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Mild pretreatment and enzymatic saccharification of cellulose with recycled ionic liquids towards one-batch process

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ABSTRACT

The development of second-generation bioethanol involves minimizing the energy input throughout the processing steps. We report here that efficient ionic liquid pretreatments of cellulose can be achieved with short duration times (20 min) at mild temperature (45 °C) with $[\mathrm{Emim}]^+[\mathrm{MeO(H)PO_2}]^-$ and at room temperature (25 °C) with $[\mathrm{Emim}]^+[\mathrm{CH_3COO}]^-$. In these conditions, yields of glucose were increased by a factor of 3. In addition, the recycling of these two imidazolium-based ILs can be performed in maintaining their efficiency to pretreat cellulose. The short time and mild temperature of cellulose solubilization allowed a one-batch processing of $[\mathrm{Emim}]^+[\mathrm{MeO(H)PO_2}]^-$ IL-pretreatment and saccharification. In the range from 0 to 100% IL in an aqueous enzymatic medium, the glucose yields were improved at IL proportions between 10 and 40%. The maximum yield at 10% IL is very promising to consider one batch process as efficient as two-step process.

1. Introduction

Lignocellulosic biomass originating from wood or agricultural residues constitutes a promising raw material for the production of second-generation bioethanol avoiding direct competition with food supply (Graham-Rowe, 2011; Sims, Mabee, Saddler, & Taylor, 2010). Moreover, bioethanol obtained from renewable sources of feedstock leads to greenhouse gas reductions and meets some criteria of sustainability (Balat, 2011; Sun & Cheng, 2002). In terms of sustainability and eco-friendly processes, the most promising pathway to produce bioethanol is the bioconversion of lignocellulosic materials through saccharification and fermentation as against the chemical route (Orozco, Ahmad, Rooney, & Walker, 2007). Many studies have demonstrated the value of enzymatic hydrolysis in aqueous or non-conventional media to produce fermentable sugars from insoluble cellulose (Gupta & Lee, 2009; Jones & Vasudevan, 2010). In addition to the mild processing conditions, the enzymatic method overcomes the drawbacks of acid hydrolysis such as product degradation and the formation of microbial inhibitors. However, the efficiency of enzymatic hydrolysis is limited due to the complex structure of lignocellulose and its variability. To overcome this barrier, lignocellulose must be pretreated in order to alter its supramolecular structure and make cellulose more accessible to cellulases. Different methods have been proposed including physical (mechanical milling), physico-chemical (steam explosion), biological (microorganisms) and chemical (alkaline, acid) pretreatments (Chandra et al., 2007). In the research field, an emerging pretreatment is the dissolution of (ligno-)cellulosic biomass in hydrophilic ionic liquids (ILs) (Liebert & Heinze, 2008; Mora-Pale, Meli, Doherty, Linhardt, & Dordick, 2011; Swatloski, Spear, Holbrey, & Rogers, 2002; Vitz, Tina, Claudia, & Ulrich, 2009; Zavrel, Bross, Funke, Büchs, & Spiess, 2009). This dissolution is induced by the formation of electron donor–electron acceptor complexes between cellulose oxygens and the constitutive cations of ILs on the one hand and between cellulose hydrogens and the constitutive anions of ILs on the other hand (Zhu et al., 2006). These interactions lead to the disruption of the intra/inter-chain H bond network of cellulose and thus to its dissolution. After regeneration from ILs, cellulose exhibits a weakening in its crystallinity and/or a disruption of its fiber organization (Mäki-Arvela, Anugwom, Virtanen, Sjöholm, & Mikkola, 2010). As a result, the IL-pretreated celluloses are more accessible to cellulases and can be efficiently hydrolyzed into fermentable sugars, as described in recent studies (Dadi, Varanasi, & Schall, 2006; Dadi, Schall, & Varanasi, 2007; Husson et al., 2011; Zhao et al., 2009). ILs are non-volatile recyclable solvents and some have low toxicity (Shill et al., 2011; Sun et al., 2009; Zavrel et al., 2009). However, in the literature, most of the IL pretreatments of cellulose prior to its enzymatic hydrolysis are performed at a temperature close to or higher than 100 °C (Li, Asikkala, Filpponen, & Argyropoulos, 2010; Li, Knierim, et al., 2010; Samayam & Schall,

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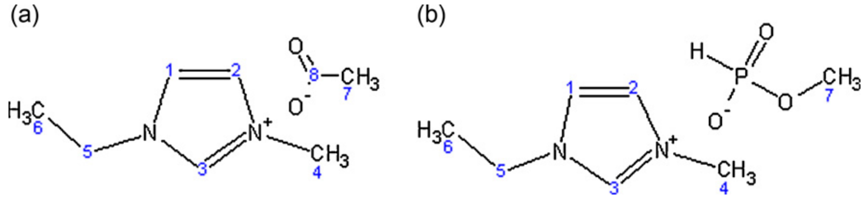

Fig. 1. Chemical structure and numbering of carbon atoms of the two distinct ionic liquids: 1-ethyl-3-methylimidazolium acetate ($[Emim]^+[CH_3COO]^-$) (a) and 1-ethyl-3-methylimidazolium methylphosphonate ($[Emim]^+[MeO(H)PO_2]^-$) (b).

2010; Zhao et al., 2009) except for phosphonate-based ILs that can dissolve cellulose under mild conditions (Fukaya, Hayashi, Wada, & Ohno, 2008). Furthermore, at weak concentrations in the reaction media, some ILs do not seem to affect cellulase activities drastically (Wang, Radosevich, Hayes, & Labbé, 2011; Zhao et al., 2009). However, a recent study has demonstrated that an efficient cellulase activity can be preserved at high hydrophilic IL ($[Bmim]^+[Cl]^-$) concentration by coating enzymes with some hydrophobic ILs (Lozano, Bernal, Bernal, Pucheault, & Vaultier, 2011). This IL-tolerance of cellulases enables efficient enzymatic hydrolysis to be carried out in diluted IL-aqueous systems thus combining pretreatment and hydrolysis in a one-batch process, which could be an alternative industrial process to acid pretreatment and hydrolysis (Jones & Vasudevan, 2010; Kamiya et al., 2008; Yang et al., 2010). The use of IL-pretreatment prior to enzymatic degradation or enzymatic degradation in IL/water media is still at an early stage. However, for ILs to be used in bio-refineries, a low-energy IL-pretreatment must be developed and IL-recycling procedure should be considered (Klein-Marcuschamer, Simmons, & Blanch, 2011). The classical way consists in evaporating, under vacuum, the anti-solvent previously used to regenerate IL-pretreated cellulose. For instance, previous studies demonstrated the feasibility to reuse imidazolium-based ILs for lignocellulosic biomass pretreatment with a decrease of its efficiency. As suggested by authors, this would be due to a progressive accumulation in IL of soluble components as lignin at each pretreatment (Dibble et al., 2011; Li, Asikkala, et al., 2010; Li, Knierim, et al., 2010; Shill et al., 2011).

