CELLULOSE PRETREATMENT WITH 1-METHYL-3-METHYLIMIDAZOLIUM DIMETHYLPHOSPHATE FOR ENZYMATIC HYDROLYSIS

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This work has been focused on a cellulose pretreatment process using 1-methyl-3-methylimidazolium dimethylphosphate ([DMIM]DMP) for subsequent hydrolysis over cellulase. Different operational variables (the amount of [DMIM]DMP, the pretreatment temperature, the pretreatment period) affecting the pretreatment were investigated. Additionally, the crystallinity index (CI) was characterized by FT-IR spectroscopy. The CI values including CI(IR), CI(IR-C**I**) and CI(IR-C**II**) were calculated. When correlated with these values, the concentrations of total reducing sugar (TRS) released after the pretreatment of microcrystalline cellulose (MC) were found to show a distinct relationship with the [CI(MC-C**I**) – CI(IR-C**I**)] values, and the result was verified by X-ray diffraction (XRD). Consequently, the optimum pretreatment conditions of crystalline transformation (from cellulose **I** to cellulose **II**) characterized by XRD are different from those of hydrolysis. This result suggests that other factors, in addition to cellulose crystallinity, affect the yields of glucose and total reducing sugar obtained by hydrolysis.

Keywords: enzymatic hydrolysis, crystallinity index, ionic liquid, pretreatment

INTRODUCTION

The rapid consumption of fossil fuel resources has motivated extensive research on biofuels and biochemicals.[1,2] Cellulose is the most abundant carbohydrate component of biomass and it is of particular interest as a kind of renewable and sustainable material for providing monomeric sugars for fermentation into fuels and biochemicals.[3] However, owing to the extensive network of inter- and intra-molecular hydrogen bonding between its fibrils, cellulose is difficult to dissolve in either water or most organic solvents. It is, thus, recalcitrant to hydrolyzing into individual glucose subunits.[4,5] Therefore, the cellulose pretreatment process is indispensable to make cellulosic materials more susceptible to hydrolysis.[6] Up to now, numerous methods have been used for cellulose pretreatment to enhance the digestibility of cellulosic materials.[7,9] Although the pretreatment methods, including alkali, acid, steam explosion and metal complex, are effective, they are highly energy-demanding processes and often require harsh conditions.[10] Consequently, an effective and economical pretreatment method is prospective.

In recent years, a new type of non-volatile solvent, namely ionic liquids (ILs), has been investigated as a powerful solvent in dissolving cellulose.[11,12] The cellulose regenerated from ILs has an essentially amorphous and porous structure. Thus, it can be easily degraded by cellulase.[13] Overall, this process is easier to operate, more environmentally friendly and less energy-demanding than current commercial dissolution methods, such as the viscose method and the acid pretreatment process.[14,15] As summarized in recent papers,[16,17] studies have mainly been focused on two chloride-based ILs – 1-butyl-3-methylimidazolium chloride ([bmim]Cl) and 1-allyl-3-methylimidazolium chloride ([amim]Cl). However, both ILs have

shortcomings: the former is a corrosive, toxic and extremely hygroscopic solid, while the latter is a viscous liquid bearing reactive side-chains. Thus, Zhou *et al*.[18] filed a patent on the synthesis of imidazolium-based dialkylphosphate ILs, which have the advantages of good thermal stability and easy manufacture on a commercial scale. However, the data on the pretreatment of cellulose with phosphate-based ILs are limited. Kamiya *et al*.[19] delivered initial information on the potential use of 1-ethyl-3-methylimidazolium diethylphosphate ([emim]DEP) as a solvent for lignocellulosic materials. Li *et al*.[20] reported that the digestibility of [emim]DEP-treated cellulose was twice that of water-treated one.

In this study, microcrystalline cellulose was pretreated with [DMIM]DMP and subsequently hydrolyzed over cellulase. In the pretreatment step, several operational variables affecting the pretreatment, including the pretreatment period, the pretreatment temperature and the amount of [DMIM]DMP, were investigated. Crystallinity index values were tracked by FT-IR. The correlation between these values and the concentrations of TRS obtained from the pretreatment process was evaluated. Until now, there has been no remarkable research correlating these two variables. Furthermore, from the XRD patterns, the crystalline transformation after the [DMIM]DMP pretreatment was determined.

