EFFICIENT ENZYMATIC IN SITU SACCHARIFICATION OF CELLULOSE IN AQUEOUS-IONIC LIQUID MEDIA BY MICROWAVE PRETREATMENT

Lu Li,[a,b] Shi-Tao Yu,[a,]* Fu-Sheng Liu,[a] Cong-Xia Xie,[c] and Cui-Zhi Xu[a]

Several representative ionic liquids (ILs) were synthesized, and [Emim]OAc was chosen as environment-friendly solvent for enzymatic in situ saccharification in view of its biocompatibility with both natural and microcrystalline cellulose, as well as its enzymatic activity. With the microwave pretreatment of natural and microcrystalline cellulose, directly enhancing the in situ enzymatic saccharification, the rate was compared versus an untreated control by the detection of dinitrosalicylic acid (DNS). It is suggested that the molecular structure of cellulose in the process of pretreatment was changed, e.g. intramolecular hydrogen bonds were broken (detected by FT-IR), and the crystallinity (monitored by SEM and XRD) changed significantly from a crystalline to an amorphous pattern. These changes of cellulose led to an increase of reducing sugar conversion during cellulose enzymatic hydrolysis.

Keywords: Ionic liquids; Cellulose; Enzymatic hydrolysis

*a: College of Chemical Engineering, Qingdao University of Science and Technology, 53 Zhengzhou Road, Qingdao 266042, Peoples Republic of China; b: Pulp & Paper Engineering State Key Laboratory, South China University of Technology, Guangzhou 510640, Peoples Republic of China; c: Key Laboratory of Eco-Chemical Engineering, Ministry of Education, College of Chemistry and Molecular Engineering, Qingdao University of Science and Technology, 53 Zhengzhou Road, Qingdao 266042, Peoples Republic of China ; * Corresponding author: yushitaoqst@126.com*

INTRODUCTION

Limited reserves of fossil fuels and global climate changes have drawn increased attention to the use of renewable biomaterials for energy production (Xie et al. 2007). Cellulose, the most abundant renewable resource in the world, has been a focus of much of this attention. The cellulose-containing materials and their derivatives have been widely used in our society (Klemm et al. 2005; Ragauskas et al. 2006). Of the liquid energy sources, ethanol produced by the fermentation of glucose is a promising biomass-derived fuel. Ethanol production from edible agricultural feedstocks is potentially problematic, so recent research has focused on ethanol production from cellulose (Jones et al. 1984). To this end, chemical hydrolysis of cellulose to glucose using inorganic acids has been employed for many years, although a by-product is formed during this process that inhibits the subsequent fermentation reaction; also the environmental load by acidification is a problem (Xie et al. 2007). Alternatively, enzymatic hydrolysis processes can alleviate these obstacles (Schafer et al. 2007). However, hydrolysis of cellulose to glucose in aqueous media catalyzed by the cellulase enzyme system suffers from slow

reaction rates due in large part to the highly crystalline structure of cellulose and inaccessibility of enzyme adsorption sites.

In principle these problems can be avoided by dissolving the cellulose in a suitable solvent to facilitate the access of cellulase to cellulosic substrates. Ionic liquids (ILs), a new class of cellulose-dissolving solvents and a new reaction medium for biocatalysis, are a potential system for the enzymatic hydrolysis of cellulose (Swatloski et al. 2002; Van and Sheldon 2007). However, the significant decrease in cellulase activity in the presence of cellulose-dissolving ILs requires that a cumbersome recovery process is necessary to retrieve the regenerated cellulose produced by the pretreatment of cellulose with ILs prior to enzymatic hydrolysis (Li et al. 2009, 2010; Zhao et al. 2009).