Our purpose is both to minimize the energy intake of pretreatment with ILs and to investigate the feasibility of their recycling by studying the effects on the enzymatic hydrolysis of pure cellulose. Indeed, many studies have shown large differences in the impact of IL-pretreatment depending on the biomass used due to varying amounts of lignin and hemicellulose (Guragain, De Coninck, Husson, Durand, & Rakshit, 2011). It is necessary to know the prerequisites of the cellulose substrate itself. Thus, the aim of the present work is to investigate the influence of both recycling and temperature/duration of pretreatments on ILs capacity to improve the enzymatic degradability of cellulose. Two ILs were selected for this study: 1-ethyl-3-methylimidazolium acetate ($[Emim]^+[CH_3COO]^-$), classically used at high temperature, and newly developed 1-ethyl-3-methylimidazolium methylphosphonate ($[Emim]^+[MeO(H)PO_2]^-$), which has recently been proposed as an efficient alternative to $[Emim]^+[CH_3COO]^-$ (Husson et al., 2011). To our knowledge, the impact of methylphosphonate-based IL recycling on its efficiency to pretreat cellulose has never been described in the literature. Pure cellulose was chosen to highlight the impact of ILs solely on the substrate of cellulases. For all tested conditions of pretreatment, the crystallinity and the surface topography of cellulose samples were characterized by CP-MAS ^{13}C NMR and scanning electron microscopy (SEM), respectively, and related to their degradation by cellulase. Hence, IL integrity and residual water content were evaluated after each recycling. Finally, described for the first time, the compatibility between cellulase and $[Emim]^+[MeO(H)PO_2]^-$ was studied to verify the feasibility of combining the IL-pretreatment and enzymatic hydrolysis of cellulose in a one-batch process.

2. Materials and methods

2.1. Chemicals and enzyme

Cotton cellulose exhibiting a high-purity degree was furnished by Sigma–Aldrich (Steinheim, Germany) and was characterized by sizes ranging from 125 to 400 μm (*i.e.* long fibers of cellulose) (Örmeci & Vesilind, 2000).

Cellulase was from *Trichoderma reesei* (EC 3.2.1.4) supplied by Sigma–Aldrich (Steinheim, Germany) which presents a specific activity of 5 U/mg; one unit liberates 1.0 μmol of glucose from cellulose in 1 h at pH 5.0 at 37 °C (2 h incubation time). 1-ethyl-3-methylimidazolium acetate ($[Emim]^+[CH_3COO]^-$) and 1-ethyl-3-methylimidazolium phosphonate ($[Emim]^+[MeO(H)PO_2]^-$), with a purity higher than 98%, were acquired from Solvionic SA (Verniole, France) (Fig. 1). Anhydrous sodium acetate (99%) came from Fluka Sigma–Aldrich (Steinheim, Germany), sodium hydroxide solution (46/48%) from Fisher Scientific (Illkirch, France) and acetic acid (99%) from Carl Roth (Lauterbourg, France). Standard glucose and cellobiose were purchased from Sigma–Aldrich (Steinheim, Germany).

2.2. Ionic liquid-based pretreatments of cellulose and regeneration step

Cellulosic substrate (2%, w/v) was added to each IL, and incubated in an oil bath at different temperatures (25 °C, 45 °C, 65 °C and 110 °C) with vigorous stirring for times varying from 20 min to 300 min. The control consisted of the incubation of cellulose (2%, w/v) in ultrapure water (deionized water with a resistivity of 18.3 MΩ cm, Barnsted Easy Pure RF) at 110 °C for 40 and 300 min to verify the thermo-stability of hydrated cellulose. After incubation, each cellulose solution was cooled in an ice bath. The cellulose was precipitated by adding ultrapure water (2:1, v/v, water/IL) to the mixture with vigorous stirring for 30 min in ice bath to increase the polarity of the medium then centrifuging it (10,733 × g, 20 min, 4 °C with an Allegra 64R Beckman Coulter rotor: F0850). A regeneration step of the pretreated celluloses was undertaken to prevent cellulase deactivation by high IL concentrations in the reaction media as suggested in recent studies (Jones & Vasudevan, 2010; Kamiya et al., 2008; Zhao et al., 2009). The cellulose flocks were collected by vacuum filtration, thoroughly washed with ultrapure water and then freeze-dried. The absence of hornification, potentially induced during freeze-dried step, was checked by solid state NMR (Köhnke, Lund, Brelid, & Westman, 2010; Luo & Zhu, 2011). Permeate solution (diluted aqueous-IL) was recovered for further IL-recycling and reuse. The percentage of residual IL trapped in pretreated celluloses was estimated at 10% (w/w) (Husson et al., 2011).

2.3. Recovery and recycle of Ionic liquids

Each permeate solution of diluted aqueous-ILs was distilled by rotary evaporator (Büchi Rotavapor R-200) under vacuum at 80 °C for 2 h. ILs-recovered were stored in 4 °C under dry atmosphere for a future reuse. IL-recovered yield was determined by measurement of IL-volume as described by the equation below:

$$Y_{\text{IL-recovered}}\,(\%) = \frac{V_{\text{IL-recovered}}\,(\text{mL})}{V_{\text{IL-used for cellulose pretreatment}}\,(\text{mL})} \times 100 \tag{1}$$

After each IL-recycling, this yield was estimated superior to 90%.

100 μL of each recovered IL were withdrawn to check both their integrity and residual water content, respectively, by liquid state ^{1}H and ^{13}C NMR and Karl Fischer coulometry.

^{1}H and ^{13}C NMR spectra were acquired on a Bruker Advance 600 MHz spectrometer equipped with TXI 1.7 mm microprobe operating at 600.1727 MHz (^{1}H channel) and 150.9272 MHz (^{13}C channel). IL samples were prepared by adding D_2O (1:3 D_2O/IL, v/v) in tubes and mixed thoroughly. ^{1}H NMR spectra consisting of 16 scans of 32 k points with spectral width of 6602.113 Hz were collected with a relaxation delay of 1 s at 300 K. The residual water signal of D_2O at 4.75 ppm was used as reference. Proton decoupled ^{13}C NMR spectra consisting of 256 scans of 65 k points with spectral width of 29761.904 Hz were collected with a relaxation delay of 2 s at 300 K. Spectral assignments of recovered $[\text{Emim}]^+[\text{MeO(H)PO}_2]^-$ and $[\text{Emim}]^+[\text{CH}_3\text{COO}]^-$ were characterized and are in agreement with the literature data (Fukaya et al., 2008; Hesse-Ertelt, Heinze, Kosan, Schwikal, & Meister, 2010).