EXPERIMENTAL

Materials

Microcrystalline cellulose was purchased from Sigma-Fluka Chemical Co. Cellulase from *Trichoderma reesei* was supplied by Beijing Solarbio Science & Technology Co. Ltd. (China). All other reagents were of analytical grade. [DMIM]DMP was synthesized as follows: equal molar amounts of trimethylphosphate and N-methyl-imidazole were added to a round-bottom flask and stirred vigorously at 140 °C for 3 h.[18] The mixture was moved to a rotary evaporator and vacuum dried at 150 °C for 4 h. The product was pure, judging by ^{1}H-NMR and ^{31}P-NMR.[21]

[DMIM]DMP pretreatment

0.5 g microcrystalline cellulose and 10-50 g [DMIM]DMP were charged into a round-bottom flask equipped with a heating jacket. Then the solution was incubated at temperatures varying from 80 to 140 °C, for 0.5, 1, 2 or 4 h. Ultrapure water as anti-solvent was added to the solution for regenerating microcrystalline cellulose from [DMIM]DMP. A precipitate immediately formed. The sample was briefly centrifuged. After separation, the precipitate was washed four times to remove the residual [DMIM]DMP completely. At this point, a conductivity meter was used to determine whether the residual [DMIM]DMP was completely removed. The conductivity of the supernatant should not exceed that of ultrapure water, otherwise there was some [DMIM]DMP left. The supernatant liquids (200 ml × 5) collected at this stage were quantitatively calculated as to the TRS yield, according to the equation:

TRS yield= (Reducing sugar weight/ Initial cellulose weight) x100

Various pretreatment conditions investigated in this study have been summarized in Table 1. Furthermore, the same pretreatment experiment was repeated at least 3 times.

Enzymatic hydrolysis

Cellulose (30 mg untreated or regenerated) was incubated with cellulase (30 FPU/g cellulose) in a 3 ml citrate buffer solution (50 Mm, pH 4.8) at 50 °C for 24 h. After centrifugation, a sample of 0.2 ml reaction mixture was withdrawn from the supernatant and diluted with 3 ml DNS solution and then it was placed in boiling water for 10 min.[22] And then the test tube was cooled in an ice-water bath. The cooled solution was diluted with water to 15 ml, and its absorbance was read against the blank reagent at 540 nm, as measured by a 722-visible spectrometer. The concentration of TRS was calculated by the DNS assay using D-glucose as a standard. The exact glucose concentration was determined by the glucose HK assay method.[23] All experiments were run in triplicate. Error bars show the standard deviation of triplicate measurements.

The yields of total reducing sugars and glucose from regenerated cellulose hydrolysis were calculated as follows:

TRS yield= (Reducing sugar weight/ Regenerated cellulose weight) x100

Glucose yield= (Glucose weight/ regenerated cellulose weight) x100

Table 1
Regenerated cellulose under different pretreatment conditions and crystallinity index (CI) values characterized by FT-IR spectroscopy

Run	[DMIM]DMP amount (g)	Pretreatment temperature (°C)	Pretreatment period (h)	Regenerated cellulose (g)*	CI(IR-C**I**)**	CI(IR-C**II**)***
1	10	100	1	0.4975	1.12	0.35
2	16.6	100	1	0.4971	1.49	1.27
3	25	100	1	0.4979	1.18	0.47
4	50	100	1	0.4986	1.02	0.53
5	16.6	100	0.5	0.4926	1.95	0.52
6	16.6	100	2	0.4946	1.69	0.19
7	16.6	100	4	0.4932	2.07	0.39
8	16.6	80	1	0.4960	1.62	0.54
9	16.6	120	1	0.4977	1.35	0.36
10	16.6	140	1	0.4968	1.30	0.53

*The amount of regenerated cellulose was determined by subtracting the TRS released after the pretreatment process from the initial cellulose (0.5 g); **The values of CI(IR-C**I**) were related to the ratios of A_{1431} and $A_{897,\ 894}$, which were determined by FT-IR spectra; ***The values of CI(IR-C**II**) were related to the ratios of A_{1263} and $A_{1202,\ 1200}$, which were determined by FT-IR spectra

