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PAPER

A cyclic process for full enzymatic saccharification of pretreated cellulose with full recovery and reuse of the ionic liquid 1-butyl-3-methylimidazolium chloride†

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A sustainable cyclic process for the enzymatic saccharification of ionic liquid (IL)-pretreated cellulose, in which the IL is recovered and recycled, has been developed. Homogeneous cellulose solutions in the IL 1-butyl-3-methylimidazolium chloride ([Bmim][Cl]) were used to prepare amorphous cellulose by antisolvent precipitation with water, ethanol or equimolar water–ethanol mixtures as green molecular solvents. Several operation parameters (*e.g.*, solvent, temperature, ultrasounds, *etc.*) for both cellulose precipitation and the washing steps were tested to achieve full desorption of the IL from the cellulose backbone. In the best conditions, up to 99.7% IL was recovered, which was then successfully reused in further cellulose dissolution/precipitation cyclic processes. Furthermore, the cellulose regenerated in each cycle was an excellent substrate for enzymatic hydrolysis, permitting full hydrolysis (*i.e.*, up to 97.7% hydrolysis after 4 h at 50 °C) by the combined action of both cellulase and cellobiase enzymes, that provides a clear glucose solution. The excellent suitability of this glucose solution for growing aerobic *Saccharomyces cerevisiae* was demonstrated.

1 Introduction

The production of second generation bioethanol from non-edible biomass (*e.g.*, lignocellulosic biomass) using clean and sustainable approaches is one of the greatest challenges on the research and industrial agenda.[1] Bioethanol production from cellulosic sources consists of three consecutive steps, such as, the pretreatment of cellulose to disrupt its highly ordered and rigid structure, hydrolysis of the cellulose to fermentable sugars, and finally, the ethanol fermentation by microorganisms. Full enzymatic hydrolysis of cellulose to its glucose units can be carried out by the synergistic action of different glycohydrolases, such as cellulases (endoglucanases EC 3.2.1.4, exoglucanases EC 3.2.1.91) and cellobiase (EC 3.2.1.21). The high specificity of enzymes is the characteristic that identifies them as the greenest approach for the saccharification of cellulose.[2] However, the crystalline structure of cellulose, which is supported by multivalent inter- and intramolecular hydrogen bonds, involves a recalcitrance to its degradation by biocatalysts. Several chemical and physical methods have been employed to improve the accessibility of biomass polysaccharides to enzymatic hydrolysis (*e.g.*, ball and compression milling, dilute acids and hydrothermal treatment, bases, *etc.*), however, none are able to increase the surface area and decrystallize cellulose sufficiently to permit full enzymatic hydrolysis, short residence times and low enzyme concentrations.[3]

The pioneering work of Rogers' group, showing that some ILs, such as 1-butyl-3-methylimidazolium chloride ([Bmim][Cl]), 1-hexyl-3-methylimidazoium chloride ([Hmim][Cl]), *etc.*, are able to dissolve cellulose,[4] has opened up new opportunities for the valorisation of large amounts of waste cellulose-containing materials (*e.g.*, forest biomass). Among those the biocatalytic depolymerisation of cellulose to its glucose units, and their subsequent transformation into bioethanol by fermentation, is the most widely attempted.[5]

Although most ILs have been shown to act as excellent reaction media for enzyme-catalyzed reactions,[6] it has been widely reported that ILs that are excellent for dissolving cellulose (*e.g.*, [Bmim][Cl], *etc.*), produce fast enzyme deactivation by protein unfolding.[7] In this context, alternative approaches to overcome the negative effect of ILs-dissolving cellulose on cellulase activity and stability have been assayed for cellulose saccharification, *e.g.*, by using buffered media containing "benign" dissolved ILs at low concentrations, such as dialkylphosphate or acetate ILs,[8] or by coating immobilized cellulase with a protective shell of hydrophobic ILs, like 1-butyl-3-methylimidazolium bistriflimide,[9] the identification and application of cellulases from microbial sources others than fungus (*e.g.*, the halophilic

