Bioresource Technology 102 (2011) 1214–1219

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Microwave-assisted pretreatment of cellulose in ionic liquid for accelerated enzymatic hydrolysis

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ARTICLE INFO

Article history:
Received 22 May 2010
Received in revised form 26 July 2010
Accepted 26 July 2010
Available online 1 August 2010

Keywords:
Cellulose pretreatment
Degree of polymerization
Ionic liquids
Microwave
Enzymatic hydrolysis

ABSTRACT

For increasing cellulose accessibility to the enzymatic attack, the pretreatment is a necessary step to alter some structural characteristics of cellulosic materials. As a new pretreatment method, microwave irradiation on cellulose dissolution pretreatment with ionic liquids (ILs) was investigated in this study. Microwave irradiation not only enhanced the solubility of cellulose in ILs but also significantly decreased the degree of polymerization of regenerated cellulose after IL dissolution pretreatment, resulting in significant improvement of cellulose hydrolysis. The rate of enzymatic hydrolysis of cotton cellulose was increased by at least 12-fold after IL dissolution pretreatment at 110 °C and by 50-fold after IL dissolution pretreatment with microwave irradiation. Our results demonstrate that cellulose pretreatment with ILs and microwave irradiation is a potential alternative method for the pretreatment of cellulosic materials.

1. Introduction

Cellulose is the most abundant biomass material on earth with an estimated annual natural production of 1.5×10^{12} tons. Cellulose has been labeled as a sustainable source of raw material for the production of biofuels and platform chemicals (Gray et al., 2006). Cellulose consists of a linear chain of D-glucose subunits linked to each other by β-(1,4)-glycosidic bonds. Individual cellulose chains are joined by a network of inter- and intra-molecular hydrogen bonds and van der Waals forces, which cause cellulose to be packed into a highly ordered crystalline structure. Moreover, natural cellulose is tangled with hemicellulose and lignin. For these reasons, lignocelluloses are highly resistant to hydrolysis. Currently, much effort is being concentrated on the conversion of natural crystalline cellulose into its monomeric glucose constituents. To break down the cellulosic biomass into fermentable sugar, acid-catalyzed hydrolysis has been widely employed for the saccharification of cellulose. However, this produces several potential byproducts inhibiting subsequent fermentation. As a result, enzymatic hydrolysis is considered as the most promising alternative method.

Pretreatment is a necessary step in making resistant cellulosic biomass more accessible to enzymatic hydrolysis (Mosier et al., 2005; Zhang and Lynd, 2005). Since the effectiveness of this step is key to the efficient enzymatic hydrolysis of cellulose, several pretreatment methods have been developed, including biological, physical, physicochemical, and chemical processes (Alvira et al., 2010; Galbe and Zacchi, 2007). Each method has some drawbacks such as: (1) biological or fungal methods require long treatment time; (2) physical treatments (e.g. chipping, milling, grinding, and gamma ray irradiation) have high energy-demands and are too expensive to be used at full-scale; (3) physicochemical processes (e.g. steam explosion) are considered promising methods but require high pressure/temperature; (4) chemical processes (e.g. dilute acid, alkali, using organic solvent, ammonia fiber explosion "AFEX", etc.) involve toxic and environmentally unfriendly compounds. Therefore, the production of biofuels and platform chemicals from cellulosic biomass requires a more efficient pretreatment method that uses less energy, operates under mild conditions, and uses green solvents which are fully recyclable. Therefore, one promising alternative could be the use of ionic liquids (ILs).

ILs consist entirely of ions and melt below 100 °C. Compared to traditional molecular solvents, ILs exhibit interesting properties such as a broad liquidus temperature, high thermal stability, and negligible vapor pressure (van Rantwijk and Sheldon, 2007). ILs have been used as novel non-derivatizing media for the dissolution of carbohydrates including cellulose (Swatloski et al., 2002; Zhao et al., 2008). To date, many ILs, especially those containing halide, acetate, formate, and phosphate anions, have shown high capacity for cellulose dissolution (Ohno and Fukaya, 2009). For example, up to 10 wt.% of cellulose can be dissolved in 1-butyl-3-methylimidazolium chloride ([Bmim][Cl]) at a temperature of 100 °C, and this dissolution can be significantly increased by microwave heating (up to 25 wt.% of pulp cellulose in [Bmim][Cl]). Moreover, cellulose

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doi:10.1016/j.biortech.2010.07.108

can be easily regenerated from a solution of cellulose in ILs by the addition of an anti-solvent such as water, alcohol, or acetone. The dissolution and regeneration of cellulose from ILs is believed to be more environmentally friendly, easier to operate, and less energy consuming than current dissolution processes (Heinze and Liebert, 2001). Regenerated celluloses from ILs were found to have amorphous and porous structures, which improved the kinetics of enzymatic hydrolysis (Dadi et al., 2006; Liu and Chen, 2006).