IL-residual water content was carried out by Karl Fischer coulometry method (831 KF Coulometer, Metrohm) equipped with an oven at 140 °C (774 Oven Sample Processor, Metrohm). 50 μL of IL samples were weighed out into glass vessels and sealed. Accuracy of the measurement was verified with lactose standards. All values were corrected by subtraction of atmospheric water content. Each analysis was repeated in duplicate and expressed as mean values with standard deviations.

2.4. Enzymatic hydrolysis of cellulose in aqueous medium

The cellulase-catalyzed hydrolysis of the different cellulose substrates (untreated or pretreated) was carried out in stirred flasks. In a typical hydrolysis reaction, 200 mg of cellulose, or about 200 mg of pretreated cellulose (taking into account the amount of residual IL), were added to 9 mL of acetate buffer (50 mM, pH 4.8) and incubated for 30 min (40 °C; 175 rpm). After this preincubation step, hydrolysis was initiated by addition of 1 mL of 10 mg/mL cellulase preparation. The final concentrations of cellulose and enzyme in the reaction medium (10 mL) were 2% (w/v) and 1 mg/mL, respectively. Control experiments without enzyme were also carried out. 100 μL reaction medium were withdrawn at different times in order to determine the progress of the reaction, which was stopped by incubating the withdrawn sample at 90 °C for 20 min to deactivate the enzyme (Yeh, Huang, & Chen, 2010). Then, the sample was diluted in ultrapure water and filtered (0.2 μm) prior to analysis by high performance anion-exchange chromatography (HPAEC).

Each pretreatment and reaction was repeated in triplicate. Initial rates and substrate conversion yields were expressed as mean values with standard deviations (±).

2.5. Enzymatic hydrolysis of cellulose in diluted IL-aqueous medium

20 mg of cellulose long fibers were added to various amounts of $[\text{Emim}]^+[\text{MeO(H)PO}_2]^-$ in Eppendorf tubes. The IL-cellulose suspensions were incubated for 1 h at 40 °C with vigorous stirring. Then, acetate buffer (50 mM, pH 4.8) was directly added and the reaction was initiated by the addition of 100 μL of cellulase preparation at a concentration of 10 mg/mL. The final concentrations of cellulose and enzyme in the reaction medium (1 mL) were 2% (w/v) and 1 mg/mL, respectively. Control experiments without enzyme or without IL were also carried out. After 90 h, to reach the steady state, the reaction was stopped by thermal inactivation of enzyme as described above. Reaction media were diluted in ultrapure water and filtered (0.2 μm) prior to analysis by HPAEC. Each reaction was repeated in triplicate. Substrate conversion yields of glucose were expressed as mean values with standard deviations (±).

2.6. Quantification of glucose and cellobiose content

The production of glucose and of cellobiose was monitored by HPAEC using an analytical CarboPac PA-20 kept at 25 °C. Elution was carried out at a flow rate of 0.5 mL/min with a gradient method described previously (Husson et al., 2011). The retention times of glucose and cellobiose were 11.96 min (±0.56%) and 29.91 min (±0.39%), respectively. Quantification was based on calibration curves established using standard glucose and cellobiose. The conversion of cellulose into glucose was determined by taking into account the difference between the Molar Mass of free glucose and glucose in a polysaccharide, *i.e.* the ratio 162/180, as described by the equation below:

$$Y_{\text{glucose}}\,(\%) = \frac{[\text{Glucose}\,(\text{g}) \times 162/180]}{[\text{Cellulose}_{\text{initial}}\,(\text{g})]} \times 100 \tag{2}$$

The conversion of cellulose into cellobiose was determined in the same way by applying the ratio 324/342, as shown in the following equation:

$$Y_{\text{cellobiose}}\,(\%) = \frac{[\text{Cellobiose}\,(\text{g}) \times 324/342]}{[\text{Cellulose}_{\text{initial}}\,(\text{g})]} \times 100 \tag{3}$$

Apparent initial rates of reaction were calculated by derivation of a second-order polynomial approximation of the concentration profile, built on experimental data obtained during the first hour of enzymatic hydrolysis (Matlab 6.5, 2002, MathWorks, USA).

2.7. Cellulose substrates characterization

Crystallinity Indexes (CrI) were determined by deconvolution of the ^{13}C NMR spectra from the areas of the crystalline (86–92 ppm) and amorphous (79–86 ppm) C_4 of glucose (Husson et al., 2011). The CrIs were given at ±5%. Cross-Polarization Magic Angle Spinning Nuclear Magnetic Resonance spectroscopy (CP-MAS ^{13}C NMR) was carried out on a Bruker DRX-500 spectrometer equipped with a 4 mm probe operating at 125.7452 MHz (^{13}C channel) and 500.0800 MHz (^{1}H channel). Samples were spun with a MAS speed of 5 kHz. Calibration of ^{13}C spectra was performed externally using ethylbenzene as a reference.

The morphology of untreated and treated cellulose samples was investigated by Scanning Electron Microscopy (SEM). An environmental high-resolution electron scanning microscope QUANTA 200 FEG (FEI Company, USA) was used in low-vacuum mode (under partial vacuum pressure of water) (Husson et al., 2011).

3. Results and discussion

The feasibility of both decreases the energy intake (temperature and time) for IL-pretreatment of cellulose and IL-recycling while maintaining performances of enzymatic production of fermentable sugar was investigated. Two distinct ILs were selected for their ability to completely dissolve cellulose: $[\text{Emim}]^+[\text{CH}_3\text{COO}]^-$ used as the reference (Zavrel et al., 2009) and the newly developed $[\text{Emim}]^+[\text{MeO(H)PO}_2]^-$ (Fukaya et al., 2008). The results are

Table 1
Glucose yield after 24 h of enzymatic hydrolysis of untreated/pretreated long fibers of cellulose for various times (20–300 min).[a]

Pretreatment	Duration of pretreatment (min)	Glucose yield at 24 h (%)
$[Emim]^+[MeO(H)PO_2]^-$	20	58.5 ± 2.2
	40	56.2 ± 3.8
	60	57.3 ± 4.1
	300	57.9 ± 4.6
$[Emim]^+[CH_3COO]^-$	20	45.4 ± 0.6
	40	43.7 ± 3.1
	60	37.3 ± 3.4
	300	34.0 ± 1.2
Deionized water (control)	300	25.8 ± 0.3
Untreated		14.2 ± 0.5

[a] Enzymatic saccharification by *T. reesei* cellulase (1 mg ml^{-1}) of long fibers of cellulose (2%, w/v) was carried out in 10 mL acetate buffer (50 mM, pH 4.8) at 40 °C. Glucose concentration was monitored by HPAEC and glucose yield was calculated as: $Y_{glucose}$ (%) = [Glucose$_{t=24h}$ (g) × 0.9]/[initial cellulose (g)] × 100 and expressed as mean ± SD of triplicate.

discussed highlighting the structural properties of the treated celluloses and their enzymatic sensitivity. Thus, the conversion rate of cellulose was examined in various $[Emim]^+[MeO(H)PO_2]^-$/buffer co-solvent mixtures.