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archaeon *Halorhabdus utahensis*) with improved IL tolerance,[10] or the use of concentrated sea water as a free-IL alternative reaction medium.[11] However, the most popular approach involves the re-precipitation of cellulose from IL solutions into polar molecular solvents (*e.g.*, water, ethanol, *etc.*) in order to disrupt the crystalline structure of this polysaccharide, thus improving its subsequent enzymatic hydrolysis in buffered media.[12] The digestibility of pretreated biomass by cellulase is limited by cellulose accessibility. However, the full degradation of cellulose to its glucose monomer units has not been reported. Some authors have applied ultrasonic intensification during the pretreatment of cellulose with ILs to improve the disruption of the cellulose structure, which enhances enzymatic *in situ* saccharification of cellulose in aqueous/IL media, providing up to 95% yield after 24 h reaction.[13] Other authors carried out the enzymatic hydrolysis step of [Bmim][Cl]-pretreated cellulose (5 mg mL^{-1}) in a pH 4.8 citrate buffer system containing up to 20% of another IL, such as the tris-(2-hydroxyethyl)methylammonium methylsulfate (HEMA), which yields up to 4 mg mL^{-1} glucose after 15 h at 60 °C.[14]

To the different advantages that IL technology may provide, as regards cellulose processing in the bioethanol industry, should be added the economical and environmental sustainability of the process based on the full recovery and recycling of ILs.[1b,6,7b] Some ILs have been described as being not fully green solvents because of their low biodegradability and high (eco)toxicological properties.[15]

Several approaches to recover ILs from biomass–IL solutions have recently been proposed, *e.g.*, the use of aqueous kosmotropic salt solutions after a cellulose re-precipitation step with polar solvent to form a three-phase system forming an IL-rich phase, [16a] or the chromatographic separation of the IL and glucose from the enzymatic reaction mixture using alumina as the stationary phase, which provided up to 93% IL recovery.[16b] However, since the final destination of the hydrolyzed cellulose solutions is fermentation by yeast to produce bioethanol, the effect of ILs on the viability of yeast cells should also be taken into account. Recently, it has been reported how the residual 1-ethyl-3-methylimidazolium acetate ([Emim][OAc]) content of cellulose hydrolysates act as a primary source of inhibition on *Saccharomyces cerevisiae* growth and ethanol production.[17a] In the same way, it was observed how an engineered *Saccharomyces cerevisiae* strain, with cellulases on its cell surface, was resistant to some cellulose-dissolving ILs (*i.e.*, [Emim][OAc], [Emim][Cl], *etc.*) up to 200 mM.[17b]

In this context, the present work describes for the first time a cyclic experimental approach (see Fig. 1) based on the enzymatic hydrolysis of regenerated cellulose from [Bmim][Cl] solutions, and the recovery and reuse of this IL in successive cellulose dissolution–regeneration cycles. The experimental protocol to produce regenerated cellulose (RC) was selected as a function of both the amount of IL recovered, and the suitability of this RC for enzymatic hydrolysis. Thus, water, ethanol, and a 50% (mol/mol) water–ethanol solution were tested as green antisolvents for the precipitation of cellulose from a cellulose–[Bmim][Cl] solution, and several operation parameters, such as temperature, stirring, ultrasound, *etc.*, were assayed in an attempt to fully recover this IL for reuse in consecutive dissolution–precipitation cycles.