Recently, microwave technology has been recognized as a powerful tool for the organic synthesis and processing of polymers (Kappe et al., 2009). However, the use of microwaves for cellulose pretreatment in ILs has not yet been reported, although Swatloski et al. (2002) showed that microwave heating could increase the solubility of cellulose in [Bmim][Cl]. Therefore, in this study, the effect of microwave irradiation on cellulose dissolution pretreatment with ILs followed by enzymatic hydrolysis was investigated. To demonstrate enhanced enzymatic hydrolysis of regenerated cellulose after IL dissolution pretreatment with microwaves, the changes in degree of polymerization (DP) and crystalline structure of several celluloses having DPs in the range from 245 to 1400 were examined in two different ILs, 1-ethyl-3-methylimidazolium acetate ([Emim][OAc]) and [Bmim][Cl].

2. Methods

2.1. Materials

[Emim][OAc] and [Bmim][Cl] were purchased from C-Tri (Suwon, Korea) and dried in a vacuum at 90 °C for 24 h before use. Highly purified Sigmacell type 101-F (Sigma, USA), Avicel PH-101 (Fluka, Switzerland), Whatman filter paper 1 (Whatman, UK), and Advantec filter paper 4A (Advantec, Japan) were obtained. Pure cotton was purchased from Daewon Health and Medical Company (Cheonan, Korea). All cellulose samples were dried in an oven at 50 °C for 24 h before use. Cellulase from *Trichoderma reesei* (421 FPU/g) was purchased from Sigma–Aldrich (St. Louis, USA). All other reagents were of analytical grade.

2.2. Cellulose dissolution and regeneration

To measure solubility of cellulose, cellulose was cut into small pieces ($1 \times 1\ mm^2$) and added gradually (10.0 mg each) into a 5 mL glass vial containing 1 mL of ILs. For conventional pretreatment, 20.0 mg of cellulose ($1 \times 1\ mm^2$) was added into a 5 mL glass vial containing 1 mL of ILs. The cellulose suspension in ILs was then placed in a heating block at 110 °C for 30 min with magnetic stirring at 500 rpm. The clear and viscous IL solution containing cellulose was formed. This pretreatment process is hereafter referred to as "IL dissolution pretreatment". The IL solution containing 20.0 mg of cellulose was then placed under microwave irradiation in a CEM Discover microwave system (Matthews, USA) at a constant power of 50 W for 12 s with magnetic stirring (an energy of 30 kJ was applied per g cellulose). This condition is hereafter referred to as "IL dissolution pretreatment with microwave irradiation". During microwave irradiation, the observed maximum temperature of the IL solution containing cellulose was below 150 °C. After dissolution, 2 mL of deionized water as an anti-solvent was added to the cellulose solution for the regeneration of cellulose from ILs. A precipitate was immediately formed, after which the resulting mixture was centrifuged and supernatant containing ILs was removed in order to retrieve regenerated cellulose. The regenerated cellulose was then washed thoroughly at least five times with deionized water to remove residual ILs. The regenerated cellulose was then dried at 50 °C for 24 h for the enzymatic hydrolysis reactions.

2.3. Enzymatic hydrolysis of cellulose

A suspension of 20.0 mg (untreated or regenerated) of cellulose in 1 mL of 5 mM citrate buffer (pH 4.8) was incubated in a reaction block at 50 °C with magnetic stirring at 150 rpm. The reaction was started by the addition of 1.0 mg of *T. reesei* cellulase. Samples (20 μL) were periodically withdrawn and diluted with buffer. The released reducing sugar was measured by DNS assay (Miller, 1959). The concentration of reducing sugar in the sample was calculated from its absorbance at 540 nm using D-glucose as the standard. Glucose concentration was also determined using a Shimadzu LC-10A HPLC system (Kyoto, Japan) equipped with a Waters 410 RI detector (Milford, USA) and Bio-Rad HPX-87P column (Hercules, USA) at 85 °C. The mobile phase used was deionized water at a flow rate of 0.6 mL/min. All reactions were performed in duplicate.

