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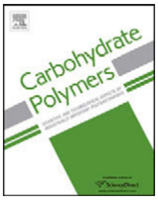

The study of factors affecting the enzymatic hydrolysis of cellulose after ionic liquid pretreatment

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ABSTRACT

Cellulose resource has got much attention as a promising replacement of fossil fuel. The hydrolysis of cellulose is the key step to chemical product and liquid transportation fuel. In this paper a serials of chloride, acetate, and formate based ionic liquids were used as solvents to dissolve cellulose. The cellulose regenerated from ILs was characterized by FTIR and X-ray powder diffraction. From the characterization and analysis, it was found that the original close and compact structure has changed a lot. After enzymatic hydrolysis, different kinds of ionic liquids (ILs) have different yields of the reducing sugar (TRS). They are 100%, 90.72%, and 88.92% from 1-butyl-3-methylimidazolium chloride ([BMIM]Cl), 1-ethyl-3-methylimidazolium acetate ([EMIM][OAc]), 1-butyl-3-methylimidazolium formate ([BMIM][HCOO]) respectively after enzymatic hydrolysis at 50 °C for 5 h. The results indicated that the yields and the hydrolysis rates were improved apparently after ILs pretreatment comparing with the untreated substrates.

1. Introduction

Cellulose is a class of biomass which widely exists in the world. Cellulose materials can be converted to a range of important industrial chemicals and can serve as a source of carbon based feedstock for ethanol fuel (Samayam & Schall, 2010). Generally, cellulose materials include three components: cellulose, hemicellulose and lignin. Cellulose is the key component of energy crops, agricultural residues and woods (Datta et al., 2010). It is a highly crystalline polymer of D-anhydroglucopyranose units joined together in long chains by β-1,4-glycosidic bonds (Miller, 1959; Swatloski, Spear, Holbrey, & Rogers, 2002; Zavrel, Bross, Funke, Büchs, & Spiess, 2009). It is extremely difficult to dissolve cellulose in water and most common organic solvents as the result of the tight hydrogen-bonding network (Hermanutz, Gähr, Uerdingen, Meister, & Kosan, 2008; Xu, Wang, & Wang, 2010) and van der Waals interactions (Atalla & VanderHart, 1984; Datta et al., 2010). That leads to notorious resistance of cellulose to hydrolysis finally.

In order to solve the problem, many methods, including biological, physical, chemical and physicochemical processes have been studied. But few of them appeared efficient. For example, the cellulose after ball-milling treatment is still an insoluble material with β-1,4-glycosidic bonds but has large parts of noncrystalline regions which can be confirmed by XRD and CP/MAS ^{13}C NMR (Zhao et al., 2005). The drawbacks of these methods have been noticed and categorized (Zhao et al., 2009).

Therefore, an effective pretreatment method seems necessary. In fact, the final purpose of pretreatment is to make the cellulose susceptible to hydrolysis. The appearance of the ionic liquids supplies a promising pretreatment routine. Ionic liquids (ILs) are a type of environmental friendly molten salts, most of which have the virtues of excellent solvency, low melting point, nonvolatility and designability. The physical and chemical properties of ILs can be adjusted to different reactions by choosing different cations, anions and substituents (Swatloski et al., 2002; Xu et al., 2010). Due to these unique qualities, ILs are considered as a kind of promising alternatives to traditional solvents. In 2002, Rogers and coworkers firstly reported [C$_4$mim]Cl could dissolve cellulose efficiently (Swatloski et al., 2002). Subsequently, more ILs were discovered to possess the superiority in dissolving cellulose materials. From these studies, it was discovered that the polymerization and crystallinity degree of the treated cellulose declined seriously by FTIR analysis (Liu & Chen, 2006) and X-ray powder diffraction analysis (Dadi, Varanasi, & Schall, 2006). Further more, ILs pretreatment could avoid the problems of excessive residence time, abundant energy consuming and equipment corruption. So, cellulose pretreatment with ionic liquid has become the hotspot in recent years. Dadi et al. pretreated the cellulose with [C$_4$mim]Cl and the initial enzymatic hydrolysis rate was approximately 50-fold higher for regenerated cellulose compared to untreated cellulose (Dadi et al., 2006). Ramakrishnan hydrolyzed the cellulose regenerated from N-methyl morpholine oxide to get 60% TRS conversion rate which