Analytical methods

FT-IR analysis

FT-IR spectra were recorded using a Nicolet iS10 spectrometer with a detector at 8 cm^{-1} resolution at 32 scans per sample. Through OMNIC 8.0 peak resolve module, the FT-IR spectra (1520-850 cm^{-1}) were resolved into 14 bands according to the characteristic bands of cellulose **I** or cellulose **II**, by using the Gaussian distribution function. The characteristic bands of cellulose **I** (cellulose **II**), including 1431(1419), 1376(1373), 1337, 1317(1319), 1282(1278), 1263, 1236(1228), 1200(1202), 1165(1162), 1114, 1058, 1032(1019), 993(983) and 897(894) cm^{-1}, were summarized by Oh *et al*.[24] The crystallinity indexes, CI(IR-C**I**) for cellulose **I** and CI(IR-C**II**) for cellulose **II**, were evaluated by the ratios of the peak areas.

XRD analysis

The crystalline structures of the cellulose samples were analyzed by X-ray diffraction. The samples were scanned by an X'Pert MDP X-ray diffraction meter (Rigaku Co.) using Nickel filtered Cu-kα radiation generated at a voltage of 40 kV and a current of 50 mA. Scans were collected with a scan speed of 6°/min from 6° to 36°.

The determination of CI (XD) by XRD was carried out by the method of Jayme and Knolle.[25] CI (XD) was calculated by the equation:

$$CI\ (XD) = 1 - h_{am}/h_{cr} = 1 - h_{am}/\ (h_{tot} - h_{am})$$

where the crystalline height (h_{cr}) is the crystalline scatter of the 002 reflection at 22.7° for cellulose **I** or 101 reflection at 20.0° for cellulose **II**, amorphous height (h_{am}) is the crystalline scatter of the reflection at 18° for cellulose **I** or reflection at 16° for cellulose **II**.[25] With amorphous halo correction, the peaks were fitted by Microcal™ORIGIN™ program.[24] The values of CI(XD) were divided into CI(XD-C**I**) for cellulose **I** and CI(XD-C**II**) for cellulose **II**. And they were calculated by the following equations:

$$CI(XD\text{-}C\mathbf{I}) = [\sum A_{CI} / \sum (A_{CI}+A_{CII})] \times CI(XD)$$

$$CI(XD\text{-}C\mathbf{II}) = [\sum A_{CII} / \sum (A_{CI}+A_{CII})] \times CI(XD)$$

where the $\sum A_{CI}$ is the sum of peak areas of cellulose **I**, $\sum A_{CII}$ is the sum of peak areas of cellulose **II**.

RESULTS AND DISCUSSION

Effects of pretreatment variables on cellulose pretreatment and hydrolysis

Effect of the [DMIM]DMP amount on the pretreatment step

The microcrystalline cellulose (0.5 g) was pretreated with different amounts of [DMIM]DMP from 10 g to 50 g at 100 °C for 1 h, and cellulose was precipitated with ultrapure water. The regenerated cellulose was subsequently hydrolyzed over cellulase at 50 °C for 24 h. As a control experiment, untreated cellulose was hydrolyzed under the same conditions. As shown in Fig. 1a, the [DMIM]DMP pretreatment process is necessary and effective for cellulose hydrolysis over cellulase. The TRS yield of the 50 g [DMIM]DMP-treated cellulose was raised to 96.4%, whereas the TRS yield of untreated cellulose was of 46.8% (Fig. 1a), that is, the yield of TRS obtained by [DMIM]DMP-treated

cellulose hydrolysis was over twice that of TRS obtained by untreated cellulose hydrolysis. The yield of glucose slightly increased to 27.3% at 16.6 g [DMIM]DMP (microcrystalline cellulose, 3% w/w) (Fig. 1b). The TRS yield released after the pretreatment was raised to 0.58% at 16.6 g [DMIM]DMP-treated and then decreased (Fig. 1c). Considering the cost of [DMIM]DMP, the optimum [DMIM]DMP amount was selected at 16.6 g. Although it released more TRS in the pretreatment process at 16.6 g [DMIM]DMP, the maximum loss of the cellulose (the yield of TRS obtained in the pretreatment process) was less than 1%.