2.4. Determination of degree of polymerization (DP)

The number-average degree of polymerization of cellulose was calculated from the ratio of the glucosyl monomer concentration, as determined by the phenol–sulfuric acid method, to the reducing-end concentration, as determined by a modified 2,2′-bicinchoninate (BCA) method (Zhang and Lynd, 2005). Cellulose samples (untreated celluloses or regenerated celluloses) were solubilized in cold phosphoric acid before measuring the reducing-end concentration.

2.5. FTIR analysis

The FTIR spectra (4000–400 cm^{-1}) were recorded by a Bruker Vertex 80V FTIR vacuum spectrometer (Ettlingen, Germany) with a resolution of 2 cm^{-1} and 32 scans per sample. Two milligram of cellulose or regenerated cellulose along with 200 mg of spectroscopic grade KBr were pressed to produce clear pellets. The background spectrum of KBr was subtracted from that of the sample spectrum.

3. Results and discussion

3.1. Enzymatic hydrolysis of cellulose

Cellulose solution was prepared by dissolving cotton cellulose in [Bmim][Cl] and [Emim][OAc] at 110 °C for 30 min without or with further microwave irradiation for 12 s. After precipitation using water as an anti-solvent, regenerated cellulose was obtained. Fig. 1(a) shows the time course of the cellulase-catalyzed hydrolysis of untreated and regenerated cellulose. Both the initial hydrolytic rate and the yield of reducing sugar were significantly enhanced after IL dissolution pretreatment, similar to that reported in the literature (Dadi et al., 2006; Liu and Chen, 2006). The amounts of reducing sugars released from the regenerated celluloses after [Bmim][Cl] and [Emim][OAc] dissolution pretreatment were 14.7 and 13.4 mg/mL after 24 h, respectively, whereas only 5.0 mg/mL of reducing sugars was released from untreated cotton cellulose. The initial rates of enzyme-catalyzed hydrolysis of regenerated cotton celluloses were at least 12 times higher than that of untreated cotton (Fig. 1(b)).

Interestingly, the cotton celluloses regenerated from IL dissolution pretreatment with microwave irradiation were hydrolyzed much faster than those without microwave irradiation. After 3.5 h of enzymatic hydrolysis, 14.4 and 13.2 mg/mL of reducing sugars were released from the celluloses regenerated from [Bmim][Cl] and [Emim][OAc] dissolution pretreatment with microwave irradiation, respectively. In contrast, only 6.4 and 5.8 mg/mL

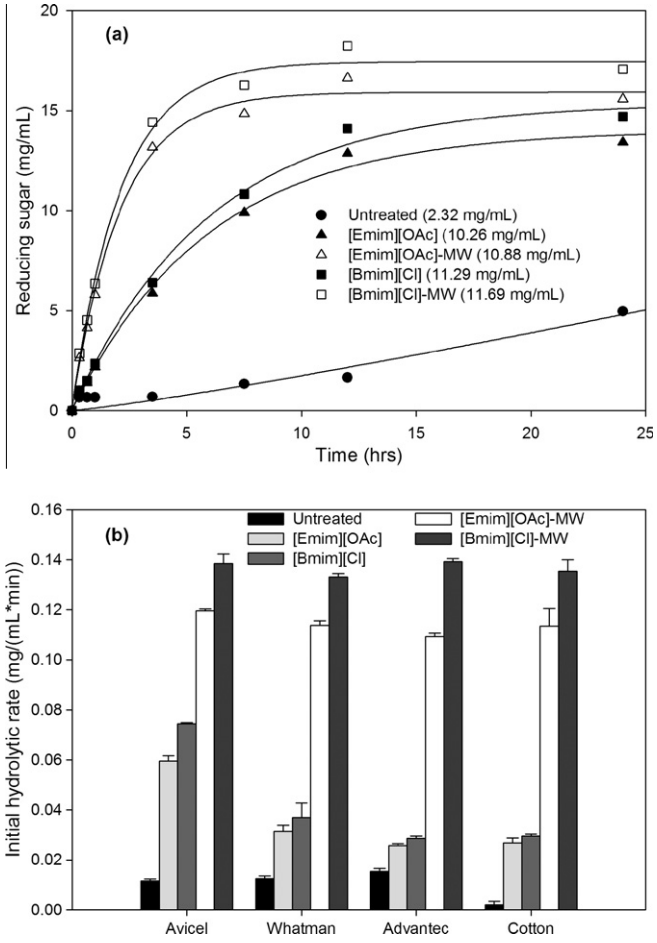

Fig. 1. (a) Reducing sugars released during enzymatic hydrolysis of untreated and regenerated cotton cellulose from ILs. Values in parentheses are glucose concentration at 24 h as determined by HPLC analysis. (b) Initial hydrolytic rate of enzymatic hydrolysis of untreated and regenerated cellulose from ILs.