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was three times as much as the untreated cellulose after enzymatic hydrolysis for 5 h (Ramakrishnan, Collier, Oyetunji, Stutts, & Burnett, 2010). Liu got 70.37% and almost 100% yield of TRS respectively from the wheat straw and steam-exploded wheat straw both of which were regenerated from [BMIM]Cl (Liu & Chen, 2006). Zhao et al. also reported that the D-glucose yield from switchgrass by ILs pretreatment could reach 96% after enzymatic hydrolysis for 24 h (Zhao, Baker, & Cowins, 2010). These results indicate that the ILs are favorable in enzymatic hydrolysis process. But why the ILs can be used to pretreat the cellulose well? Which factors affect the yield of glucose and the hydrolysis rate? Some problems still need further research. This study aims to investigate the impact of ILs on enzymatic hydrolysis and some other influencing factors. It will be helpful for choosing the proper solvent and the optimal conditions for enzymatic hydrolysis process.

2. Experimental

2.1. Materials

N-methylimidazole (99.0%) was purchased from Yancheng Kalechem Co., Ltd (Jiangsu, China). Amberlyst A-26 (OH) ion exchange resin was supplied from J&K chemical (Beijing, China). Cellulase from *Trichoderma reesei* (cellulase activity was 7.5 FPU/mg) was purchased from Imperial Jade Bio-Technology Co., Ltd (Ningxia, China). Cellulose microcrystalline (average particle size 50 μm) was purchased from Acros (NJ, USA).

2.2. Ionic liquid preparation

[BMIM]Cl and [AMIM]Cl were prepared according to the methods described in previous literatures (Huddleston, Willauer, Swatloski, Visser, & Rogers, 1998; Zhang, Wu, Zhang, & He, 2005). Carboxylic based ionic liquid was prepared by the ion exchange resin (Amberlyst-26 OH form) following these steps: Firstly, the ion exchange resin was loaded with the desired anion on the resin. A 10 wt% formic acid solution was flushed through a column which contained 100 g resin. About 300 ml solution was acquired to achieve complete substitution of the OH ions. Then the column was flushed with an aqueous solution of the chloride or bromine salt of desired cations which resulted in rapid displacement of the target anions. After the reaction completed, the aqueous solution containing the formic based ionic liquid was concentrated and dried. Residual chloride or bromide ions were determined by an ion selective electrode until the ions content in the formic based ionic liquid was less than 20 ppm. [Bmim]Cl (5.0 g) was dissolved in 50 ml distilled water and added to a column loaded with formic anions. The eluted was concentrated and dried under vacuum at 90 °C over 24 h. The same procedure can be carried out to get [EMIM][HCOO], [BMIM][OAc] and [EMIM][OAc].

2.3. Buffer and DNS solution preparation

The citrate buffer (pH 4.8, 0.05 mol/l) was prepared by mixing citric acid (conc. 4.01 g/l) and sodium citrate (conc. 8.56 g/l) in distilled water. The DNS solution was prepared according to an IUPAC method: 1416 ml distilled water, 10.6 g 3,5-dinitrosalicylic acid, 19.8 g sodium hydroxide, 306 g potassium sodium tartrate, 7.6 ml phenol melted at 50 °C, and 8.3 g sodium metabisulfite.

2.4. Cellulose dissolution and regeneration

Cellulose (1.0 g) was added into a certain amount of ILs and stirred for 20 min at a controlled temperature until they formed a transparent solution. The cellulose was regenerated by adding anti-solvent into the solution with vigorous stirring for 30 min. The regenerated cellulose was collected by a vacuum filtration and washed with anti-solvent. In view of previous study, the residual ILs would affect the yield of the reducing sugar to different degree (Zhao et al., 2009). Therefore, the regenerated cellulose needed to be washed thoroughly.