A potential approach to overcome the above drawback is to hydrolyze cellulose "in situ" while it is dissolved in ILs, which requires developing ILs compatible with both cellulose solubility and cellulase activity. Recently, Kamiya et al. (2008) reported that by adjusting the ratio of [Emim][DEP] to water, the enzymatic in situ saccharification of cellulose in aqueous-ILs media was made possible. Over 50% of the cellulose could be converted to glucose in 24 h, indicating that [Emim][DEP] shows good compatibility with cellulase. But in the aqueous-[Emim][DEP] system, the efficiency of cellulose hydrolysis into glucose is still not high enough. The efficiency of cellulose hydrolysis is related to the attainability of efficient interactions between the cellulose and cellulase; in other words, the highly crystalline structure of cellulose could not be completely opened in the ILs media, so that the efficiency of cellulose hydrolysis remained low (Swatiloski et al. 2002). In-depth studies concerning biocompatibility of ILs and process intensification of in situ enzymatic saccharification would be necessary to make the hydrolysis more applicable. In addition, as an alternative to the conventional heating technique, microwave irradiation, can provide a fast and efficient solubilization (Majetich et al. 1997). Swatiloski et al. (2002) studied the solubization of cellulose by $[C_4mim]Cl$ under microwave radiation. After regeneration, the morphology of the material was signify-cantly changed, displaying a rough, but conglomerate texture in which the fibers are fused into a relatively homogeneous macrostructure. For example, the solubility of cellulose with a DP of 1000 could be increased by 150% (Swatiloski et al. 2002).

Microwave heating is characterized by an internal heating process due to the direct absorption of energy by polar molecules. This differs significantly from conventional heating methods that are based on heat transfer. This internal heating may be responsible for the more effective breakdown of the H-bond network between the microfibrils, although care must be taken because heating occurs rapidly and can easily lead to biopolymer pyrolysis (Feng et al. 2008). In the course of our previous work on the application of microwave irradiation in organic chemistry, we demonstrated that many reactions could be run in a focused microwave oven, thereby achieving striking reductions in reaction times, better yields, and cleaner reactions than for purely conventional heating processes (Guillard et al. 1999; Frere et al. 2001).

It is reasonable to suppose that the increase of the diffusion rate and sorption of polar molecules in cellulose could be enhanced if microwaves induce orientation of hydroxyl groups by resonance absorption of microwave energy. Such orientation could therefore be maximized (maximum of dielectric loss) when the frequency of the electromagnetic waves is coincident with the frequency of macromolecular motion, or

t (T) = b/2pf_{max}, were t (T) is the relaxation time at temperature T, f_{max} is the frequency of the electrical field, corresponding to dielectric loss maximum, and b characterizes the asymmetry of the relaxation process in relation to that in liquids (for which b = 1). The relaxation time depends on the temperature according to the Arrhenius law: t (T) = t_0 exp (E/RT), were E is the activation energy. At high microwave frequencies, the temperature range corresponding to segmental motion in amorphous polymers is situated above their decomposition temperature. However, localized and thermally activated motions (secondary relaxation transitions) are characterized by a much lower temperature range. An extensive study of secondary relaxation processes in cellulose over a large frequency range (10^{-2}–10^{6} Hz) of electric field has recently been performed (Meissner et al. 2000; Delarosa et al. 2001). By using the values of b, t_0 and E, presented in these works we have calculated the temperatures corresponding to the dielectric loss maximum and, hence, to the maximum of hydroxyl group orientation at the typical frequency of microwave assisted synthesis (2.54×10^{9} Hz). As a result, temperatures of 60°C are calculated. The appropriate temperature range was used for comparative study of solvent pattern of cellulose under both microwave irradiation and conventional heating. In addition, microwave irradiation was considered an efficient pretreatment method that can help enzymes break down the carbohydrates to simple sugars (Zhu et al. 2005; Swatiloski et al. 2002). If microwave and ILs together can bring about the enzymatic saccharification of cellulose, then it is of interest to find out whether the saccharification rate could be enhanced.

In this study, several representative ionic liquids (ILs) were synthesized, and an IL having biocompatibility with both cellulose and enzyme activity was chosen. The enzymatic in situ saccharification rate in homogenous system of ILs was compared for both natural and microcrystalline cellulose either in the presence of microwave pretreatment or not, and the reason of the different results was researched by SEM, FT-IR, and XRD.

EXPERIMENTAL

General

Microcrystalline cellulose (particle size 50 μm, DP 153) was from the Chengdu Kelong Chemical Reagent Factory. Cellulase (activity 56 FPU) was from Continent Biotech (Shanghai) Co. The natural cellulose (straw, willow, and pledget) samples were laboratory-prepared by a Soxhlet extraction process, and the DP of straw, willow and pledget is 150, 143, and 147 respectively. The DNS reagent was from Qingdao Zhengye ltd. All other reagents were of analytical grade and used without further purification.