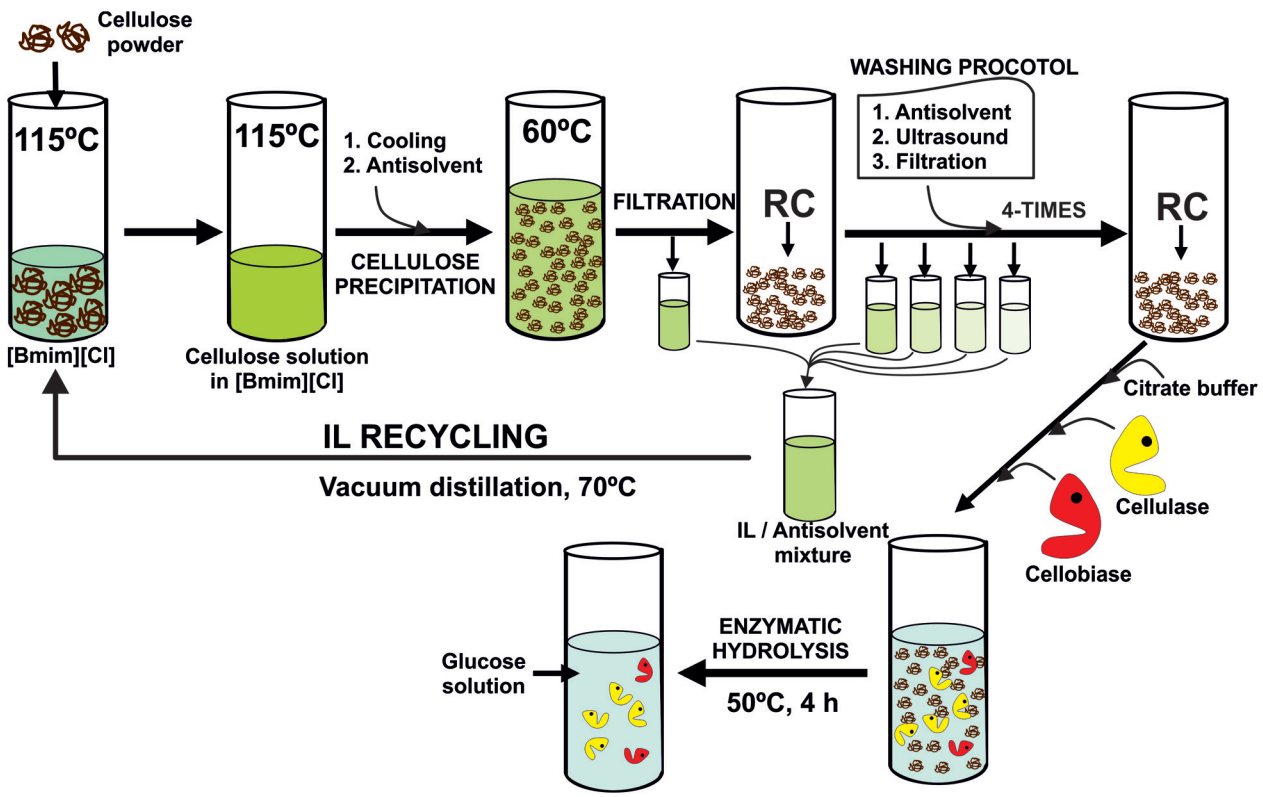

Fig. 1 Scheme of the cyclic protocol for the enzymatic saccharification of IL-pretreated cellulose and the recycling of the ionic liquid 1-butyl-3-methylimidazolium ([Bmim][Cl]). RC: regenerated cellulose. For details see the Experimental section.

2 Experimental

Cellulase from *T. reesei* (Celluclast 1.5 L, EC 3.2.1.4), and cellobiase from *Aspergillius niger* (Novozyme 188, beta-1,4-glucosidase, EC 3.2.1.21) were a gift from Novozymes S.A (Spain). Microcrystalline cellulose (20 μm powder) and other chemicals were purchased from Sigma-Aldrich-Fluka (Madrid, Spain). The IL 1-butyl-3-methylimidazolium chloride, [Bmim][Cl], (99% purity) was purchased from IoLiTec GmbH (Germany).

Prior to use, enzyme preparations were ultrafiltered to eliminate all the low molecular weight additives, as follows: 25 mL of Celluclast or Novozym 188 were diluted in 225 mL of 50 mM citrate buffer pH 4.8, and the resulting solutions were concentrated 10-fold by ultrafiltration at 8 °C using a Vivaflow 50 (Sartorious) system equipped with polysulphone membranes (10 kDa, cut-off). For each enzyme, the process was repeated three-times, leading to cellulase (0.115 U mg^{-1} protein, 147.5 mg protein per mL) or cellobiase (0.814 U mg^{-1} protein, 93.6 mg protein per mL) solutions, respectively.

Preparation of regenerated cellulose (RC)

Firstly, the [Bmim][Cl] (10 g) was introduced into a 100 mL Erlenmeyer flask, and incubated at 115 °C in a thermoblock for 15 min, until the IL was fully melted. Then, microcrystalline cellulose (1 g) was added, and the mixture incubated with mechanical stirring for 1 h at 115 °C, which gave a clear, colourless and viscous cellulose solution. This solution was then cooled to 60 °C in a glycerol thermostatic bath. The cellulose was regenerated by adding 50 mL (approx. 5-fold IL-cellulose volume) of water, ethanol or a equimolar (23.5 : 76.5, v/v) water–ethanol solution, pre-heated to 60, 70 or 80 °C, and the resulting cellulose suspension was vigorously stirred for 15 min. The RC gel was recovered by filtration through a nylon membrane (0.1 mm mesh), while the liquid fraction was collected and stored for further analysis by HPLC and IL recovery. Then, the RC gel was washed five times with 50 mL of different antisolvent solutions at room temperature so that the IL was fully desorbed from the cellulose gel. This involved two-washing steps, using the same antisolvent as for cellulose regeneration, applying 150 W ultrasounds (Ultrasons, Selecta, Spain) for 15 min; two-washing steps with ultrapure water (MilliQ–Millipore System) and mechanical stirring for 15 min; and a final washing step with 50 mM citrate buffer pH 4.8 and mechanical stirring. The five resulting washing fractions were carefully collected and stored for further analysis by HPLC and IL recovery. The moisture content of the resulting regenerated cellulose was 84% (w/w), as measured by weight loss of RC after drying in an oven for 14 h at 105 °C.

