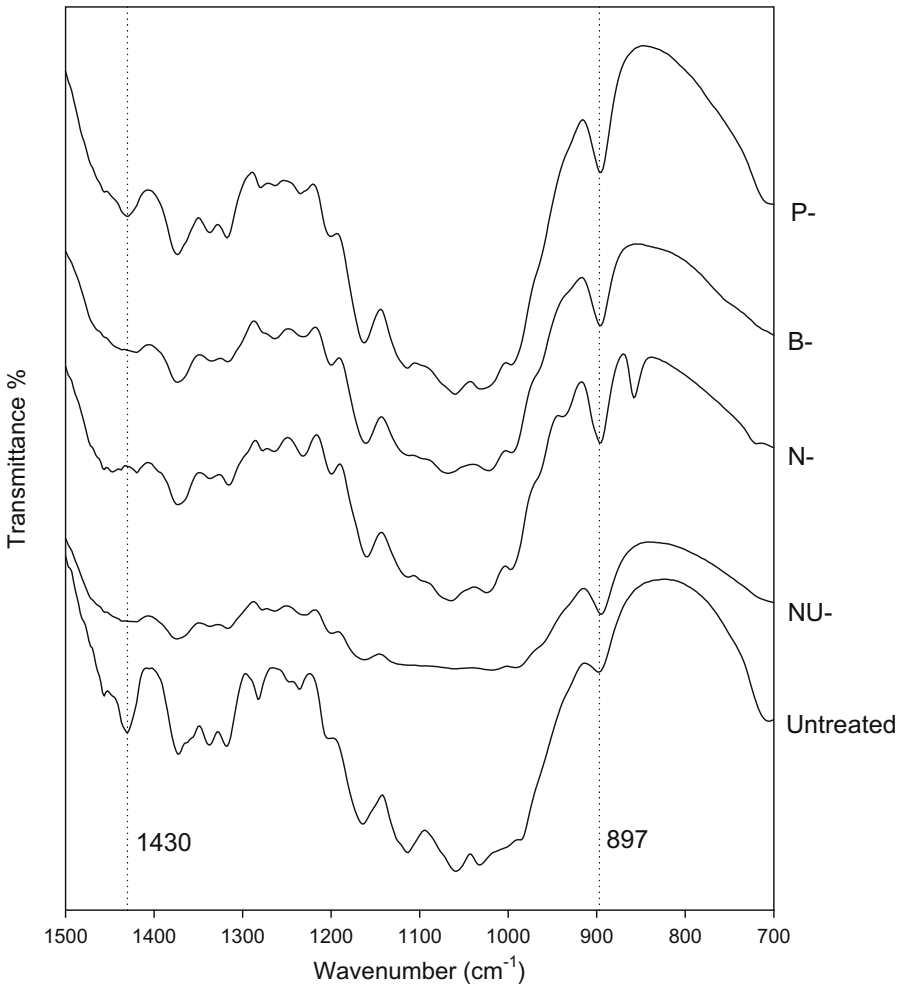

Fig. 2. FTIR spectra of untreated and regenerated cotton cellulose. NU-, N-, B-, and P-cellulose are cellulose regenerated from NaOH/Urea, NMMO, [BMIM]Cl, and 85% phosphoric acid dissolution, respectively.

Table 1
The structure features for untreated cotton and regenerated cotton from different dissolution agents.

Sample	FTIR	Crystallinity(XRD)		BET area	DP
	LOI(A1430/A897)	C_{rI}	C_{rII}	m^2/g	
Native Cellulose	1.32	0.84	0.32	n.a.	994
[a]NU-Cellulose	1.04	0.20	0.64	n.a.	1129
N-Cellulose	1.11	−0.12	0.37	n.a.	460
B-Cellulose	1.14	−0.18	0.42	n.a.	118
P-Cellulose	1.21	0.53	0.48	31.64	38

[a] NU-, N-, B-, and P-Cellulose are cellulose regenerated from NaOH/Urea, NMMO, [BMIM]Cl, and phosphoric acid dissolution, respectively. n.a. not available.

dissolution) indicates it contains a higher fraction of cellulose I as compared with other regenerated celluloses.