X-ray powder diffraction patterns of the samples were obtained on a XB-3A instrument using monochromatic CuK_α radiation (λ = 0.15418 nm). It was operated at 40 kV and 100 mA. The experimental conditions correspond to a step width of 0.02° and scan speed of 2°/min. It should be mentioned that the diffraction must be operated at narrow seam and in the diffraction region 2θ = 2 to 70°. IR spectra were recorded with a Nicolet 510P FT-IR spectrometer in the range of 2000 to 800 cm^{-1}, using KBr powder containing ca. 1 wt% of sample. SEM images were taken for both untreated and pre-

treated cellulose using a Hitachi S-5000 microscope, where samples were coated with a thin layer of gold in an automatic sputter coater before observation. The accelerating voltage was 5 kV, and images of the samples were acquired at 5000 magnification.

Methods

Synthesis of ionic liquids

The ILs shown in Fig.1 were synthesized according to the following references (Xie et al. 2008; Liu et al. 2007; Gong et al. 2005; Huddleston et al. 2001).

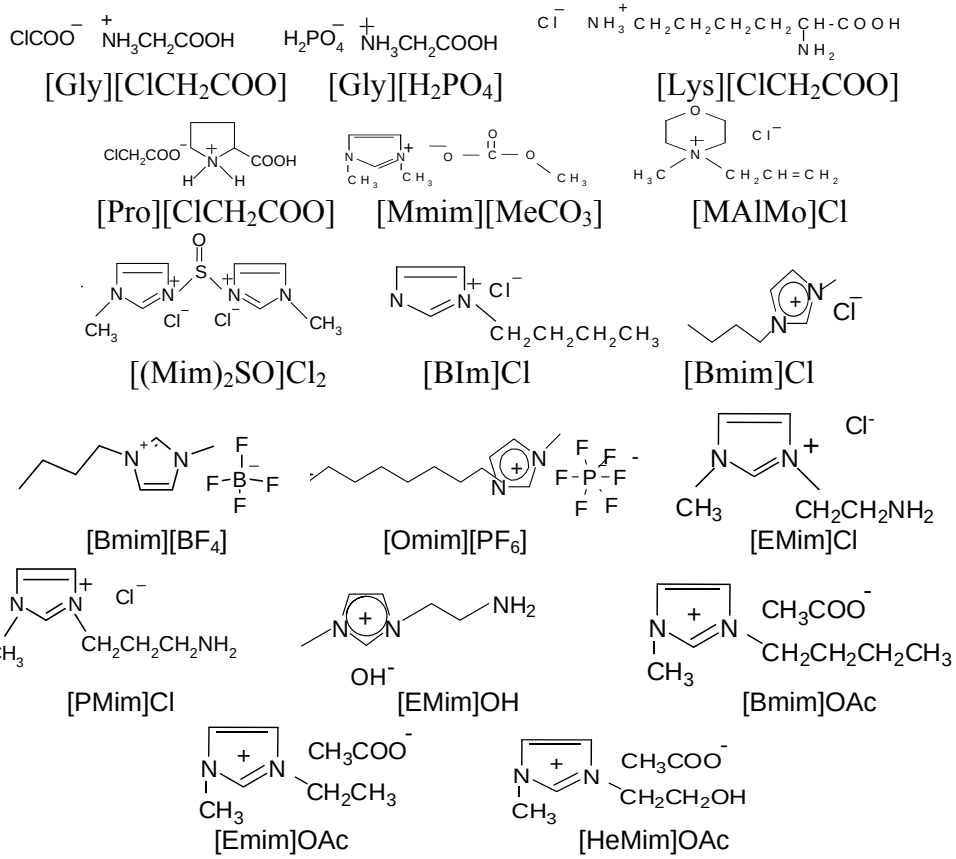

Fig. 1. Ionic liquids (ILs) prepared for use in the present study

Dissolution of cellulose in ILs

The solubility of cellulose in each IL was measured by a standardized procedure (Mazza et al. 2009). A weighed amount of IL was added to clear glass vials equipped with a magnetic stirrer and placed into a heating oil bath. Small precise amounts of microcrystalline cellulose (about 10 mg) were added discretely into the vials. The mixture was stirred for dissolution until the solution was just within the boundary of transparency. Experiments were conducted at different heating temperatures (from 40 °C to 90 °C increased by 10 °C every time), and the solubility of cellulose was calculated by the following formula:

$$\text{Solubility} = (\textit{Weight of Dissolved Cellulose/ Weight of ILs}) \times 100\% \quad (1)$$

Regeneration of cellulose

When water was added to the ILs at concentrations greater than ca. 1 wt% (approximately 0.5 mole fraction H_2O) the solvent properties were significantly impaired, and cellulose was no longer soluble. In this way the regeneration of cellulose was obtained at room temperature.