3.1. Effect of pretreatment time

To study the influence of IL-pretreatment time, the usual temperature of 110 °C was chosen as a reference (Kim et al., 2011; Shill et al., 2011; Zhao et al., 2009). These studies showed a significant improvement in the enzymatic hydrolysis efficiency of (ligno-)cellulosic samples after pretreatment by ionic liquids at 110 °C. Various samples of long fiber celluloses were pretreated by each ionic liquid for different times: 20 min, 40 min, 60 min and 300 min. The influence of IL-pretreatment time was then investigated through the efficiency of hydrolysis of these pretreated celluloses by cellulases from *T. reesei*. Based on kinetics profiles of cellulase-catalyzed hydrolysis which showed a rapid accumulation of glucose and cellobiose from 0 to 24 h (Fig. 2), glucose yields at 24 h were significant and sufficient to compare these distinct conditions of ILs-pretreatment (Table 1). For every IL and time of pretreatment tested, hydrolysis was significantly increased compared to the untreated substrate. To test the effect of long exposure time at high temperature, a control experiment was done by incubating cellulose with deionized water for 300 min. In this case, glucose yields were 1.4 times greater at 24 h than for untreated cellulose, showing a weak effect on enzymatic saccharification of cellulose (Table 1). As shown in Table 1, pretreatment for 20 min with $[Emim]^+[MeO(H)PO_2]^-$ or $[Emim]^+[CH_3COO]^-$ enhanced the conversion rate by up to a factor of 4 or 3, respectively, at 24 h compared to untreated cellulose. Our data confirmed that the IL-pretreatment of cellulosic substrates leads to a significant improvement in the efficiency of enzymatic hydrolysis by cellulases. Moreover, glucose yields obtained for longer times with $[Emim]^+[MeO(H)PO_2]^-$ were very similar (close to 58%). $[Emim]^+[MeO(H)PO_2]^-$ pretreatment time was found to have no significant effect on the efficiency of enzymatic degradation. Similar results showed that efficiency of glucose recovery was not increased with longer IL-incubation times on lignocellulosic palm biomass (Tan, Lee, & Mohamed, 2011). In the case of pretreatments with $[Emim]^+[CH_3COO]^-$, the results seemed different. Indeed, an increase in pretreatment time tended to decrease the efficiency of IL-pretreatment slightly as suggested by a glucose yield of about 45% after a pretreatment of 20 min versus 34% for 300 min, *i.e.* 1.3 times lower. This could be because, after a longer pretreatment time, the regeneration step with $[Emim]^+[CH_3COO]^-$ was less efficient than with $[Emim]^+[MeO(H)PO_2]^-$ leading to more residual IL entrapped in the cellulose. Furthermore, a long incubation time of cellulose in $[Emim]^+[CH_3COO]^-$ could lead to its undesirable acetylation by the acetate ion that may inhibit cellulase activity and explain the slight decrease of glucose yield obtained (Karatzos, Edye, & Wellard, 2012). Another hypothesis is based on a better compatibility of $Emim]^+[MeO(H)PO_2]^-$ with cellulases.

3.2. Effect of pretreatment temperature

In order to compare with our previous work on the structure and degradation of cellulose (Husson et al., 2011), the duration of 40 min was chosen to test the effect of temperature variation. Thus, IL-pretreatments of cellulose were carried out for 40 min from room temperature up to 110 °C. The effectiveness of the different pretreatment temperatures was investigated through the efficiency of hydrolysis of these celluloses by cellulase from *T. reesei*. The kinetics of enzymatic hydrolysis of the pretreated celluloses was compared with untreated cellulose and with pretreated cellulose with water at 110 °C as shown in Fig. 2. The last experiment was used as a reference to check the effect on cellulose of its exposure to the highest temperature. In addition, glucose yields at 24 or 72 h and initial apparent rates of sugars production are presented in Table 2.

Regardless of temperature, pretreatment with the commonly used $[Emim]^+[CH_3COO]^-$ (Fig. 2a and b) clearly showed higher amounts of glucose and cellobiose than those observed for untreated cellulose or cellulose pretreated with water at 110 °C. In the latter case, the release of glucose was 1.4 times greater at 24 h (1.2 times at 72 h) than for untreated cellulose, showing a weak effect of incubation in water at 110 °C on cellulose digestibility by cellulase. Likewise, this weak effect was illustrated by the initial apparent rates of glucose and cellobiose production (Table 2). $[Emim]^+[CH_3COO]^-$ pretreatment of cellulose led to yields of glucose nearly 3 times greater than for untreated cellulose (Table 2) regardless of temperature, although the efficiency was slightly decreased at 25 °C. Thus, temperature does not seem to have a significant effect on the efficiency of IL-pretreatment of pure cellulose, in contrast to cellulosic samples containing lignin like sugarcane bagasse described in a recent study (Sant'Ana da Silva, Lee, Endo, & Bon, 2011). The same experiments performed with $[Emim]^+[MeO(H)PO_2]^-$ (Fig. 2c and d) gave similar results except for the pretreatment carried out at 25 °C for which glucose yields were only, respectively, 2 and 1.5 times greater at 24 and 72 h than for untreated cellulose. The lower initial apparent rates of glucose and cellobiose production can also be mentioned in comparison with those obtained after pretreatment with $[Emim]^+[CH_3COO]^-$ at 25 °C (Table 2). Otherwise, the $[Emim]^+[MeO(H)PO_2]^-$ pretreatment of cellulose at 45 °C, 65 °C and 110 °C led to glucose yields 3–3.7 times greater than for untreated cellulose (Table 1). It can also be noticed that during the first 10 h of enzymatic degradation, cellobiose accumulated in larger amounts at 65 °C and 110 °C than with the $[Emim]^+[CH_3COO]^-$ pretreatment for which the temperature had no effect (Fig. 2b and d). Moreover, the initial apparent rates of glucose and cellobiose production were significantly increased by IL-pretreatments (Table 2). However, in the case of celluloses pretreated by each IL, a drastic increase in temperature (110 °C) did not appear to improve significantly the initial apparent rates of sugar production. It seems that the optimal initial apparent rates were obtained after IL-pretreatment in mild conditions (45–65 °C) for $[Emim]^+[MeO(H)PO_2]^-$ and at 25 °C for $[Emim]^+[CH_3COO]^-$.