of reducing sugars were released from the celluloses regenerated from [Bmim][Cl] and [Emim][OAc] dissolution pretreatment alone, respectively. The initial hydrolytic rates of regenerated cotton celluloses after IL pretreatment with microwave irradiation (0.114 and 0.136 mg/(mL min) for [Emim][OAc] and [Bmim][Cl], respectively) were at least 4 times higher than those of regenerated celluloses after IL pretreatment with conventional heating only (0.027 and 0.030 mg/(mL min) for [Emim][OAc] and [Bmim][Cl], respectively). However, compared to that of untreated cotton cellulose (0.002 mg/(mL min)), these initial hydrolytic rates were surprisingly 50 times faster. In addition, the amount of reducing sugars released from regenerated celluloses after [Bmim][Cl] and [Emim][OAc] dissolution pretreatment with microwave irradiation were 17.1 and 15.6 mg/mL after 24 h, respectively. This implies that an approximately 3-fold enhancement in the cellulose hydrolysis yield can be achieved using IL dissolution pretreatment with microwave irradiation compared to that of untreated cotton cellulose (5.0 mg/mL after 24 h).

3.2. Effect of dissolution conditions on structural properties of regenerated cellulose

In the enzyme-catalyzed hydrolysis of cellulose, both enzyme-related factors such as product inhibition, thermal stability, and adsorption and substrate-related factors such as cellulose crystallinity and degree of polymerization (DP) have great effects on the kinetics of cellulose hydrolysis (Chandra et al., 2007; Kumar and Wyman, 2009; Mansfield et al., 1999). Since *T. reesei* cellulase was only used in this study, substrate-related factors were compared with untreated cellulose to understand the enhanced hydrolysis of regenerated cellulose from ILs.

As shown in Table 1, IL dissolution pretreatment with microwave irradiation led to a remarkable reduction in the DP of cellulose. For example, following dissolution in [Emim][OAc] and [Bmim][Cl] with microwave irradiation, regenerated cotton experienced 96% and 97% decreases in DP, respectively, whereas only 19% and 26% reductions in DP were observed in cotton regenerated from IL dissolution pretreatment with conventional heating. This phenomenon was also found in the Avicel, Whatman, and Advantec celluloses, which have DPs in the range from 245 to 1400. All regenerated celluloses after IL dissolution pretreatment with microwave irradiation had similar DPs (50–59 for [Emim][OAc] and 40–45 for [Bmim][Cl]). Therefore, it is not surprising that all of the regenerated celluloses after IL dissolution pretreatment with microwave irradiation exhibited similar initial hydrolytic rates, as shown in Fig. 1(b) (0.109–0.120 mg/(mL* min) for [Emim][OAc] and 0.133–0.139 mg/(mL* min) for [Bmim][Cl], respectively).

This result might be explained by the difference between microwave heating and conventional heating. Microwave heating pertains to the internal heating of materials, whereas conventional heating pertains to the conduction of heat. Moreover, the microwave heating of materials is closely related to the polarization of materials. Polar solvents absorb microwave irradiation extremely efficiently, resulting in rapid heating. However, non-polar solvents absorb little or no microwave radiation, resulting in no heating. Thus, ILs with high polarity could be easily heated by microwave irradiation (Feng and Chen, 2008; Kappe et al., 2009).

Table 1
Solubility and structural features of celluloses dissolved in ILs.