Microwave pretreatment experiments

Five milligram of celluloses was dissolved in 0.5 mL of six different ILs to equal concentration. Then the cellulose solutions were pretreated with the help of a microwave generator (XH-200A, Kunshan Sonication Co., China) at a frequency of 2.54×10^6 kHz. The temperature was maintained at 60°C during the microwave heating experiments. Cellulose solutions treated at 60°C by conventional heating for 30 min served as the control group.

Enzymatic in situ saccharification of cellulose in aqueous-ILs media

Sodium acetate buffer (pH 4.8) was added to the cellulose solutions prepared by microwave pretreatment to make a total 0.5 mL solution containing various concentrations of ILs (20%,v/v). Enzymatic reaction was initiated by addition of cellulase to the aqueous-ILs mixture. The enzymatic solution was vortex mixed, and then the enzymatic hydrolysis was carried out in a constant temperature incubator shaker at 100 rpm. The efficiency of enzymatic saccharifcation was evaluated by quantifying the glucose released after 24 h. The reducing sugar yield was determined by the dinitrosalicylic acid (DNS) method every 1 h (for the period of the first to the 10^{th} h), 2 hs (10-20 h), and 4 hs (20-40h). The enzymatic hydrolysis rate was calculated as follows:

$$\text{Enzymatic hydrolysis rate (\%)} = \text{reduced sugar} \times 0.9 / (\text{sample weight} \cdot \text{cellulose content}) \times 100\% \quad (2)$$

RESULTS AND DISCUSSION

Selection of Ionic Liquids

Main results are shown in Table 1, comparing the ability of different ILs to dissolve cellulose under matched conditions.

It can be seen that acidic ILs could not dissolve cellulose, and only neutral and basic ILs could dissolve cellulose (except for [Bmim][BF_4] and [Omim][PF_6]) from Table 1. The [BIm]Cl, [Bmim][Cl], [EMim]OH, and [Emim]OAc showed better solubilizing capability. However, Cl^- and OH^- could poison cellulase and deactivate it (Table 2); the ILs of OAc^- became the main research target. Compared with the solubility of cellulose and the biocompatibility with cellulase of three ILs of OAc^-, [Emim]OAc was judged to have the best solubility and biocompatibility with cellulase (Tables 1 and 2).

Table 1. The Influence of Different Ionic Liquids for the Dissolution of Cellulose

Ionic liquids	Content of cellulose in solvent			
	Micro	Straw	Willow	Pledget
[Gly][$ClCH_2COO$]	-	-	-	-
[Gly][H_2PO_4]	-	-	-	-
[Lys][$ClCH_2COO$]	-	-	-	-
[Pro][$ClCH_2COO$]	-	-	-	-
[Mmim][$MeCO_3$]	-	-	-	-
[MAlMo]Cl	1.1	0.9	0.8	1.0
[$(Mim)_2SO$]Cl_2	2.1	2.0	1.9	1.8
[BIm]Cl	6.9	6.7	6.8	6.5
[Bmim][Cl]	7.3	7.2	7.0	7.1
[Bmim][BF_4]	-	-	-	-
[Omim][PF_6]	-	-	-	-
[EMim]Cl	3.4	3.2	3.0	3.3
[PMim]Cl	3.9	3.4	3.5	3.8
[EMim]OH	5.2	4.9	4.8	5.0
[Bmim]OAc	2.3	2.0	2.1	2.2
[Emim]OAc	4.7	4.5	4.5	4.6
[HeMim]OAc	2.9	2.6	2.7	2.8

It was suggested that Emim had the smallest steric hindrance, which made it easy to enter the inside of cellulose (Heinze et al. 2008). Therefore, [Emim]OAc is able to break the intermolecular and intramolecular hydrogen bonds of cellulose and make the structure of cellulose loose. By this means it accelerates cellulose dissolution in the [Emim]OAc. The mechanism of dissolving cellulose was according to the theory of EDA (Swatloski et al. 2002). In addition, the loose structure of cellulose in [Emim]OAc made cellulase easy to contact with glycosidic bonds in order to accelerate hydrolysis. Therefore, [Emim]OAc was selected for more careful research, as described below.