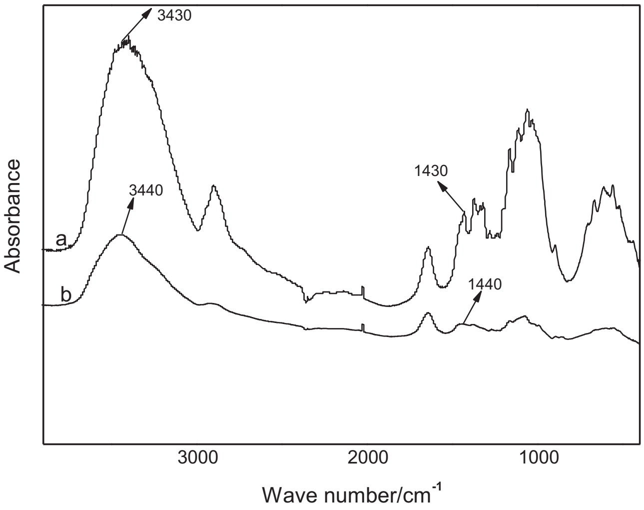

Fig. 1. The FTIR spectrum of the regenerated cellulose from [BMIM]Cl and the original cellulose. a, Original cellulose; b, regenerated cellulose.

2.5. Enzymatic hydrolysis of cellulose

A suspension solution of 1.0 g cellulose and 0.015 g cellulase were added into 150 ml critic buffer. The enzymatic hydrolysis was carried out in a water bath. At a time interval, the reaction solution 1.0 ml was withdrawn to a test tube and 3.0 ml DNS solution was added. Then the test tube was sealed and placed in the boiling water for 15 min. The test tube was cooled to room temperature in an ice bath. The reducing sugar (TRS) yield was determined by DNS method using glucose as a standard (Miller, 1959). The enzymatic hydrolysis rate was calculated as follows:

$$\text{Enzymatic hydrolysis rate}\,(\%) = \frac{\text{reduced sugar} \times 0.9}{\text{sample weight}} \times 100$$

3. Results and discussion

3.1. Characterization of regenerated cellulose

The FTIR spectrum of the cellulose regenerated from [BMIM]Cl and the original cellulose is shown in Fig. 1. In curve a, the peak at 1430 cm^{-1} can be assigned to bending vibration of CH_2. This band is strong in crystalline cellulose, and weak in amorphous cellulose (Nelson & O'Connor, 1964). So, the curve b reveals obviously that the crystallinity of the regenerated cellulose is much lower than the original cellulose. The broad absorption in the range from 3000 cm^{-1} to 3500 cm^{-1} is due to stretching vibration of intermolecular O–H forming among cellulose molecules. Comparing curve b to a, the broad absorption gets much weaker, which may suggests the intermolecular hydrogen bonds are broken greatly. Hermetically sealed construction of cellulose tends to amorphism which can supply higher accessible surface areas and more binding sites for enzyme than the untreated cellulose. The declined crystallinity and the incompact structure of regenerated cellulose could be the important reason of the increase of the hydrolysis rate.

The X-ray diffraction patterns of the cellulose films are shown in Fig. 2. The original cellulose is cellulose I as indicated by the

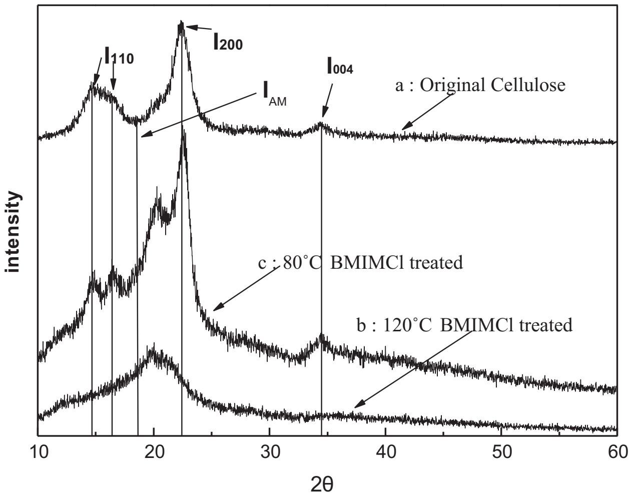

Fig. 2. The XRD spectrum of the regenerated cellulose (pretreated by [BMIM]Cl at 80 °C and 120 °C) and the original cellulose.