Cellulose	Solubility (w/v%)[a]		DP[b]	FTIR	
	[Emim][OAc]	[Bmim][Cl]		LOI[c]	TCI[d]
Avicel	22	18	(245) 226, 205	(1.28) 0.34, 0.38	(0.63) 0.30, 0.33
Avicel-MW	28	24	(245) 51, 40	(1.28) 0.36, 0.41	(0.63) 0.32, 0.35
Whatman	14	8	(963) 924, 870	(1.21) 0.82, 0.99	(0.61) 0.28, 0.22
Whatman-MW	22	15	(963) 55, 42	(1.21) 0.77, 0.81	(0.61) 0.24, 0.25
Advantec	12	8	(1312) 1071, 705	(1.18) 0.76, 0.88	(0.58) 0.41, 0.40
Advantec-MW	19	14	(1312) 57, 44	(1.18) 0.71, 0.80	(0.58) 0.38, 0.38
Cotton	8	6	(1376) 1110, 1021	(1.26) 0.34, 0.37	(0.71) 0.30, 0.22
Cotton-MW	15	12	(1376) 59, 45	(1.26) 0.31, 0.36	(0.71) 0.29, 0.24

[a] Celluloses were added gradually (10.0 mg each time) in 1 mL of ILs at 110 °C under magnetic stirring of 500 rpm until clear viscous solution obtained. Experiments of cellulose dissolving in ILs with microwave irradiation were carried out with microwave power of 50 W at pulse of 5 s.
[b] The value in parentheses was DP of untreated cellulose. The data were showed in order of regenerated cellulose from [Emim][OAc] and [Bmim][Cl], respectively.
[c] LOI: lateral order index were determined as the ratio of two absorption bands of α1430 cm^{-1}/α897 cm^{-1} from FTIR spectra.
[d] TCI: total crystallinity index which is the ratio of α1372 cm^{-1}/α2900 cm^{-1}.

Since the dissolution of cellulose in a solvent depends on the solvent properties as well as the structural properties of cellulose, reduced DP can explain the higher solubilities of regenerated celluloses after IL dissolution pretreatment with microwave irradiation compared to those after conventional heating only (Table 1). Table 1 also shows that [Emim][OAc] was more effective for cellulose dissolution than [Bmim][Cl]. Generally, the solubility of cellulose in ILs strongly depends on the interaction between the hydroxyl group of cellulose and the anions of the ILs, which serve as hydrogen-bonding acceptors. The ability of ILs to act as hydrogen-bonding acceptors is determined by their physicochemical properties, especially hydrogen-bonding basicity (β). According to the Kamlet–Taft parameters of [Bmim] salts, acetate anion has stronger hydrogen-bonding basicity (1.09) than that of chloride anion (0.87) (Ohno and Fukaya, 2009). Moreover, [Emim][OAc] (162 cP at 25 °C) has relatively lower viscosity than [Bmim][Cl] (1100 cP at 25 °C) (Bonhote et al., 1996; Li et al., 2006). As a result, [Emim][OAc] can easily dissolve a higher amount of cellulose than [Bmim][Cl].

Changes in the crystallinity of celluloses regenerated from IL dissolution pretreatment were also investigated by FTIR spectroscopy in the 400–4000 cm^{-1} region (FTIR spectra not shown), which is commonly used to characterize the polymorphs of highly crystalline cellulose (Nelson and O'Connor, 1964a). The adsorption band at 1430 cm^{-1} assigned to the CH_2 scissoring motion was strong in type I crystalline (cellulose I) and very weak in type II crystalline (cellulose II) and amorphous cellulose. However, the adsorption band at 897 cm^{-1} assigned as C–O–C stretching at the β-(1,4)-glycosidic linkage was weak and broad in cellulose I but strong and sharp in cellulose II and amorphous cellulose. Due to the sensitivities of the two absorption bands at α1430 cm^{-1} and α897 cm^{-1}, the absorbance ratio (α1430 cm^{-1}/α897 cm^{-1}), known as the lateral order index (LOI) (Hurtubise and Krassig, 1960), indicated the cellulose I fraction in the cellulose structure (Oh et al., 2005). Furthermore, the ratio of the two absorption bands between α1372 cm^{-1} (C–H bending) and α2900 cm^{-1} (C–H and CH_2 stretching), known as the total crystallinity index (TCI), was also used to measure the crystallinity of cellulose material (Nelson and O'Connor, 1964b). According to the FTIR spectra (not shown), the absorption bands at α1430 and α1372 cm^{-1} for untreated cotton were significantly decreased in cotton celluloses regenerated from ILs. However, little difference was found between regenerated cellulose after IL dissolution pretreatment only and that after microwave irradiation. In other words, the structure of cellulose I in untreated cotton was transformed into an amorphous or cellulose II structure after regeneration from ILs. Therefore, the LOI and TCI were significantly decreased in all regenerated cellulose samples (Table 1). According to the LOI value, cotton celluloses regenerated from IL dissolution pretreatment had 18–75% lower crystallinity than untreated celluloses, which provided higher surface accessibility for the enzyme. These results also explain the enhancement of enzymatic hydrolysis in cellulose regenerated from ILs. However, there were little differences in LOI and TCI between regenerated cotton celluloses after IL dissolution pretreatment with or without microwave irradiation. It suggests that reduced DP rather than crystallinity is responsible for enhanced enzymatic hydrolysis in regenerated celluloses from ILs dissolution pretreatment with microwave irradiation compared to those with conventional heating only.