Effect of pretreatment temperature on the pretreatment step

Cellulose was treated in 16.6 g [DMIM]DMP at varied temperatures from 80 to 140 °C for 1 h, the regenerated cellulose was subsequently hydrolyzed by cellulase at 50 °C for 24 h. As seen in Fig. 2a, the TRS yield obtained by hydrolysis increased up to 98.0% with the pretreatment temperature. The yield of TRS appeared to be significantly affected by the temperature from 80 to 120 °C, though the TRS yield was slightly higher at 140 °C. The glucose yield rose to 29.8% at 120 °C and then glided down (Fig. 2b). The yield of TRS obtained after the pretreatment process decreased to a valley at 120 °C (Fig. 2c). Consequently, the optimum pretreatment temperature was selected at 120 °C. As explained above, the decrease of glucose concentration was attributed to the higher conversion of microcrystalline cellulose into the TRS in the pretreatment process.

Effect of pretreatment time on pretreatment step

The pretreatment time was varied from 0.5 h to 4 h for the samples treated with 16.6 g [DMIM]DMP at 100 °C. As shown in Fig. 3a, the yield of TRS obtained by hydrolysis over cellulase increased up to 96.4% with the pretreatment time, while the glucose yield reached 37.2% at 2 h, but decreased after 2 h (Fig. 3b). The yield of TRS released after the pretreatment was of 1.09% at 2 h and then increased (Fig. 3c). This implies that the more TRS released in the pretreatment process, the lower the glucose concentration obtained by the hydrolysis reaction. In order to obtain higher TRS and glucose for fermentation into biofuels and biochemicals, the optimum pretreatment time was 2 h.

Correlation of CI values of [DMIM]DMP-treated cellulose with TRS concentrations

The crystallinity indexes of the pretreated celluloses were characterized by FT-IR spectroscopy. The different chemical environments of hydrogen bonding in cellulose **I** structure or cellulose **II** structure can be measured by FT-IR spectroscopy.[26]

Therefore, FT-IR absorption gives some useful information related to the change of hydrogen bonding during crystalline transformation. As reported in recent years, $A_{1431}/A_{897,894}$ is related to the proportion of cellulose **I** and, the $A_{1263}/A_{1202,1200}$ represents the proportion of cellulose **II**.[27,30]

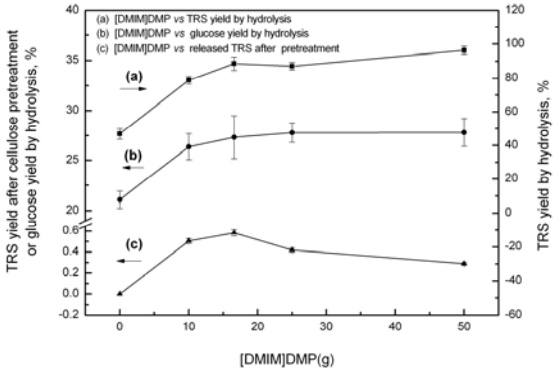


Figure 1: Effect of various amounts of [DMIM]DMP on the cellulose pretreatment step; (a) the yield of TRS obtained by the hydrolysis of cellulose (pretreated with various amounts of [DMIM]DMP at 100 °C) over cellulase at 50 °C for 24 h; (b) the yield of glucose obtained by the hydrolysis of the corresponding [DMIM]DMP-treated cellulose over cellulase at 50 °C for 24 h; (c) the yield of TRS released after cellulose pretreatment with [DMIM]DMP at 100 °C, 1 h