typical diffraction peaks at $2\theta = 14.8°$, 16.3°, and 22.6°. Previous literatures have studied on this problem (Bang, Lee, Park, & Cho, 1999; Zhang et al., 2005). After dissolution and subsequent coagulation with water, the regenerated cellulose exhibits the typical diffraction patterns of cellulose II at $2\theta = 20°$, and 21.7°, which are a typical diffraction pattern for cellulose II (Bang et al., 1999). These results indicate that the transformation from cellulose I to cellulose II occurs after the dissolution and regeneration in [BMIM]Cl. This phenomenon was also reported in some other known solvent systems such as [AMIM]Cl (Zhang et al., 2005) for cellulose. Compared to the original cellulose, the intensity of diffraction peaks of these regenerated cellulose films reduced significantly, which indicated the crystallinity of these regenerated cellulose films was much lower than the original cellulose. This phenomenon means [BMIM]Cl can rapidly break intermolecular and intramolecular hydrogen bonds and destroy the original crystalline form in the dissolution process.

3.2. *Effect of anti-solvent chosen to regenerate cellulose*

Methanol, ethanol, and deionized water were used as anti-solvents to regenerate cellulose from cellulose/ILs solution. From Fig. 3, it can be seen that H_2O is the most beneficial for enzymatic hydrolysis from the perspective of the high TRS yield. The order is: $H_2O > MeOH > EtOH$. The effects of anti-solvent on the yield of TRS are different for different cellulose/ILs solution. The cellulose regenerated from [BMIM]Cl by deionized water gives slightly higher TRS yield than by methanol and ethanol after enzymatic hydrolysis for 8 h. But both of the TRS yields from [EMIM][OAc] and [EMIM][HCOO], especially from the [EMIM][HCOO], have apparent declines by different anti-solvents. So, although ILs could be reclaimed easily from the organic solvents, such as MeOH or EtOH for their low boiling points, H_2O is a better choice to be used as the anti-solvent because of its economical and environmental friendly property and the final high yield of TRS.

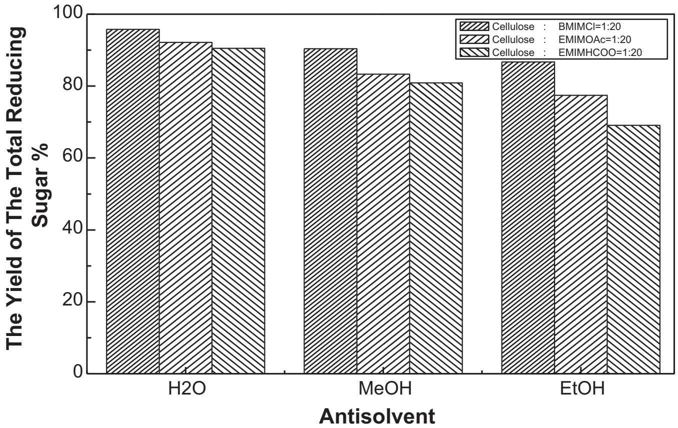

Fig. 3. The yield of total reducing sugar after enzymatic hydrolysis of the regenerated cellulose (different anti-solvents were used to precipitate cellulose from cellulose/IL solution) and the original cellulose for 8 h.