Table 2. Influence of Different Ionic Liquids for Cellulase

ILs	cellulose /g	Enzymatic hydrolysis rate /%			
		Micro	Straw	Willow	Pledget
[MAlMo]Cl	0.2	0	0	0	0
[$(Mim)_2SO_4$]Cl_2	0.2	0	0	0	0
[BIm]Cl	0.2	0	0	0	0
[Bmim][Cl]	0.2	0	0	0	0
[EMim]Cl	0.2	0	0	0	0
[PMim]Cl	0.2	0	0	0	0
[Emim]OH	0.2	0	0	0	0
[Bmim]OAc	0.2	4.2	3.1	4.3	2.2
[Emim]OAc	0.2	19.8	7.7	11.5	4.7
[HeMim]OAc	0.2	3.5	1.4	3.8	2.4

Hydrolysis and Glucose Formation Rates of Different Cellulose Systems

The enzymatic hydrolysis rate of microcrystalline and natural cellulose was studied using microwave pretreatment (or not) in [Emim]OAc system (Fig. 2). Cellulose dissolved in ILs was pretreated by microwave to investigate its effect on enzymatic saccharification of cellulose. As shown by the results in Fig. 2, microwave treatment gave rise to a significant improvement in the conversion of cellulose to glucose.

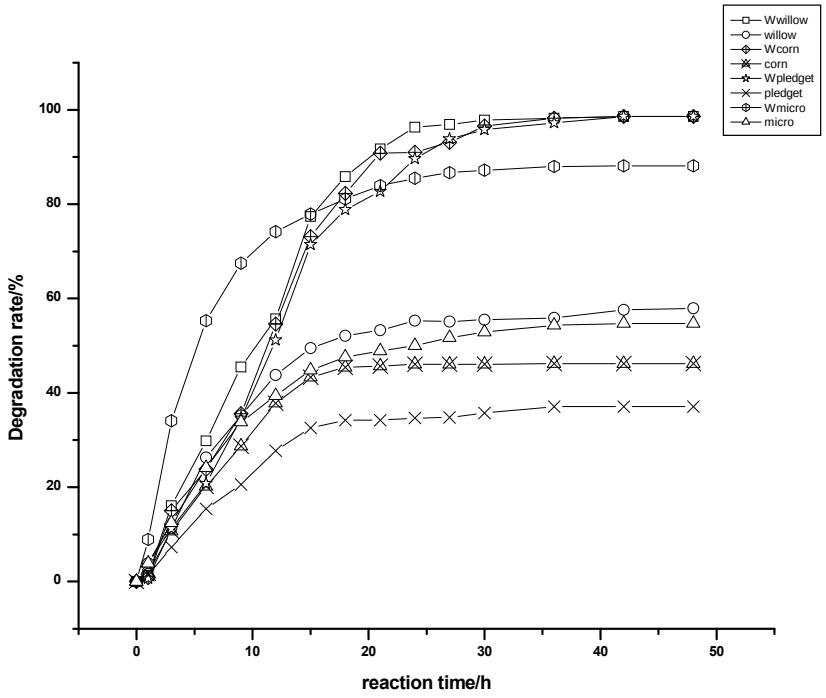

Fig. 2. Hydrolysis and glucose formation rates of different cellulose system (W-microwave pretreatment)

In contrast to cellulose with conventional heating pretreatment, the conversion of cellulose pretreated by microwave heating increased notably, especially in the first 4 h. The conversion of cellulose (microcrystalline) treated with microwave reached 90% in ILs after 24 h, while the corresponding results showed 50% for cellulose treated with conventional heating, suggesting that microwave pretreatment definitely contributed to saccharification.

To explain why microwave heating enhanced enzymatic hydrolysis of cellulose, the FT-IR spectra of cellulose/ILs pretreated by conventional and microwave heating were analyzed. The FT-IR spectroscopy data in Figs. 3 indicated that the peaks near 3346, 2900, 1637, 1431, and 1163 cm^{-1} for the data of the cellulose (A) , and cellulose pretreated with conventional heating(B) were basically the same as the characteristic cellulose peaks for the data of the cellulose pretreated with microwave(C) (Liu et al. 2007). The band representative of the bridge stretching of O-H groups in cellulose pretreated with microwave was near 3346 cm^{-1}, as shown in the FT-IR spectrum C. The intensity of the band was weakened. This may be due to the weakening of hydrogen bonds in the cellulose molecule or the emergence of cooperation in the process of dissolution. The band near 1431 cm^{-1} disappeared. The band near 1455 cm^{-1} is representative