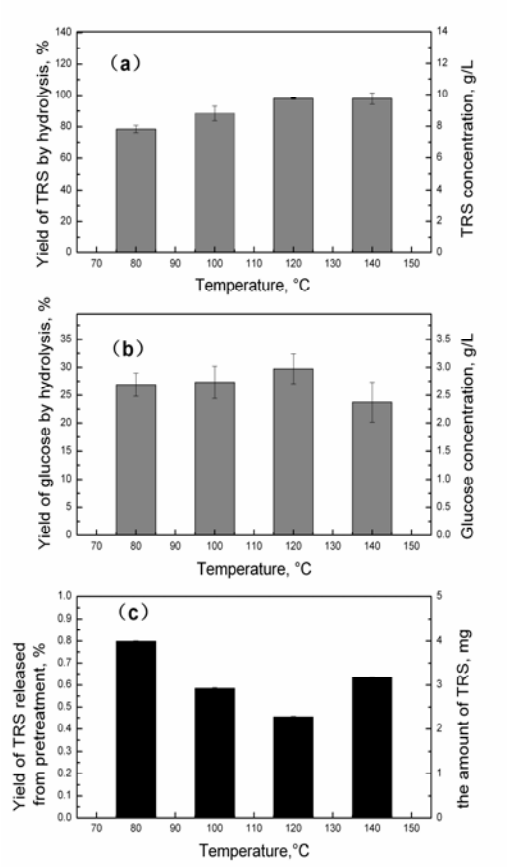


Figure 2: Effect of different pretreatment temperatures on the cellulose pretreatment step: (a) the yield of TRS obtained by the hydrolysis of [DMIM]DMP-treated cellulose (pretreated with 16.6 g [DMIM]DMP for 1 h at different temperatures) over cellulase at 50 °C for 24 h; (b) the yield of glucose obtained by the hydrolysis of the corresponding [DMIM]DMP-treated cellulose over cellulase at 50 °C for 24 h; (c) the yield of TRS released after cellulose pretreatment with 16.6 g [DMIM]DMP for 1 h

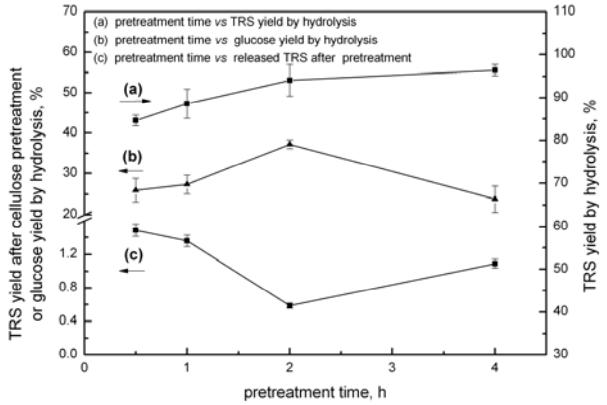


Figure 3: Effect of various pretreatment time periods on the cellulose pretreatment step: (a) the yield of TRS obtained by the hydrolysis of the [DMIM]DMP-treated cellulose (16.6 g [DMIM]DMP at 100 °C) over cellulase at 50 °C for 24 h; (b) the yield of glucose obtained by the hydrolysis of the corresponding [DMIM]DMP-treated cellulose over cellulase at 50 °C for 24 h; (c) the yield of TRS released after cellulose pretreatment with 16.6 g [DMIM]DMP, 100 °C

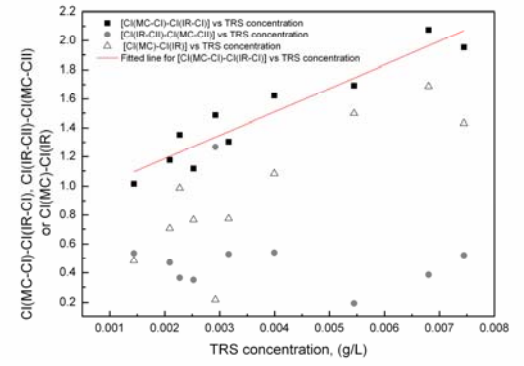
The TRS released after the pretreatment would result from the microcrystalline cellulose (MC). Thus, the differences between the CI values of microcrystalline cellulose (CI(MC), CI(MC-C**I**), CI(MC-C**II**)) and those of [DMIM]DMP-treated cellulose samples were calculated in order to investigate the effect of pretreatment conditions. As shown in Fig. 4, the TRS yield was found to show a linear relationship with the [CI(MC-C**I**) – CI(IR-C**I**)] value. The [CI(IR-C**II**) – CI(MC-C**II**)] values have leveled off with various TRS values. That is, the TRS released in the pretreatment process resulted from cellulose **I**. Since the CI value of the cellulose was the sum of the CI values of cellulose **I** and cellulose **II**, the trend of [CI(MC) – CI(IR)] values was similar to that of [CI(MC-C**I**) – CI(IR-C**I**)] values.