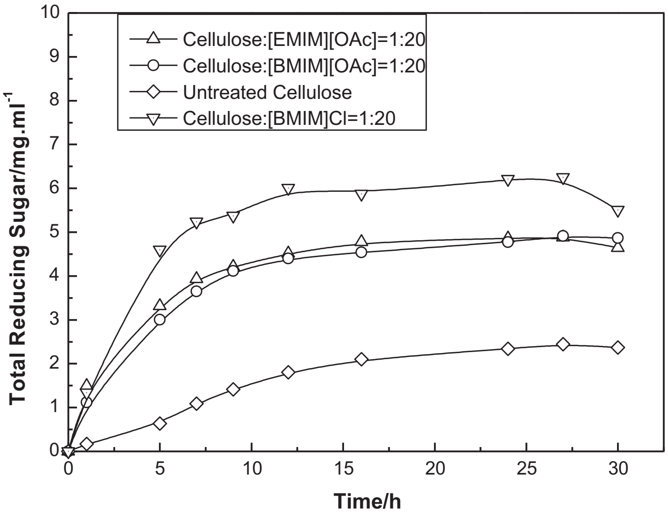

Fig. 4. Enzymatic hydrolysis rate curves of the regenerated cellulose (cellulose was pretreated in ionic liquids at 80 °C) and the original cellulose.

3.3. *Effect of pretreatment temperature*

As seen in Figs. 4 and 5, the yields of TRS from the regenerated cellulose appear to be affected significantly by temperature. The simple reason is that the ILs dissolved the cellulose more sufficient at higher temperatures. In fact, the effects of temperatures are very complicated. In [BMIM]Cl system, the TRS concentration reached 8.49 mg/ml at 120 °C for 5 h, while it was only 4.60 mg/ml at 80 °C. The hydrolysis rate at 120 °C was a little less than twice the rate

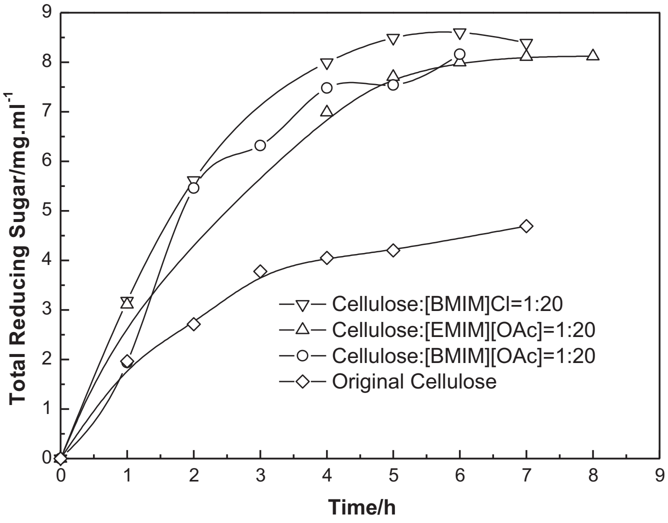

Fig. 5. Enzymatic hydrolysis rate curves of the regenerated cellulose (cellulose was pretreated in ionic liquid at 120 °C) and the original cellulose.

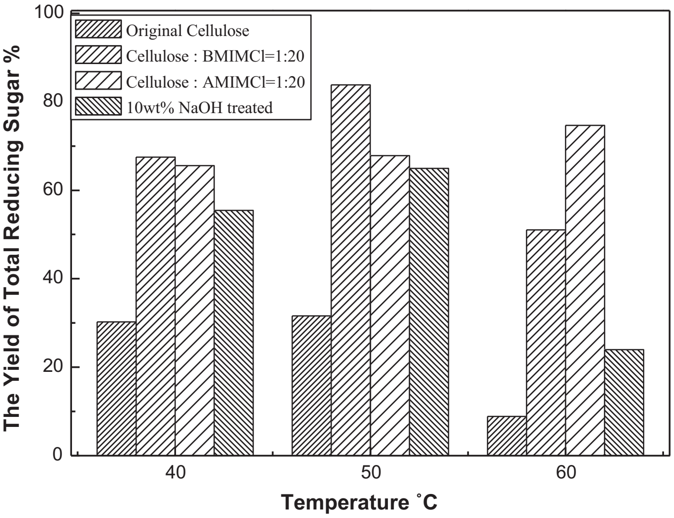

Fig. 6. The yield of total reducing sugar after enzymatic hydrolysis of the regenerated cellulose and the original cellulose for 24 h at 40 °C, 50 °C, 60 °C respectively.