These results confirm that IL-pretreatment of cellulose leads to the highest glucose yields following enzymatic hydrolysis. $[Emim]^+[MeO(H)PO_2]^-$ exhibits several advantages in comparison

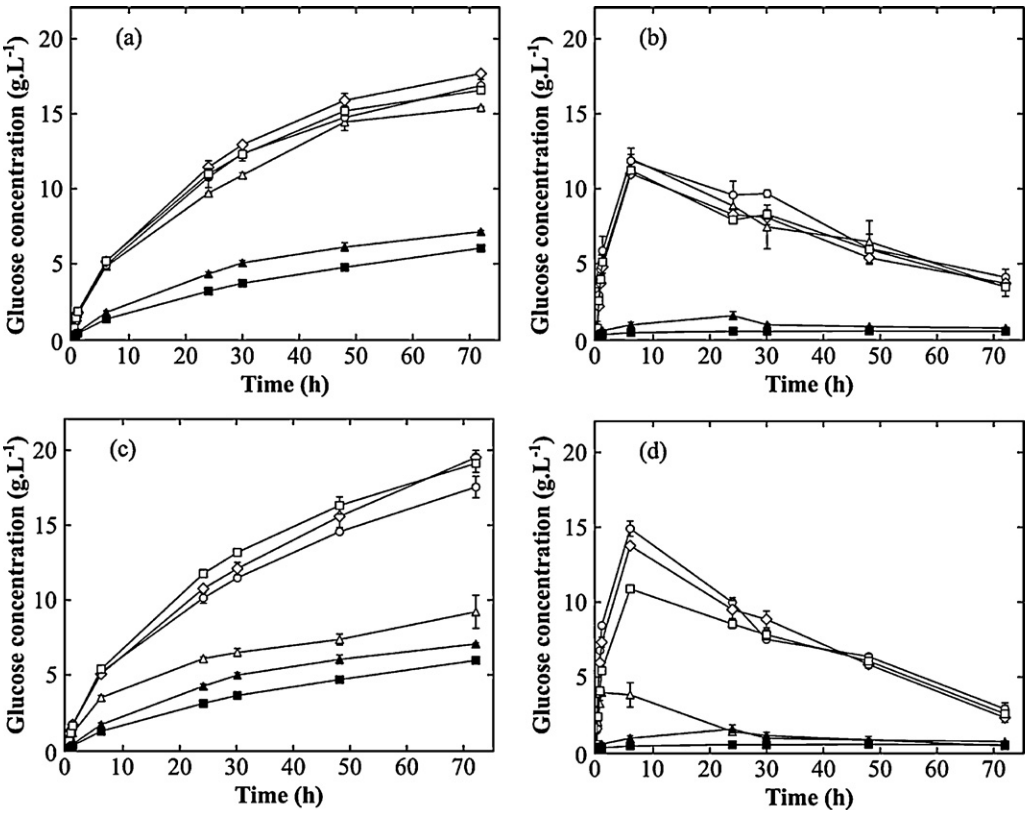

Fig. 2. Quantitative monitoring of glucose (left) and cellobiose (right) production during cellulase-catalyzed hydrolysis of long fibers of cellulose. (■) Untreated cellulose, (▲) cellulose pretreated with deionized water at 110 °C (control), cellulose pretreated with $[Emim]^+[CH_3COO]^-$ (a and b) or $[Emim]^+[MeO(H)PO_2]^-$ (c and d) at 25 °C (△), 45 °C (○), 65 °C (◇) or 110 °C (□). Error bars represent standard deviations of mean values.

with $[Emim]^+[CH_3COO]^-$ such as a lower viscosity and a lower cost due to a one-pot synthetic procedure (Fukaya et al., 2008). For these reasons, it could be a useful alternative to other commonly used ILs and could be preferable for pretreating biomass efficiently prior to enzymatic hydrolysis on a larger scale. However, the remarkable discovery is that these pretreatments on pure cellulose can be carried out at mild temperatures to achieve a significant glucose yield (around 70–80%). Compared to other reports on IL-pretreatment and to confidential data from our lab on industrial lignocellulosic samples of wheat straw and *Miscanthus*, it is clear that, in addition to the effect of IL on lignin extraction (Pu, Jiang, & Ragauskas, 2007), there is an effect on the cellulose supramolecular structure that occurs at low temperature and increases the enzymatic degradability.

3.3. Structural changes associated with cellulose pretreatment

The hypotheses to explain the improvement in enzymatic hydrolysis involve the structural changes occurring during pretreatments. The crystallinity indexes (CrI) of the untreated cellulose and IL-pretreated celluloses were investigated by solid-state ^{13}C NMR and presented in Table 2. For the untreated

Table 2
Yields and initial rates of enzymatic hydrolysis of untreated/IL-pretreated celluloses at different temperatures for 40 min and their respective crystallinity indexes (CrI).[a]

Pretreatment	Temperature of pretreatment (°C)	Glucose yield (%)		Initial apparent rate (mg L^{-1} min^{-1}) of sugars production		Crystallinity index (%) ±5%
		At 24 h	At 72 h	Glucose	Cellobiose	
$[Emim]^+[MeO(H)PO_2]^-$	25	27.7 ± 0.4	41.8 ± 4.9	29.0 ± 0.6	112.8 ± 7.4	54.5
	45	45.9 ± 1.1	79.3 ± 3.2	47.3 ± 0.5	238.0 ± 5.9	59.0
	65	48.8 ± 0.5	88.2 ± 2.2	46.3 ± 2.8	231.1 ± 16.0	nd
	110	53.3 ± 0.3	86.4 ± 2.7	34.2 ± 4.2	128.8 ± 8.1	59.3
$[Emim]^+[CH_3COO]^-$	25	43.7 ± 0.1	69.4 ± 0.3	37.4 ± 3.8	163.1 ± 26.7	76.3
	45	48.4 ± 2.1	75.9 ± 1.9	38.7 ± 1.2	163.4 ± 3.0	66.4
	65	51.4 ± 1.4	79.6 ± 0.2	30.8 ± 0.1	119.1 ± 7.3	nd
	110	49.4 ± 1.2	74.6 ± 0.2	40.3 ± 0.3	139.2 ± 10.4	73.1
Deionized water (control)	110	19.4 ± 0.5	32.1 ± 0.4	9.1 ± 1.4	21.9 ± 3.2	nd
Untreated		14.3 ± 0.3	27.2 ± 0.3	6.6 ± 1.0	15.1 ± 3.5	71.7