In addition to FTIR, XRD was also employed to investigate the crystallinity of the regenerated cellulose samples. As shown in Fig. 3, the native cotton cellulose has typical cellulose I diffraction angles around 22.6°, 16.4°, and 14.7°. Once regenerated from NaOH/Urea dissolution (NU-cellulose), the XRD pattern showed a broad and overlapped peak with diffraction angles around 21.7° and 20.1°, and a small peak with diffraction angles around 12.1°. Since these diffraction angles are the characteristic angles for cellulose II crystal structure (Gilbert, 1994), the NU-cellulose was considered to have a cellulose II structure. This agrees with the general finding that mercerization (alkaline treatment) will transform cellulose I into cellulose II crystal structure. For the cellulose regenerated from [BMIM]Cl and NMMO dissolution (B-cellulose and N-cellulose), a quite flat diffraction pattern was obtained which indicates an amorphous structure. In contrast, distinct peaks with diffraction angles around 22.6°, 20.1°, 16.4°, and 14.7° were obtained for the P-cellulose. This implies that the crystal structure of P-cellulose is consisted of cellulose I and II. The empirical crystallinity index, Cr, which represents the relative crystalline content in a cellulose is also shown in Table 1. For the cellulose I content, the native cotton cellulose has a highest C_{rI} value around 84%. NaOH/urea dissolution reduces the C_{rI} value to around 21%. The NMMO and [BMIM]Cl dissolution significantly decrease the C_{rI} of N-cellulose and B-cellulose to a value lower than 0%. In contrast, the P-cellulose has a C_{rI} value around 54% indicates a considerable amount of cellulose I was maintained. The C_r value for cellulose II follows the order of NU-cellulose (64%) >>P-cellulose (48%) >B-cellulose (42%)>N-cellulose (37%)> native cellulose (32%). This indicates that NU-cellulose, as expected, possesses a higher fraction of cellulose II structure. Even though qualitatively the XRD patterns of N- and B-cellulose appear completely amorphous, the C_{rII} values of 37–42% represent that an appreciable amount of cellulose II remained as their crystalline structure. It has been reported that cellulose I is much more difficult to be hydrolyzed in comparison with cellulose II or amorphous cellulose (Hong, Ye, & Zhang, 2007; Rahkamo, Viikari, Buchert, Paakkari, & Suortti, 1998). The mostly amor-

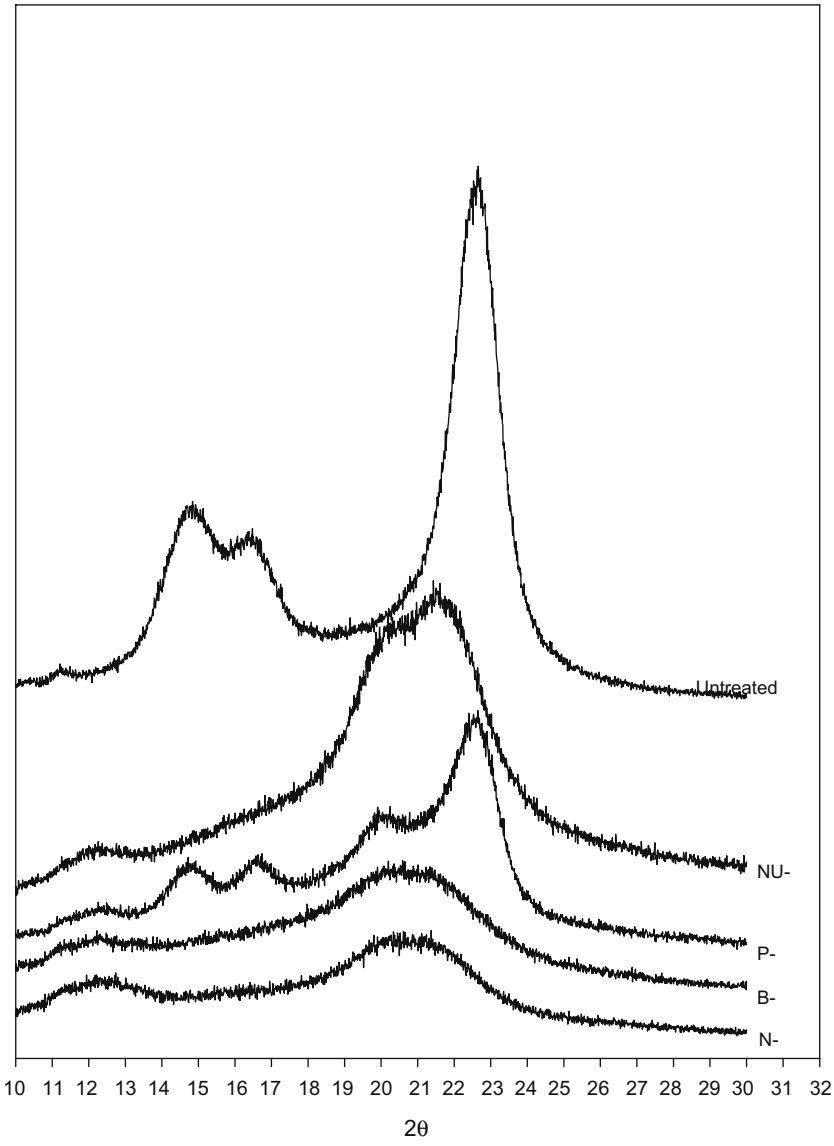

Fig. 3. XRD patterns of untreated and regenerated cotton cellulose. NU-, N-, B-, and P-cellulose are cellulose regenerated from NaOH/Urea, NMMO, [BMIM]Cl, and 85% phosphoric acid dissolution, respectively.