of the bridge deforming of CH_2 groups in cellulose, which means that hydrogen bonds were broken. Additionally, there is a small absorption peak in the fingerprint area about the 894 cm^{-1} band, which is characteristic of β-linkages. This peak, which is generated by the deformation of the CH_2 and C-O-H, did not appear in the case of cellulose pretreated with conventional heating. This change implies that the crystalline type of cellulose changes in the process of dissolution. The decreased crystallinity of cellulose was beneficial to the enzymatic hydrolysis rates. In addition, the viscosities [η] of samples pretreated with microwave were much lower than the corresponding values for pretreatment with conventional heating. This indicated that cellulose dissolved by microwave displayed a lower molecular weight than that dissolved by conventional heating (Swatloski et al. 2002). More disruption must occur during microwave treatment, resulting in lessening of crystallinity. These results are in accord with the FT-IR characterization.

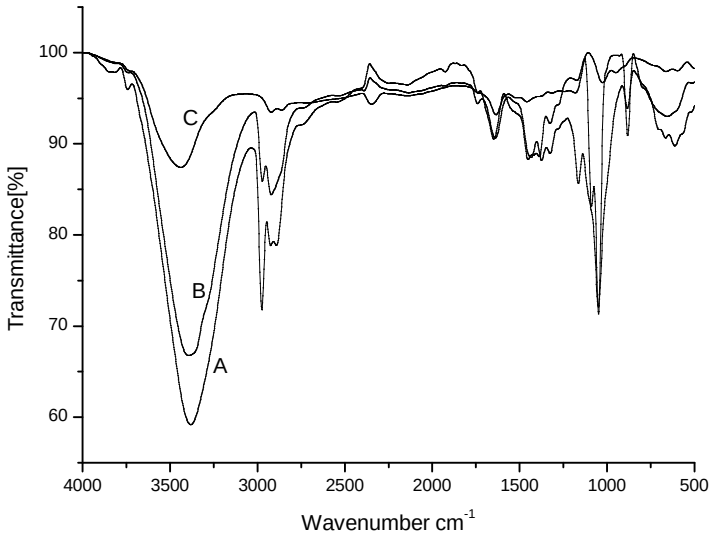

Fig. 3. FT-IR spectra of microcrystalline cellulose (A); treated with conventional heating (B); cellulose treated with microwave (C)

SEM analyses were also employed to investigate the changes in the structure of cellulose regenerated from ILs under conventional and microwave heating pretreatment, respectively. SEM graphs suggested that in the original microcrystalline cellulose the major component present was ordered and condensed fibrils (Fig. 4a). After being regenerated from ILs (Fig. 4b), the surface became rough and swollen. When the cellulose was subjected to microwave pretreatment, the structure of the regenerated cellulose was further loosened and became less compact (Fig. 4c), illustrating the disruption of linkages in cellulose to a certain extent.

The same results were obtained from the results of XRD (Fig. 5). The structure of cellulose was examined by XRD. It can be seen that the crystallization of cellulose decreased substantially. There was a group of low-intensity, wide-distribution and flat-type diffraction peaks located at 2θ=15.44~21.64°. These characteristics are in accord with crystalline cellulose of type Ⅱ (Liu et al. 2007). More accessible external and

internal surface area of cellulose was accessible as binding sites for cellulase, leading to the enhancement of enzymatic saccharification.

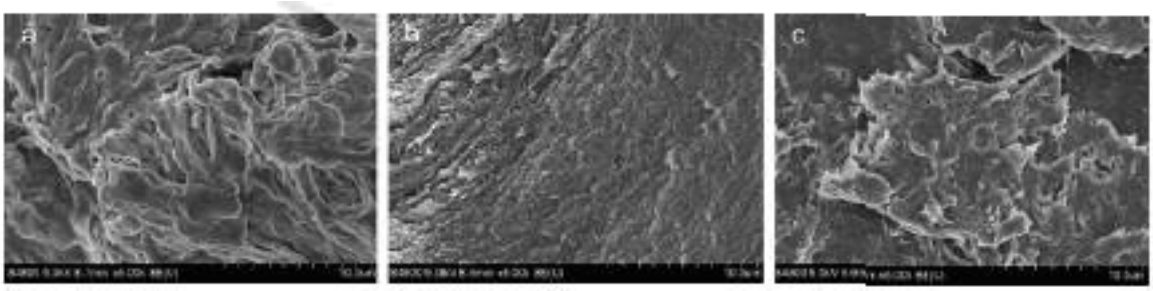

Fig. 4. SEM of microcrystalline cellulose (a); treated by conventional heating (b); treated by microwave (c)