The result was verified by XRD analysis. With amorphous halo correction, XRD spectra peaks were fitted in the characteristic bands of cellulose **I** (14.7°, 16.8°, 20.5°, 22.7°) and cellulose **II** (12.1°, 20.0°, 21.9°) by Microcal^{TM}ORIGIN^{TM} program.[31,33] The correlation between CI(XD-C**I**) and CI(IR-C**I**) is shown in Fig. 5. The coefficient of 0.94 determined the CI(IR-C**I**) had a good linear relationship with CI(XD-C**I**). That is, the results characterized by the FT-IR assay are correctable.

Characterization of [DMIM]DMP-pretreated cellulose

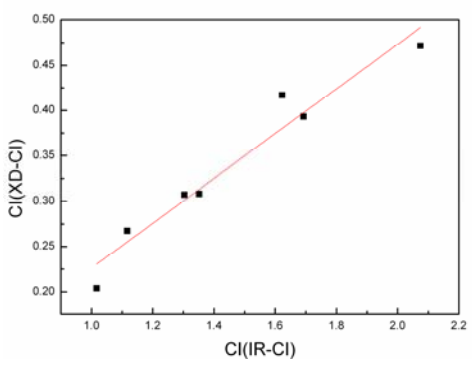
The XRD patterns of untreated sample and the [DMIM]DMP-treated samples under various pretreatment conditions are presented in Fig. 6. The characteristic peak of cellulose **I** at 22.7° disappeared. A broad asymmetric peak consisting of a doublet at 20.0° and 21.9° appeared, and a new peak emerged at 12.1°. These changes indicated the transformation from cellulose **I** to cellulose **II** over the [DMIM]DMP pretreatment.

The change of full width of half maximum (FWHM) is suggesting changes in crystallite size, misalignment of crystals. As shown in Fig. 7a, the curves similarly followed the trend of first rising to the highest point, then decreasing to a constant value, indicating increased ordering of the cellulose **II** lattice or an increase in the size of the cellulose **II** crystallites after the [DMIM]DMP pretreatment. As seen in Fig. 7b, the main peak shifted rapidly from cellulose **I** (22.7°) to cellulose **II** (20.0°) before 1 h, and then it leveled off. The main peak positions shifted to 20.0 only after 50 g [DMIM]DMP and 140 °C, that is, loosening crystallinity would work better if cellulose is pretreated with 50 g [DMIM]DMP at 140 °C for 1 h.



$R^2 = 0.89$

Figure 4: Correlation between concentration of total reducing sugars released in [DMIM]DMP pretreatment and the differences of CI values between microcrystalline cellulose (CI(MC), CI(MC-C**I**) or CI(MC-C**II**)) and [DMIM]DMP-treated cellulose (CI(IR), CI(IR-C**I**) or CI(IR-C**II**))



$R^2 = 0.94$

Figure 5: Correlation between CI(XD-C**I**) and CI(IR-C**I**)

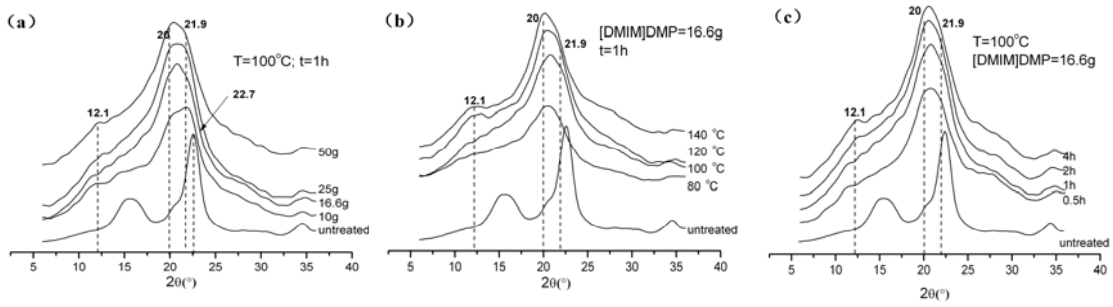


Figure 6: XRD patterns of untreated cellulose and cellulose samples pretreated in (a) various amounts of [DMIM]DMP at 100 °C for 1 h; (b) various pretreatment temperatures with 16.6 g [DMIM]DMP for 1 h; (c) various pretreatment time at 100 °C with 16.6 g [DMIM]DMP