at 80 °C. As to [BMIM][OAc] and [BMIM][HCOO] systems, the differences of TRS yields were larger than twice between 120 °C and 80 °C. Previous studies have reached the conclusion that chloride-containing ILs tend to be more viscous than those containing acetate and are more thermally stable than those ILs containing formate (Kosan, Michels, & Meister, 2008; Shill et al., 2011). It can speculate that the viscosity of the acetate and formate based ILs came down more quickly as the temperature increased than chloride based ILs. So the TRS yields in [BMIM][OAc] and [BMIM][HCOO] systems increased more with the temperatures increasing than that in [BMIM]Cl system as well. However, the effects of the anions in the ILs on the cellulose dissolution are also tremendous. It was found that the solubility of cellulose increases almost linearly with increasing hydrogen bond accepting ability of anions in the ionic liquids (Xu et al., 2010). Chloride based ILs could dissolve cellulose efficiently because of the formation of hydrogen bonding between the hydroxyl protons of cellulose and the chloride anion of the IL. Under the interaction of the factors involved above, we got the trendlines shown in Figs. 4 and 5.

3.4. The effect of temperature on enzymatic hydrolysis of the regenerated cellulose

As shown in Fig. 6, the TRS released from the regenerated cellulose and the original cellulose at 50 °C were much higher than at 40 °C and 60 °C. By this token, cellulase can perform the best activity at 50 °C. The thermal stability of the cellulase may be related to the yield of the cellulose. When the temperature increases, the TRS yield declines.

3.5. Effect of ILs amount on the enzymatic hydrolysis

The reactions were performed at mass ratios of 1:10, 1:15 and 1:20 for cellulose to IL. As shown in the table, the effects of the amount of ILs on hydrolysis were different for different ILs. But they took on the same trends approximately. The hydrolysis rates accelerated when the amount of ILs used in the pretreatment step increased. The more ILs was used, the more sufficiently the ILs interacted with the cellulose in the studied range. When the mass ratio of cellulose/ILs is 1:20, the TRS yield of the regenerated cellulose from 1-butyl-3-methylimidazolium chloride ([BMIM]Cl) has reached almost 100%, which means the cellulose could be converted completely. From the economic point, the cost also needs to be controlled besides the high yield. Excessive consumption of ionic liquids will increase the cost of the process. Over all, the optimum mass ratio of cellulose/ILs is 1:20. No matter how much the ILs was used, the TRS yields of the cellulose treated with the ILs were discovered much higher than the cellulose untreated with ILs (61.37%) at 120 °C. The reasonable explanation is that the anions in the ILs could break the hydrogen bonds among the cellulose molecules, which changed the microcrystalline cellulose to amorphous structure. Besides that, the pretreatment increased the internal surface area of cellulose, which is favorable for cellulose to absorb enzyme. This result suggests that the cellulose pretreatment with ILs is necessary and effective for enzymatic hydrolysis (Table 1).

Table 1
TRS from cellulose in different ILs, reaction conditions: cellulose (1 g) dissolved in 20 g of IL at 120 °C for 20 min, cellulase (0.05 mol/l, 150 ml), hydrolysis temperature.

Type of ILs	TRS yield (%)
[BMIM]Cl	100[a], 97.95[b], 90.34[c]
[EMIM][OAc]	90.72[a], 57.04[b], 57.04[c]
[BMIM][OAc]	88.79[a], 81.92[b], 81.04[c]
[BMIM][HCOO]	88.92[a], 76.38[b], 69.84[c]
[EMIM][HCOO]	72.08[a], 68.97[b], 61.84[c]
–	61.37

–, The cellulose was not pretreated with ILs.
[a] Mass ratio of cellulose/IL was 1:20.
[b] Mass ratio of cellulose/IL was 1:15.
[c] Mass ratio of cellulose/IL was 1:10.