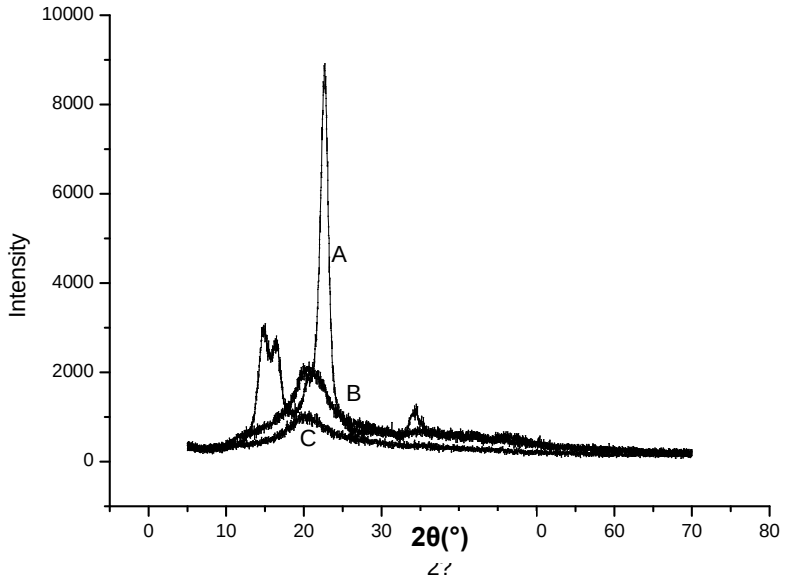

Fig. 5. XRD patterns of microcrystalline cellulose (A); treated by conventional heating (B); and cellulose treated by microwave(C)

CONCLUSIONS

Different ILs were investigated in this study, and ten of them were able to dissolve cellulose. In particular, [EMIM]OAc showed favorable solubility and biocompatibility simultaneously. Thus, the [EMIM]OAc reaction system was identified as promising for in situ enzymatic saccharification of cellulose. The results of analysis showed that pretreatment by microwave decreased the crystallization of cellulose, which might contribute to increased rate of enzymatic hydrolysis of cellulose.

ACKNOWLEDGMENTS

The authors would like to acknowledge the National Natural Science Foundation of China (31070520), Shandong Province Office of Education (J09LG16), Open Foundation of Chemical Engineering Subject (Qingdao University of Science and Technology), and Open Foundation of Pulp & Paper Engineering, State Key Laboratory, South China University of Technology (200912) for funding this work.

REFERENCES CITED

Delarosa, A., Heux, L., and Cavaillé, J. Y. (2001). "Secondary relaxations in poly(allyl alcohol), PAA, and poly(vinyl alcohol), PVA. II. Dielectric relaxations compared with dielectric behaviour of amorphous dried and hydrated cellulose and dextran," *Polymer* 42, 5371-5379.

Feng, L., and Chen, Z. (2008). "Research progress on dissolution and functional modification of cellulose in ionic liquids," *J. Mol. Liq.* 142, 1-5.

Frère, S., Thiéry, V., and Besson, T. (2001). " Microwave acceleration of the Pechmann reaction on graphite/ montmorillonite K10: Application to the preparation of 4-substituted 7-aminocoumarins," *Tetrahedron Lett.* 42, 2791-2794.

Gong, H. S., Yue, Q. C., and Yan, Q. P. (2005). "Amino-functionalized ionic liquid as a nucleophilic scavenger in solution phase combinatorial synthesis," *Comb. Chem.* 7, 561-566.

Guillard, J., and Besson, T. (1999). "Synthesis of novel dioxinoben- zothiazole derivatives," *Tetrahedron* 55, 5139-5144.

Heinze, T., Dorn, S., and Schoebitz, M. (2008). "Interactions of ionic liquids with polysaccharides – 2: Cellulose," *Macromol. Symp.* 262, 8-22

Huddleston, J. G., Visser, A. E., Reichert, W. M., Willauer, H. D., Broker, G. A., and Rogers, R. D. (2001). "Characterization and comparison of hydrophilic and hydrophobic room temperature ionic liquids incorporating the imidazolium cation," *Green Chem.* 3, 156-164.