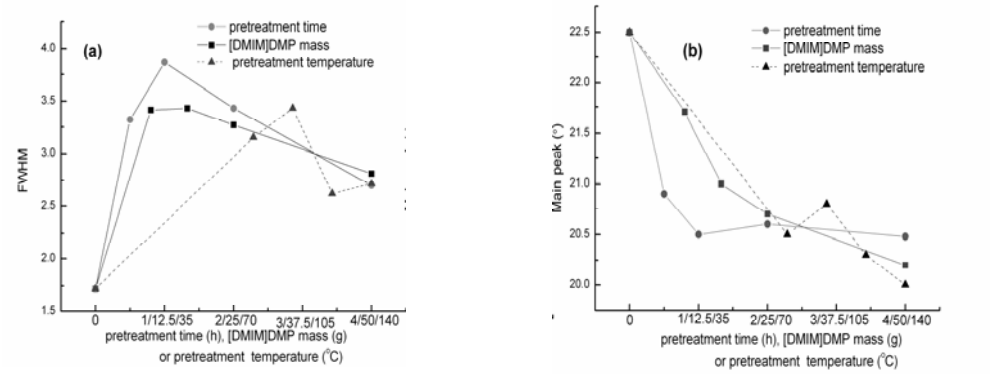


Figure 7: (a) FWHM of main peak of the XRD patterns of cellulose pretreated under different conditions; (b) main peak of the XRD patterns of cellulose pretreated under different conditions (solid dots represent samples pretreated with 16.6 g [DMIM]DMP at 100 °C for different pretreatment time; solid squares – samples pretreated at 100 °C for 1 h with different amounts of [DMIM]DMP; solid triangles – samples pretreated with 16.6 g [DMIM]DMP for 1 h at different pretreatment temperature

*The sections of the X axis tick label separated by slashes represent pretreatment time/the amount of [DMIM]DMP/pretreatment temperature, respectively, for example the tick label "1/12.5/35" represents pretreatment time of 1 h/pretreatment [DMIM]DMP – 12.5 g/pretreatment temperature – 35 °C

**The first data of the temperature curves represents untreated cellulose

Comparatively, this condition is different from the optimum pretreatment condition of hydrolysis which was investigated above. That is, the yield of TRS and glucose by hydrolysis may be governed by other characteristics, such as available surface area, degree of polymerization and particle size of the regenerated cellulose, in addition to cellulose crystallinity.[34,35]

CONCLUSIONS

The present study demonstrated that the pretreatment with [DMIM]DMP is necessary and effective for enzymatic hydrolysis. The optimum pretreatment conditions are the following: 0.5 g cellulose in 16.6 g [DMIM]DMP at 120 °C for 2 h. The TRS concentrations released after the pretreatment process were found to show a linear relationship with the [CI(MC-C**I**) – CI(IR-C**I**)] values. Additionally, the optimum pretreatment conditions of crystalline transformation are different from those of hydrolysis, which is suggesting that other factors impact the yield of TRS or glucose for hydrolysis, in addition to cellulose crystallinity, although at present, we have no direct evidence of that.