3.3. Effect of residual IL on regenerated cellulose

It was reported that *T. reseei* cellulase could be inactivated by a high concentration of [Bmim][Cl] and [Emim][OAc] (Turner et al., 2003; Zhao et al., 2009). During the regeneration of cellulose from ILs, some residual ILs might have become trapped in the cellulose chains despite thorough washing. Thus, residual ILs in regenerated cellulose might affect enzymatic hydrolysis. Following complete hydrolysis of regenerated cellulose, some quantity of [Bmim][Cl] was detected in the hydrolytic reaction solution (Fig. 2(a)). In cotton cellulose regenerated from IL dissolution pretreatment, the highest amount of residual [Bmim][Cl] was 15.6 wt.%. This can be explained by the highly complex structure of cotton cellulose, which consists of twisted, ribbon-like fibers (Zhao et al., 2009). On the other hand, regardless of the type of cellulose, the amounts of residual [Bmim][Cl] in regenerated celluloses after IL dissolution pretreatment with microwave irradiation were around 4 wt.%, which is at least 2-fold lower than those prepared without microwave irradiation. These results suggest that microwave irradiation not only reduces the DP of cellulose dissolved in ILs but also decreases the amount of residual ILs in regenerated celluloses. The reduced amount of residual ILs in regenerated cellulose could therefore enhance the efficiency of ILs for the pretreatment of cellulose as well as that of the subsequent enzymatic hydrolysis step (Fig. 1(a) and (b)). As shown in Fig. 2(b), *T. reesei* cellulase was inactivated when the concentration of [Bmim][Cl] was higher than 5 wt.%. In the presence of 20 wt.% [Bmim][Cl], only 15% of *T. reesei* cellulase activity remained. This result also supports our results shown in Fig. 1(a).