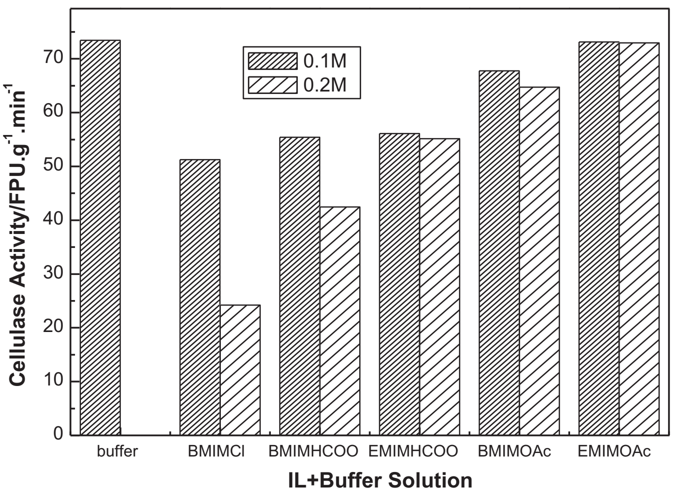

Fig. 7. Effect of IL concentration on the cellulase inactivation.

3.6. Effect of residual ILs on hydrolysis

In 2003, Turner et al. discovered cellulase would lose activity in the presence of [BMIM]Cl (Turner, Spear, Huddleston, Holbrey, & Rogers, 2003). The problem also existed in a serials of acetate based ILs (Zhao et al., 2009). This paper also investigated the relation between ILs and cellulase inactivation.

0.1 g cellulase was dissolved in 1 l citrate buffer solution (pH 4.8, 0.05 mol/l). Then took 100 ml out respectively to two beakers and added 0.01 mol IL to one, 0.02 mol IL to the other one. 15 ml IL/buffer solution mixture was respectively removed to two tubes and a piece of filter paper in a certain size was added to each tube. The hydrolysis process was carried out at 50 °C for 1 h. The concentration of TRS was measured by DNS method and the filter paper unit (FPU) was calculated as follows:

$$\mathrm{FPU} = \frac{\text{reducing sugar}\,(\mu\text{mol})}{\text{used cellulase}\,(\text{g}) \times \text{time}\,(\text{min})}$$

Fig. 7 was plotted according to the calculated result above. From Fig. 7, it was observed that Cl ion based ILs deactivated cellulase more easily than formate and acetate based ILs. Based on this point, the acetate based ILs are more beneficial for hydrolysis process because of their less influence on cellulase inactivation. Further more, the higher of the concentration of residual ILs, the severer the cellulase inactivation.

4. Conclusion

Cellulose was dissolved in ILs including [EMIM][OAc], [BMIM][OAc], [BMIM][HCOO], [EMIM][HCOO], [BMIM]Cl, [AMIM]Cl, followed by a regeneration process and was hydrolyzed by cellulase. The regenerated cellulose was characterized by FTIR and XRD. The crystallinity of the cellulose dropped a lot and the structure has changed from cellulose I to cellulose II. It was observed that cellulose treated with ILs was more easily hydrolyzed than that untreated or treated with NaOH solution. Many factors influence the regeneration and hydrolysis process. The effects of the anti-solvents, the temperatures of pretreatment and enzymatic hydrolysis, the ILs amounts and the residual ILs were studied in our work. We got the favorable reaction conditions from this study. The suggested procedure is: the cellulose dissolved in the ILs at 120 °C for 20 min and regenerated by adding anti-solvent H_2O. The regenerated cellulose was hydrolyzed at 50 °C. The highest yield (100%) of TRS was obtained from the enzymatic hydrolysis of cellulose regenerated from [BMIM]Cl at 50 °C for 5 h, while 88.92% and 90.72% yield of TRS could be reached from the cellulose regenerated from formate and acetate based ILs. From the work we have done, it was obvious that the ILs pretreatment could enhance the hydrolysis rate and the yield of TRS. It confirms further that some kinds of ionic liquids have a promising application in biomass clean energy.

Acknowledgements

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