[a] Enzymatic saccharification by *T. reesei* cellulase (1 mg ml^{-1}) of untreated/pretreated long fibers of cellulose (2%, w/v) was carried out in 10 mL acetate buffer (50 mM, pH 4.8) at 40 °C. Glucose concentration was monitored by HPAEC and glucose yield was calculated as: $Y_{glucose}$ (%) = [Glucose (g) × 0.9]/[initial cellulose (g)] × 100 and expressed as mean ± SD of triplicate. CrIs were determined by deconvolution of CP-MAS ^{13}C NMR. nd: not determined.

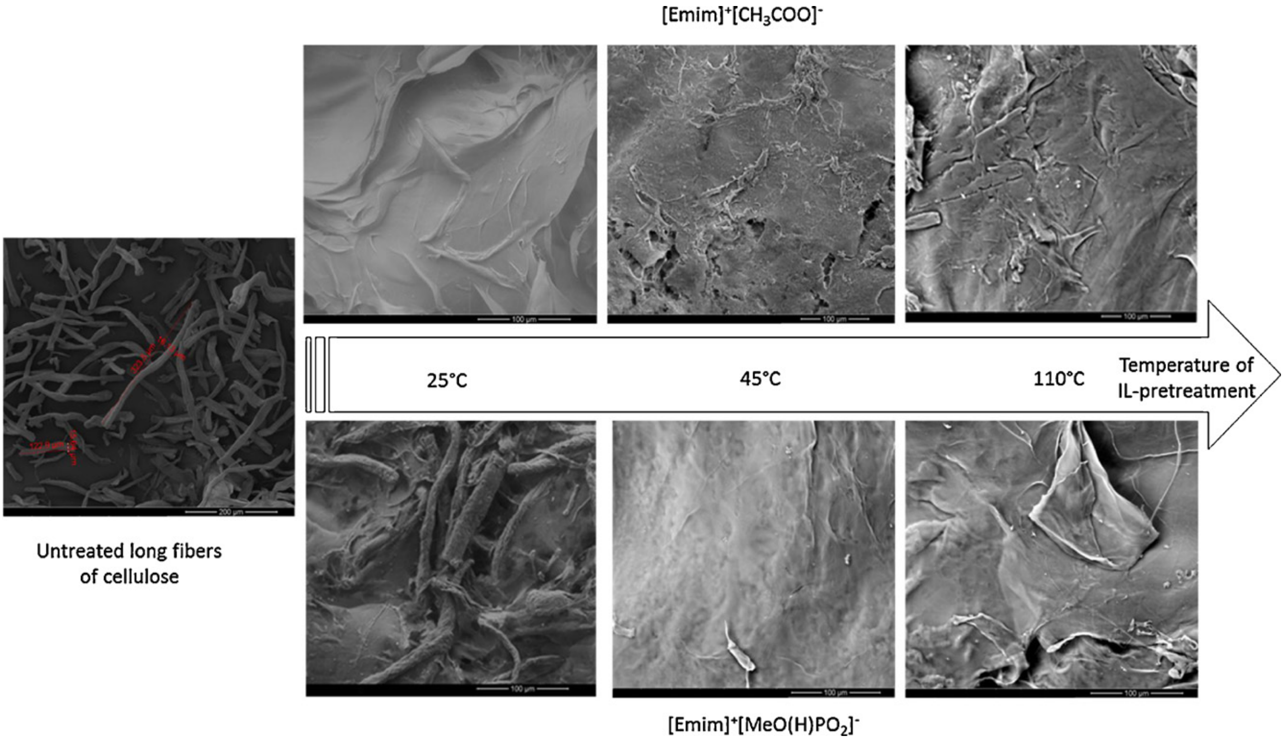

Fig. 3. SEM micrographs of untreated/pretreated long fibers of cellulose with the two distinct IL at 25 °C, 45 °C and 110 °C for 40 min.

cellulose, the CrI was 71.7%. After cellulose pretreatment with $[Emim]^+[MeO(H)PO_2]^-$, the CrIs were 54.5%, 59.0% and 59.3% at 25 °C; 45 °C and 110 °C, respectively. The $[Emim]^+[MeO(H)PO_2]^-$ pretreatments seemed to lead to a slight decrease in the CrI of the regenerated celluloses at all temperatures tested although their digestibility was drastically improved. However, it should be pointed out that the lowest glucose yield was obtained after IL-pretreatment at room temperature despite the similar CrI. This demonstrates that CrI is not the best parameter to use to indicate the enzymatic degradability of cellulose, as already suggested in a previous investigation (Husson et al., 2011). After cellulose pretreatment with $[Emim]^+[CH_3COO]^-$, the CrIs were 76.3%, 66.4% and 73.1% at 25 °C; 45 °C and 110 °C, respectively. $[Emim]^+[CH_3COO]^-$ pretreatments seemed to have no effect on the CrI of regenerated celluloses at all temperatures used. This could be due to recrystallization during the regeneration step as suggested by Samayam et al. (2011). Despite this restored crystallinity, the pretreated cellulose exhibited a higher enzymatic degradability than untreated cellulose at both room and high temperature. These results again suggest that the CrI is not the most reliable parameter to explain the enzymatic hydrolysis efficiency.

SEM images of untreated and pretreated celluloses by each distinct IL at 25 °C, 45 °C and 110 °C are reported in Fig. 3. Untreated cellulose exhibits a highly ordered organization of well-separated fibers which could be related with weak glucose yields obtained, respectively, 14.3 and 27.2% at 24 and 72 h.

At temperatures above 25 °C, the two distinct IL-pretreatments led to a total disruption of fibers organization and to smooth surfaces. However, at room temperature, fibers are still largely present after $[Emim]^+[MeO(H)PO_2]^-$ pretreatment while only few residual fibers are detected after $[Emim]^+[CH_3COO]^-$ pretreatment. These observations correlate well with the yields of glucose obtained for each IL-pretreatment temperature (Table 2). In fact, the presence of lasting fibers pretreated cellulose at 25 °C may limit its enzymatic degradability (glucose yield at 24 h, respectively, around 28 and 44% after $[Emim]^+[MeO(H)PO_2]^-$ and $[Emim]^+[CH_3COO]^-$ pretreatment). At the opposite, a smooth cellulose surface allows to an efficient cellulase accessibility illustrated by significant improved glucose yields. An *in situ* SEM observation of cellulose in contact with $[Emim]^+[MeO(H)PO_2]^-$ at 40 °C revealed first a swelling of fiber leading to its collapse (see video in supplementary data). Thus, we have confirmed that one essential condition to improve the enzymatic degradability of cellulose is the disruption of its fiber organization.