Jones, J. L., and Semrau, K. T. (1984). "Wood hydrolysis for ethanol production-previous experience and the economics of selected processes," *Biomass* 5(2), 109-135.

Kamiya, N., Matsushita, Y., Hanaki, M., Nakashima, K., Narita, M., Goto, M., and Takahashi, H. (2008). "Enzymatic in situ saccharification of cellulose in aqueous-ionic liquid media," *Biotech. Lett.* 30, 1037-1040.

Klemm, D., Heublein, B., and Fink, H. P. A. (2005). "Cellulose: Fascinating biopolymer and sustainable raw material," *Angew Chem. Int. Ed.* 44, 2-37.

Li, Q., He, Y. C., Xian, M., Jun, G., Xu, X., Yang, J. M., and Li, L. Z. (2009). "Improving enzymatic hydrolysis of wheat straw using ionic liquid 1-ethyl-3-methyl imidazolium diethyl phosphate pretreatment," *Bioresource Tech.* 100, 3570-3575.

Li, C., Knierim, B., Manisseri, C., Arora, R., and Scheller, H. V. (2010). "Comparison of dilute acid and ionic liquid pretreatment of switchgrass: Biomass recalcitrance, delignification and enzymatic saccharification," *Bioresource Tech*. 101, 4900-4906.

Liu, C. F., Sun, R. C., and Zhang, A. P. (2007). "Preparation and characterization of phthalated cellulose derivatives in room-temperature ionic liquid without catalysts." *Agric. Food Chem.* 55(6), 2399-2406.

Majetich, G., and Wheless, K. (1997). "Microwave heating in organic chemistry: An update," In: *Microwave–Enhanced Chemistry, Fundamentals, Samples Preparation and Application*., Haswell, S. J. (ed.), American Chemical Society, Washington, DC, 1997, pp. 455.

Mazza, M., Catana, D., Vaca-Garcia, C., and Cecutti, C. (2009). "Influence of water on the dissolution of cellulose in selected ionic liquids," *Cellulose.*16, 207-215.

Meissner, D., Einfeld, J., and Kwasniewski, A. (2000). "Contributions to the molecular origin of the dielectric relaxation processes in polysaccharides - the low temperature range," *J. Non-Cryst. Solids.* 275, 199-209.

Ragauskas, A. J., Williams, C. K., and Davison, B. H. (2006). "The path forward for biofuels and biomaterials," *Science* 311, 484-488.

Schäfer, T., Borchet, T. W., and Nielsen,V. S. (2007). "Industrial enzymes," *Adv. Biochem. Eng. Biotechnol*. 105, 59-131.

Swatloski, R. P., Spear, S. K., and Holbrey, J. D. (2002). "Dissolution of cellulose with ionic liquids," *Chem.Soc*. (124), 4974-4975.

Swatloski, R. P., Spear, S. K., Holbrey, J. D., and Rogers, R. D. (2002). "Dissolution of cellose with ionic liquids," *J. Am. Chem. Soc.* 124, 4974-4975.

Van, R. F., and Sheldon, R. A. (2007). "Biocatalysis in ionic liquids," *Chem. Rev.* 107, 2757-2785.

Xie, H., King, A., Kilpelainen, I., Granstrom, M., and Argyropoulos, D. S. (2007). "Thorough chemical modification of wood-based lignocellulosic materials in ionic liquids," *Biomacromolecules.* 8, 3740-3748.

Xie, Y., Shang, X. X., Jiang, F. X., and Hu J. G. (2008). "Antigrowth effects of chitosan and its derivatives on human hepatocellular carcinoma cell line," *J. Clinical Rehabilitative Tissue Engin. Res.* 12(23), 4579-4582.

Zhao, H., Jones, C. L., Baker, G. A., Xia, S. Q., Olubajo, O., and Person, V. N. (2009). "Regenerating cellulose from ionic liquid for an accelerated enzymatic hydrolysis," *J. Biotech.* 139, 47-54.

Zhu, S. D., Wu, Y. X., Yu, Z. N., Liao, J. T., and Zhang,Y. (2005). "Pretreatment by microwave/alkali of rice straw and its enzymic hydrolysis," *Pro. Biochem.* 40(9), 3082-3086.

Article submitted: December 4, 2010; Peer review completed: April 12, 2011; Revised version received: July 20, 2011; Further revisions received and article approved: Sepember 12, 2011; Published: September 15, 2011.