Recovery of 1-butyl-3-methylimidazolium chloride

The [Bmim][Cl] content of each washing liquid sample was separated by vacuum distillation at 70 °C and 74 hPa for 4 h. Then, all the fractions containing the recovered IL were jointly introduced into a 100 mL Erlenmeyer flask and incubated for 16 h in an oven at 80 °C to dryness. The dry [Bmim][Cl] recovered was repeatedly used to dissolve cellulose in further experiments, as described above.

HPLC analysis of [Bmim][Cl]

The IL concentration of all the washing fractions was determined in a Shimadzu HPLC equipped with a multi-channel (LC-20AD) pump and DAD (SPD-M20A) detector, using a Synergi Polar-RP 150 × 4.6 mm column (Phenomenex) packed with polar end-capped particles (4 μm, pore size 80 Å) as the stationary phase. The analyses were performed under isocratic conditions (0.75 mL min^{-1} flow rate) using a 70 : 30 (v/v) 5 mM phosphate buffer (KH_2PO_4–H_3PO_4) pH 3.0/acetonitrile as the mobile phase.[18] The elution profiles were monitored at 218 nm, and identification and quantification of the [Bmim][Cl] peak (2.8 min retention time) was made by the corresponding calibration straight line using acetophenone (11 min retention time) as the internal standard.

Enzymatic hydrolysis of regenerated cellulose (RC)

The 2% (w/v) RC suspension used as the substrate was prepared by introducing 500 mg of wet RC (80 mg dry RC) into a screw-capped vial with a Teflon-lined septum (5 mL total capacity), containing 4 mL of 50 mM citrate buffer pH 4.8. The mixture was maintained under magnetic stirring in a glycerol bath at 50 °C until a homogeneous suspension was observed. The reaction was then started by adding both cellulase (120 μL, 147.5 mg prot. mL^{-1}) and cellobiase (60 μL, 93.6 mg prot. mL^{-1}) glycohydrolases and was magnetically stirred for 4 h. At regular time intervals, 65 μL-aliquots were taken and suspended in 0.1 M bicarbonate buffer, pH 9.8 (1.035 mL) to stop the reaction, the samples were then centrifuged at 13 000 rpm for 5 min. The resulting clear phase was used to quantify glucose and cellobiose by HPLC, and the total reducing sugars by the dinitrosalicylic acid (DNS) method. One unit of cellulase activity was defined as the amount of enzyme that produces 1 μmol of reducing sugars per minute. One unit of cellobiase activity was defined as the amount of enzyme that hydrolyzes 1 μmol of cellobiose per minute. All experiments were carried out in duplicate.

Microbial growth test

Growth assays were performed using a lyophilised commercial *Saccharomyces cerevisiae* strain (Maurivin-PDM, AB Mauri, Australia) in YPD media [2% (w/v) glucose, 2% (w/v) peptone and 1% (w/v) yeast extract].[17,19] The pH of the media were adjusted to 6.5 before sterilising. A starter culture was first prepared by adding 20 mg commercial *S. cerevisiae* lyophilised powder to 40 mL standard YPD medium, which was incubated under shaking (300 rpm) for 16 h at 30 °C in aerobic conditions. Then, a new YPD medium (40 mL) was prepared by dissolving peptone and yeast extract in a 2% (w/v) glucose solution in 5 mM citrate buffer pH 4.8, previously obtained by enzymatic hydrolysis of RC, as described above. Prior to use, this glucose solution was ultrafiltered using a Vivaflow 50 (Sartorious) system equipped with polysulphone membranes (10 kDa, cut-off) to eliminate soluble enzymes. As a growing control, another standard YPD medium was prepared by dissolving commercial glucose, peptone and yeast extract in 5 mM citrate buffer pH 4.8. Then, the pH of both growing media was adjusted to 6.5 before