3.4. Effect of IL-recycling

The effect of IL-recycling on the efficiency to pretreat the cellulose prior to enzymatic hydrolysis was investigated. The mildest conditions of pretreatment (40 min, 45 °C) were chosen based on the results described before. Five recycling and reuses have been done for each distinct IL. Table 3 presents glucose yields at 24/72 h of enzymatic saccharification and initial apparent rates of glucose production obtained with recycled IL-pretreatments and control experiment. Knowing that water in IL can prevent cellulose solubilization (Swatloski et al., 2002), residual water content of each recycled IL was also presented in this table.

Whatever the number of $[Emim]^+[CH_3COO]^-$ recycling, pretreatments led to similar improved yields of glucose, respectively, around 43 and 66% at 24 and 72 h in comparison with that obtained for the untreated cellulose substrate (12.7 ± 0.1% at 24 h and 25.1 ± 0.3% at 72 h). Table 3 showed also that this tendency was even more marked by the initial apparent rates of glucose production (increase by a factor 7 after IL-pretreatment). Whatever the number of $[Emim]^+[CH_3COO]^-$ reuses, all pretreated celluloses exhibited a smooth surface without any residual fibers as illustrated by SEM micrographs (Fig. 4). This is well correlated with their better enzymatic digestibility as already described before. Furthermore, residual water content of this IL remained constant independently of the recycling (around 5%, w/w) and would not seem to affect significantly $[Emim]^+[CH_3COO]^-$ pretreatment efficiency (Table 3).

In the same way, regardless the number of reuses, $[Emim]^+[MeO(H)PO_2]^-$ pretreatment allowed a significant improvement of enzymatic saccharification efficiency in

Table 3
Effect of recycling IL on enzymatic hydrolysis performances of untreated/IL-pretreated celluloses and on IL-residual water content.

Pretreatment	Number of reuses[a]	IL-residual water content (%, w/w)	Glucose yield (%)		Initial apparent rate ($mg\,L^{-1}\,min^{-1}$) of glucose production
			At 24 h	At 72 h	
$[Emim]^+[MeO(H)PO_2]^-$	Not recycled	0.325 ± 0.005	42.7 ± 0.7	60.1 ± 0.6	41.8 ± 1.2
	1	2.405 ± 0.025	33.6 ± 1.1	49.9 ± 1.6	37.6 ± 2.3
	2	3.075 ± 0.015	34.3 ± 4.0	49.3 ± 5.1	41.6 ± 3.8
	3	2.905 ± 0.033	35.6 ± 2.0	50.0 ± 3.9	45.7 ± 3.6
	4	2.690 ± 0.090	36.5 ± 2.9	52.9 ± 4.4	51.8 ± 3.8
	5	2.710 ± 0.123	37.5 ± 1.1	56.2 ± 1.4	49.5 ± 1.6
$[Emim]^+[CH_3COO]^-$	Not recycled	4.940 ± 0.008	41.3 ± 3.1	63.5 ± 4.3	39.1 ± 3.6
	1	4.650 ± 0.003	41.2 ± 1.7	65.0 ± 2.4	42.8 ± 1.3
	2	5.250 ± 0.003	41.2 ± 3.9	64.2 ± 5.0	42.9 ± 4.5
	3	4.775 ± 0.013	42.5 ± 2.4	65.9 ± 3.7	42.1 ± 1.5
	4	4.940 ± 0.030	44.4 ± 2.8	69.0 ± 3.1	43.9 ± 2.9
	5	4.705 ± 0.015	46.2 ± 3.8	68.9 ± 4.8	39.2 ± 2.6
Untreated	–		12.7 ± 0.1	25.1 ± 0.3	5.9 ± 0.1

[a] IL-recycling by distillation under vacuum.

comparison with untreated cellulose (Table 3). However, efficiency of $[Emim]^+[MeO(H)PO_2]^-$ decreased slightly after the first recycling. Indeed, glucose yields were 33.6 ± 1.1% versus 42.7 ± 0.7% at 24 h without recycling. These weaker saccharification performances remained constant for the four next recycling experiments (around 36% at 24 h). These results could be explained by the presence of persistent cellulose fibers after IL-pretreatment from the first recycling (Fig. 4). This diminution of recycled $[Emim]^+[MeO(H)PO_2]^-$ pretreatment efficiency was estimated close to 15%. But NMR analyses demonstrated that no alteration of $[Emim]^+[MeO(H)PO_2]^-$ molecular structure occurred after each recycling. Nevertheless, its residual water content is increased after the first recycling then remained equivalent until the last recycling (around 3%, w/w, after IL-recycling versus 0.325 ± 0.005%, w/w, without recycling). This increase of IL-residual water content could affect cellulose solubility during IL-pretreatment step and would explain therefore the slight decrease of glucose yield obtained after enzymatic hydrolysis.

3.5. Enzymatic hydrolysis of cellulose in diluted IL-aqueous systems

IL-pretreatments of cellulose at 45 °C sufficiently altered its supramolecular structure to improve its enzymatic saccharification. This mild temperature is very close to the optimal temperature of cellulase activity. Based on these results, it appeared rational