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REFERENCES

[1] H. W. Blanch, P. D. Adams, K. M. Andrews-Cramer, W. B. Frommer, B. A. Simmons and J. D. Keasling, *ACS Chem*. *Biol*., **3**, 17 (2008).
[2] T. L. Richard, *Science*, **329**, 793 (2010).
[3] G. Cheng, P. Varanasi, C. L. Li, H. B. Liu, Y. B. Melnichenko, B. A. Simmons, M. S. Kent and S. Singh, *Biomacromolecules*, **12**, 933 (2011).
[4] A. P. Dadi, S. Varanasi and C. A. Schall, *Biotechnol*. *Bioeng*., **95**, 904 (2006).
[5] M. Murakami, Y. Kaneko and J. Kadokawa, *Carbohydr*. *Polym*., **69**, 378 (2007).
[6] R. P. Chandra, R. Bura, W. E. Mabee, A. Berlin, X. Pan and J. N. Saddler, *Adv*. *Biochem*. *Eng*. *Biotechnol*., **108**, 67 (2007).
[7] R. Rinaldi and F. Schüth, *ChemSusChem*, **2**, 1096 (2009).
[8] D. Scordia, S. L. Cosentino, J. W. Lee and T. W. Jeffries, *Biomass Bioenerg*., **35**, 3018 (2011).
[9] R. Del, F. Luis, R. P. Chandra and J. N. Saddler, *Biotechnol*. *Bioeng*., **108**, 1549 (2011).
[10] N. Mosier, C. Wyman and B. Dale, *Bioresource Technol*., **96**, 673 (2005).
[11] M. P. Mauricio, M. L. Luciana, T. V. Doherty, R. J. Linhardt and J. S. Dordick, *Biotechnol*. *Bioeng*., **108**, 1229 (2011).
[12] K. Shill, S. Padmanabhan, Q. Xin, J. M. Prausnitz, D. S. Clark and H. W. Blanch, *Biotechnol*. *Bioeng*., **108**, 511 (2011).
[13] A. P. Dadi, C. A. Schall and S. Varanasi, *Appl*. *Biochem*. *Biotechnol*., **137**, 407 (2007).
[14] T. Heinze, T. Liebert, *Progr*. *Polym*. *Sci*., **26**, 1689 (2001).
[15] N. C. Silva, G. V. Betancur, M. P. Vasquez, B. G. Edelvio and N. J. Pereira, *Appl*. *Biochem*. *Biotechnol*., **163**, 928 (2011).
[16] H. Zhao, G. A. Baker, Z. Song, O. Olubajo, T. Crittle and D. Peters, *Green Chem*., **10**, 696 (2008).
[17] H. Zhao, C. L. Jones, G. A. Baker, S. Xia, O. Olubajo and V. N. Person, *J*. *Biotechnol*., **139**, 47 (2009).
[18] Y. H. Zhou, A. J. Robertson, J. H. Hillhouse and D. Baumann, Phosphonium and imidazolium salts and methods of their preparation, WO 2004/016631 A1, Cytec Inc. Ontario, Canada, 2004.
[19] N. Kamiya, Y. Matsushita, M. Hanaki, *et al*., *Biotechnol*. *Lett*., **30**, 1037 (2008).
[20] Q. Li, Y. C. He, M. Xian *et al*., *Bioresource Technol*., **100**, 3570 (2009).
[21] R. Q. Xie, Y. F. Zhang and X. Y. Li. *Beijing Fuzhuang Xueyuan Xuebao*, accepted (2011).
[22] G. L. Miller, *Anal*. *Chem*., **31**, 426 (1959).
[23] R. L. Bondar and D. C. Mead, *Clin*. *Chem*., **20**, 586 (1974).
[24] S. Y. Oh, D. I. Yoo, Y. Shin, *et al*., *Carbohydr*. *Res*., **340**, 2376 (2005).
[25] H. A. Krässig, in "Cellulose: Structure, accessibility and reactivity", Polymer Monographs VII, edited by Gordon and Breach Science Publishers, 1993, pp. 88-90, 100-107, 128-125.
[26] J. Blackwell and R. H. Marchessault, in "Cellulose and Cellulose Derivatives", edited by N. Bikales and LE Segal, Wiley, 1971, pp. 1.
[27] R. T. O'Connor, E. F. Dupre and E. R. Mccall, *Text*. *Res*. *J*., **28**, 542 (1958).
[28] X. Colom and F. Carrillo, *Eur*. *Polym*. *J*., **38**, 2225 (2002).
[29] M. Akerholm, B. Hinterstoisser and L. Salmen, *Carbohydr*. *Res*., **339**, 569 (2004).
[30] C. Y. Liang and R. H. Marchessault, *J*. *Polym*. *Sci*., **39**, 269 (1959).
[31] A. Thygesen, J. Oddershede, H. Lilholt, A. B. Thomsen and K. Stahl, *Cellulose*, **12**, 563 (2005).
[32] S. Kumar, R. Gupta, Y. Y. Lee and R. B. Gupta, *Bioresource Technol*., **101**, 1337 (2010).
[33] M. Ago, T. Endo and T. Hirotsu, *Cellulose*, **11**, 163 (2004).
[34] R. Dasari and R. Berson, *Appl*. *Biochem*. *Biotechnol*., **137**, 289 (2007).
[35] Y. H. P. Zhang and L. R. Lynd, *Biotechnol*. *Bioeng*., **88**, 797 (2004).