Table 1 Effect of cellulose regeneration protocol on [Bmim][Cl] recovery as a function of antisolvent and washing step conditions[a]

		Washing steps					
Entry	Cellulose precipitation with antisolvent (AS)	1. AS + US	2. AS + US	3. H_2O	4. H_2O	5. CB	Total IL recovery (%)
1	78.0 (H_2O, 60 °C)	10.7	6.4	nd	nd	nd	95.1
2	80.0 (H_2O, 70 °C)	8.4	6.8	nd	nd	nd	95.2
3	82.0 (H_2O, 80 °C)	6.2	7.2	nd	nd	nd	95.4
4	67.1 (EtOH, 60 °C)	21.6	8.9	nd	nd	nd	97.6
5	93.7 (H_2O–EtOH, 60 °C)	4.4	1.4	0.2	<0.1	nd	99.7
6	91.8 (H_2O–EtOH, 70 °C)	5.3	1.4	0.3	0.1	nd	98.9
7	89.9 (H_2O–EtOH, 80 °C)	5.0	1.2	0.4	0.2	nd	96.7

[a] AS + US, antisolvent plus ultrasound for 15 min at room temperature; CB, 50 mM citrate buffer pH 4.8; nd, not detected; see Experimental section for details of samples.

sterilising. Growth assays were run by adding 0.1 mL of the above *S. cerevisiae* starter and shaking (300 rpm) at 30 °C for 30 h in aerobic conditions. At regular intervals, the optical densities (O.D.) of both cultures were determined at 600 nm (OD_{600}) by appropriate dilution in a spectrophotometer (Novaspec II, LKB-Pharmacia).

HPLC analysis of sugars

The glucose and cellobiose concentrations of the enzymatic reaction samples were determined in a Shimadzu HPLC equipped with a multi-channel (LC-20AD) pump, oven and light scattering (ELSD-LT II) detector. A Rezex RCM-monossacharide-Ca^{+2} column (300 × 7.8 mm, Phenomenex) was used as the stationary phase at 60 °C. Analyses were performed in isocratic conditions (0.6 mL min^{-1} flow rate) using water as a mobile phase. The glucose (11 min retention time) and cellobiase (9 min retention time) peaks were identified and quantified from the corresponding calibration straight lines, using xylitol (23 min retention time) as the internal standard.

3 Results and discussion

The regeneration of cellulose from IL solutions is a key step in weakening its crystallinity and/or disrupting its fibre organization, resulting in a more accessible substrate for enzymatic hydrolysis into fermentable sugars, and subsequent transformation into bioethanol.[12] However, the recovery and reuse of ILs in cellulose processing for the biofuel industry could be considered as an essential task for ensuring the sustainability of any proposed approach in this field. Taking into account that [Bmim][Cl] has been described as one of the best ILs for dissolving lignocellulosic materials,[4,5] a 10% cellulose solution in this IL was chosen as starting material to prepare RC, while water, ethanol or an equimolar water–ethanol mixture, were used as antisolvents to precipitate cellulose. In preliminary experiments, it was observed how the RC texture was hard and lumpy when the precipitation step was carried out at room temperature, while a fine and soft RC powder was obtained at higher temperatures, *i.e.*, 60, 70 or 80 °C. Seven different experimental protocols were followed to produce RC, and each RC sample was washed five times in order to attain full IL recovery (see Fig. 1). For all RC cases, the first two washing steps were carried out using the respective antisolvent applied during the precipitation step, as well as the intensification effect of ultrasounds. Then, the resulting RCs were washed by water (twice), and then by 50 mM citrate buffer pH 4.8. Table 1 shows the [Bmim][Cl] recovery yield for each step in the seven approaches tested to produce RC. As can be seen, the use of water as the antisolvent agent to precipitate cellulose (entry 1) permitted a direct 78% IL recovery at 60 °C, which slightly increased to 82% when the temperature was raised to 80 °C (entries 2 and 3). In these cases, the application of two consecutive washing steps, intensified by the action of ultrasound, allowed improvement of the total IL recovery to 95.1–95.4%. The application of additional washing steps with water and citrate buffer did not provide further improvements in IL recovery. The use of ethanol as the antisolvent (entry 4) provided the worst results during direct cellulose precipitation at 60 °C (67.1% IL yield), which rose to 97.5% after two consecutive washing steps with the same antisolvent and intensification by the action of ultrasound. Once again, this yield was not improved by additional washing steps with both water and citrate buffer. Higher temperatures were not assayed because of the boiling point of ethanol (78 °C). However, the best results were obtained by using the equimolar ethanol–water mixture as the antisolvent at 60 °C (entry 5), resulting directly in a 93.7% IL recovery yield. The increase in temperature during the precipitation step produced a decrease in the IL recovery yield (entries 6 and 7). The application of the proposed washing protocol to the resulting RC samples improved the total IL recovery yield up to 99.7% (entry 5), clearly demonstrating the suitability of the proposed methodology to reach full recovery of ILs in cellulose processing. These results cannot be explained simply by the ability of either the water or ethanol protic solvents to shift [Bmim] and [Cl] ions from the RC, because neither is able to achieve full recovery when used as pure solvent (entries 1 to 4). In this context, Lindman *et al.* suggested that cellulose is significantly amphiphilic and that the hydrophobic interactions are important for explaining its solubility pattern.[20] Thus, the full IL recovery obtained for the equimolar water–ethanol mixture could be explained by both the role of water and ethanol as very strongly hydrogen-bonded molecules, as well as, the hydrophobic properties of the aliphatic moiety of the ethanol molecule, behaving together a useful green solvent for IL recovery. Furthermore, the role of ultrasound in the two washing steps could also be considered as the key, because of the demonstrated efficiency for both the disruption and dissolution of cellulose fibres.[13,21]