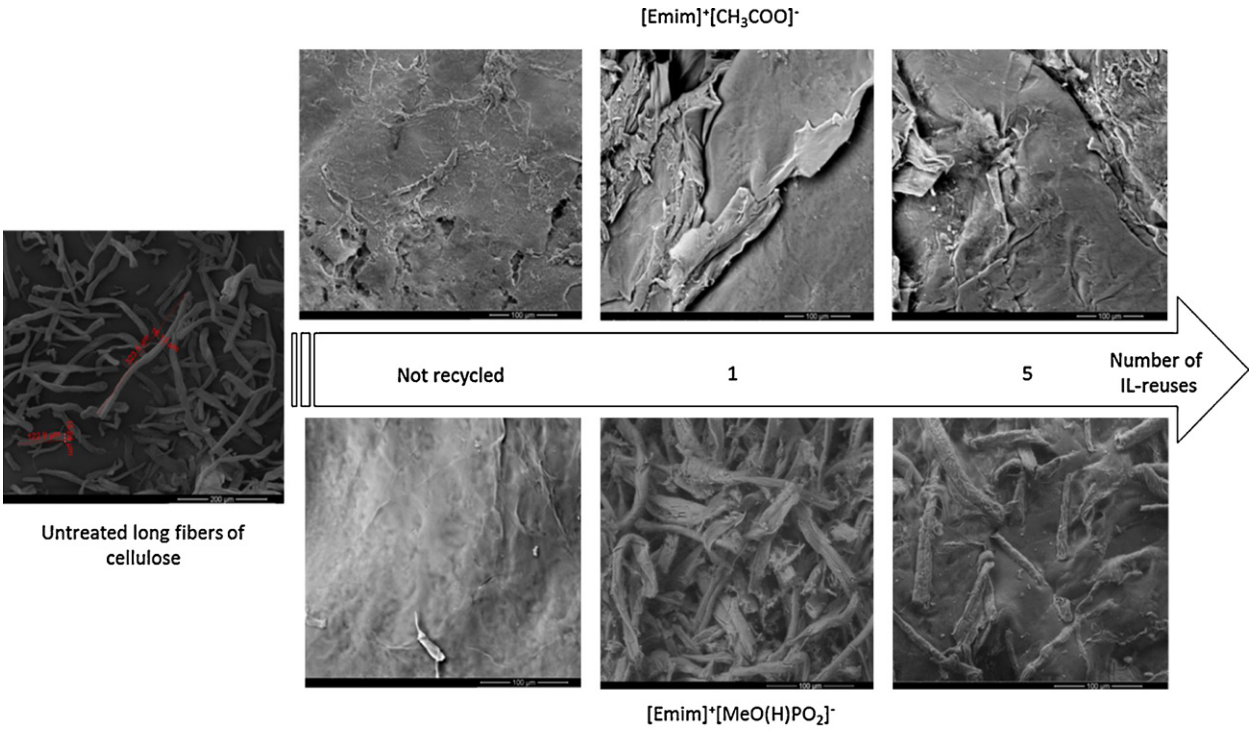

Fig. 4. SEM micrographs of untreated/pretreated long fibers of cellulose at 45 °C for 40 min with the two distinct IL before and after recycling.

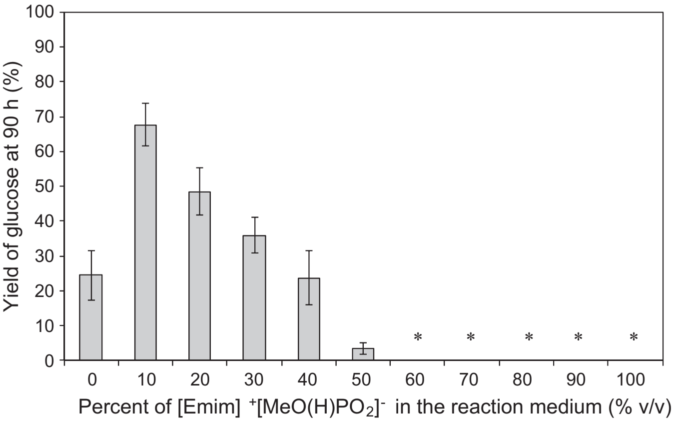

Fig. 5. Yields of glucose obtained at 90 h (thermodynamic equilibrium) by cellulose-catalyzed hydrolysis of cellulose long fibers in various diluted [Emim]$^+$[MeO(H)PO$_2$]$^-$-aqueous systems. (*) The glucose concentration was not detectable.

to consider carrying out the IL-pretreatment step and enzymatic hydrolysis in a one-batch process. In this context, the compatibility between the cellulase of *T. reesei* and [Emim]$^+$[MeO(H)PO$_2$]$^-$ was investigated for the first time. Enzymatic hydrolysis of cellulose was directly carried out in buffer containing various amounts of this IL at 40 °C. No glucose production was detected in the absence of enzyme whatever the amount of IL in the reaction medium. Fig. 5 presents the glucose yield obtained by cellulase-catalyzed hydrolysis at 90 h in the different IL-aqueous systems. An IL concentration of 10% (v/v) in the reaction medium led to a maximal increase in the glucose yield (68 ± 6% versus 25 ± 7% obtained in classical aqueous medium without pretreatment). At IL concentrations higher than 10% (v/v), a progressive decrease occurred until a yield of 24 ± 8% was obtained at 40% (v/v) IL concentration. Above IL concentrations of 40% (v/v), the hydrolytic activity of *T. reesei* cellulase was drastically affected (glucose yield less than 5%). At these high IL concentrations, the cellulases may establish hydrogen bonds with the constitutive phosphonate anions of the IL and would be totally inactivated as suggested in previous studies concerning similar enzymes in the presence of [Emim]$^+$[CH$_3$COO]$^-$ (Zhao et al., 2009; Wang et al., 2011). However, for moderate IL concentrations (around 10%, v/v, in our study), the improved glucose yields could be due to a compromise between a better accessibility of cellulose due to its partial solubilization and a minimized inactivation of cellulases. These results agree with previous studies concerning cellulase activity in diluted alkylphosphate anion-based IL-aqueous systems (Chen, Lee, Wei, Hsieh, & Yu, 2009; Jones & Vasudevan, 2010; Kamiya et al., 2008). Although the yield was lower than that obtained by a separate IL-pretreatment, this is a promising way to carry out efficient enzymatic degradation of cellulose in diluted IL-aqueous systems and to overcome the delicate step of cellulose regeneration from the IL.

4. Conclusions

The feasibility of significantly minimizing the energy intake of [Emim]$^+$[MeO(H)PO$_2$]$^-$ and [Emim]$^+$[CH$_3$COO]$^-$-pretreatments by decreasing both the time and temperature was demonstrated. The higher enzymatic digestibility of pretreated celluloses was due to the loss of their fiber organization rather than to a decrease in their crystallinity index. Thus, short IL-pretreatments of cellulose can be carried out at mild temperature to achieve better high yields of glucose. In addition, the possibility of performing efficient celluloses pretreatments with recycled ILs has also been proved for these two imidazolium-based ILs. For these reasons, [Emim]$^+$[CH$_3$COO]$^-$ and [Emim]$^+$[MeO(H)PO$_2$]$^-$ could be successful candidates to pretreat efficiently lignocellulosic biomass in mild conditions. Finally, the compatibility of *T. reesei* cellulase with [Emim]$^+$[MeO(H)PO$_2$]$^-$ at a concentration of up to 40% in the reaction medium constitutes a promising result to consider the development of a one-batch process avoiding the water regeneration step.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2012.05.101.

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