phous and cellulose II structure, therefore, can explain the enhancement observed on NU-, N-, and B-cellulose hydrolysis (Fig. 1). The P-cellulose, on the other hand, had a quite high C_r value for both cellulose I and II. This indicates that the high crystallinity of P-cellulose is mainly consisted of cellulose I and II. With the high content of cellulose I (C_{rI} value of 54% vs. 84% for native cellulose), P-cellulose was expected to have a hydrolysis rate and yield lower than other regenerated celluloses. On the contrary, as shown in Fig. 1, P-cellulose demonstrates a highest hydrolysis rate and yield. Evidently, the crystallinity and crystal type of cotton cellulose alone can not explain the observed hydrolysis enhancement.

3.3. Effect of surface area and degree of polymerization

In order to explain the high hydrolysis rate attained by the high crystalline P-cellulose, the surface area and degree of polymerization of all regenerated cellulose samples were examined. As shown in Table 1, only the specific surface area of P-cellulose could be detected (~30 m^2/g) which is approximately 30-fold higher than that of the microcrystalline Avicel (0.55–1.04 m^2/g) (Kocherbitov, Ulvenlund, Kober, Jarring, & Arnebran, 2008). All other samples have a specific surface area too small to be detected by our BET system (detection limit ~0.1 m^2/g). It has been reported that the particle size of regenerated cellulose strongly depends on its regeneration rate (Nelson & Deng, 2007). The faster regeneration rate is, the smaller particle size will be obtained. Therefore, instant regeneration was carried out by adding 60 ml water at one time into the 10 g stirred cellulose dopes. In comparison with other regenerated celluloses, P-cellulose demonstrates a much smaller average particle size (ca. 2.7 μm) as determined by laser particle analyzer (Photal LPA-3100, Ostuska Electronic, Osaka, Japan).

Except for NU-cellulose, the DP of regenerated cellulose decreased significantly. N-, B-, and P-cellulose have a DP approximately 1/2, 1/10, 1/30 of that of the untreated cotton cellulose, respectively. This indicates the cellulose chain will be degraded to certain extent during the harsh dissolution condition (130 °C for NMMO and [BMIM]Cl, 50 °C for 85% phosphoric acid). Since the DP was determined by measuring the insoluble cellulose reducing power, the lower DP represents the existence of a higher amount of reducing ends that will provide more sites for exo-cel-

lulase to initiate the cleavage of cellulose. The mostly amorphous structure and reduced DP of B- and N- cellulose, therefore, lead to a high enzymatic hydrolysis enhancement. It is interesting to be noted that P-cellulose not only has a high fraction of crystalline structure but also possesses a very low DP. The significantly lowered DP was evidently resulted from the extensive hydrolysis of cellulose by the 85% phosphoric acid during dissolution. The high crystallinity of P-cellulose could also be explained based on its low DP because the smaller cellulose molecule is the easier to be packed into crystal structure during its regeneration. A similar result has also been reported that microcrystalline cellulose with crystalline index high as 0.8 could be prepared by dissolving cellulose in phosphoric acid at 55 °C (Wei et al., 1996). Even though P-cellulose maintains a higher fraction of crystalline structure, the synergetic effect of high specific surface area and low DP on cellulose cleavage by cellulase leads to a highest hydrolysis rate and yield obtained (Fig. 1). It is generally believed that crystalline structure of cellulose determines its easiness of enzymatic hydrolysis. But, according to our results it can be concluded that the synergetic effect of surface area and DP plays a more important role than that of crystalline structure on enhancing cellulose hydrolysis by cellulase.

4. Conclusion

Significant enhancement on reducing sugars releasing rate and hydrolysis yield could be achieved during enzymatic hydrolysis of celluloses regenerated from NaOH/Urea, NMMO, [BMIM]Cl and 85% phosphoric acid dissolution preparations. The significant hydrolysis enhancement of NU-cellulose was mainly resulted from crystal structure change from cellulose I to an easier digestive cellulose II structure. The enhancement on N- and B-cellulose hydrolysis was mainly due to their amorphous structure. In contrast, the P-cellulose had a high crystalline structure but the synergetic effect of the highest specific surface area and lowest DP leads P-cellulose still could achieve a highest hydrolysis rate and yield among these four regenerated cellulose.

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