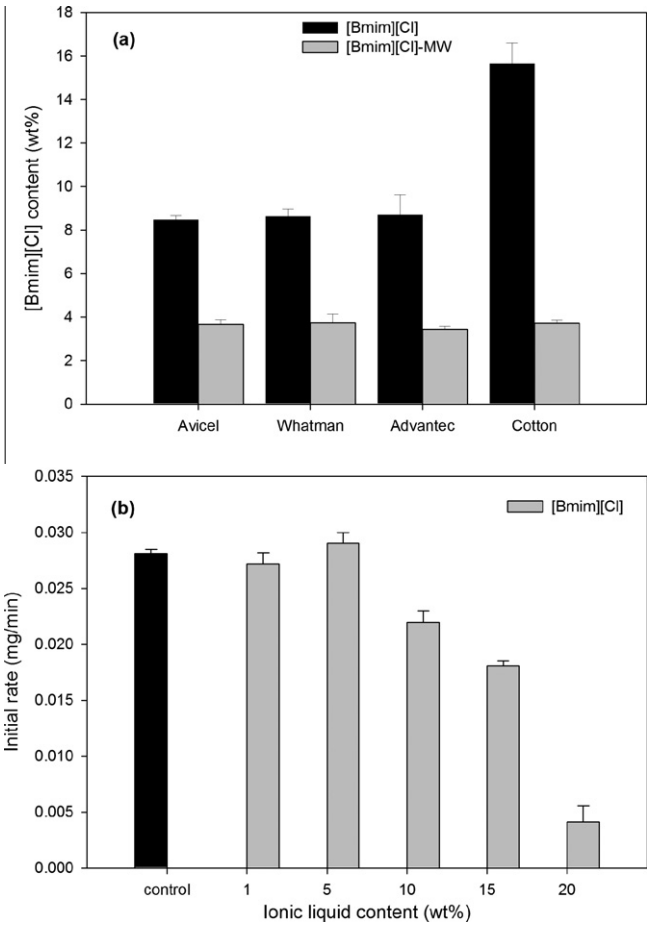

Fig. 2. (a) Residual amount of [Bmim][Cl] in cellulose regenerated from [Bmim][Cl]. Residual [Bmim][Cl] was calculated based on the amount of Cl^- in the reaction solution following complete enzymatic hydrolysis. The amount of Cl^- was determined by the silver chromate method (Toral et al., 2007). (b) Initial rate of enzymatic hydrolysis of amorphous cellulose in the presence of ILs. Enzymatic hydrolysis reactions were performed using 1.0 mg of *T. reesei* cellulase and 20.0 mg of amorphous cellulose (Sigmacell) in 1 mL of 5 mM citrate buffer (pH 4.8) containing predetermined amount of ILs at 50 °C.

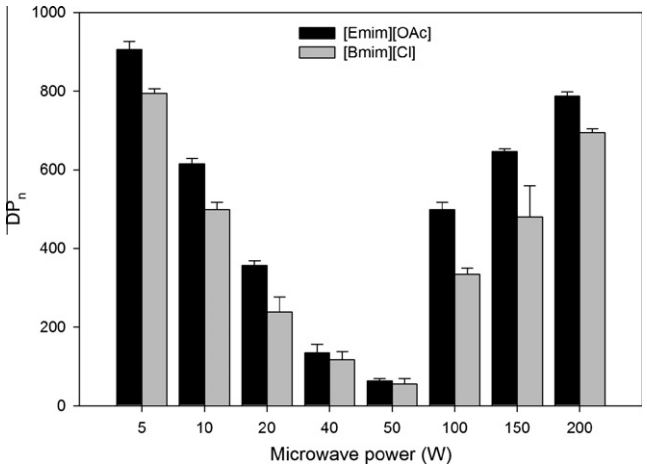

Fig. 3. Effect of microwave power on the DP of regenerated cotton cellulose from ILs. The experiment was carried out using 20.0 mg of cotton cellulose in 1 mL of ILs at a specific microwave power (600 J/mL of cellulose solution).

3.4. Optimization of cellulose pretreatment in ILs by microwave heating

Since microwave irradiation during cellulose dissolution in ILs could efficiently reduce the DP of cellulose, further optimization of the microwave irradiation conditions was attempted. Theoretically, microwave irradiation causes internal heating (in core volumetric heating). Specifically, the whole volume is simultaneously heated by direct coupling of microwave energy with the molecules present in the reaction mixture (Kappe et al., 2009). In order to investigate the effect of microwave power on the pretreatment of cellulose dissolved in ILs, the same amount of energy was introduced into the cellulose solution in ILs at different microwave power levels. An energy of 600 J which was calculated from the input microwave power and operating time was applied to 1 mL of cellulose solution (in our preliminary experiments on cellulose with microwave irradiation, 600 J/mL of IL/cellulose solution in ILs did not cause any decomposition of cellulose in ILs). As shown in Fig. 3, an increase in microwave power up to 50 W resulted in a decrease in the DP of cellulose dissolved in ILs. However, a further increase in microwave power resulted in an increase in the DP of cellulose dissolved in ILs. As a result, the optimal microwave power for cellulose treatment was found to be 50 W.

For the large-scale application of ILs in the biomass hydrolysis process based on ILs, development of efficient IL recovery methods is a prerequisite. Except evaporation, membrane filtration (Fernández et al., 2008), ion-exclusion chromatography (Binder and Raines, 2010), and electromagnet (Lee et al., 2007) are reported so far to recycle ILs, but these methods are complex and are tested in a lab scale. Therefore, further research on IL recovery methods should be investigated in detail for the viability of biomass processing with ILs.

4. Conclusions

Significant enhancement of the release of reducing sugars in both rate and yield was achieved during the enzymatic hydrolysis of cellulose regenerated from IL dissolution pretreatment with microwave irradiation. This was mainly due to a decrease in DP by microwave irradiation, in addition to the changes in the crystal structure by ILs dissolution pretreatment. In addition, microwave irradiation enhanced the dissolution of cellulose in ILs and reduced the amount of residual ILs in regenerated celluloses. In conclusion, IL dissolution pretreatment with microwave irradiation can be used as a potential alternative method for the pretreatment of cellulosic materials.

Acknowledgements

This work was financially supported by the Ministry of Knowledge Economy, Korea through the Cleaner Production Technology Development Project. This work was also supported in part by Inha University, Korea.

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