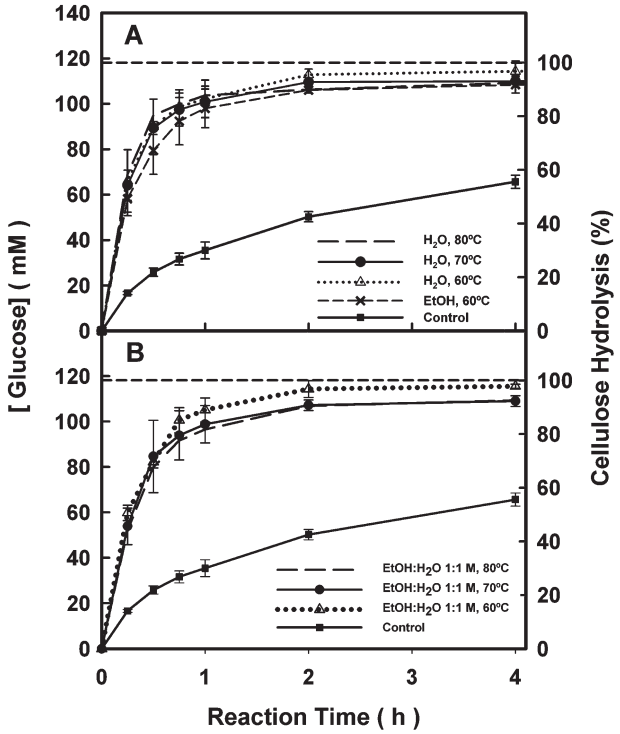

Fig. 2 Time-course profiles of both cellulose hydrolysis degree, and total reducing sugars released by the combined action of cellulase and cellobiase using regenerated cellulose (RC) as substrate. The different RCs were obtained from 2% (w/w) cellulose solutions in [Bmim][Cl] using water or ethanol (A), or a 1 : 1 (mol : mol) ethanol–water solution (B), as antisolvents at different temperatures. The control reaction was carried out using microcrystalline cellulose as substrate (see details in the Experimental section).

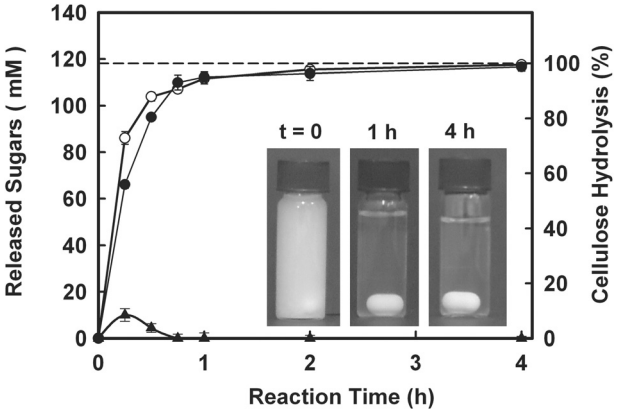

Fig. 3 Time-course profiles of total reducing sugars (○), glucose (●) and cellobiase (▲) released by the combined action of cellulase and cellobiase, using the regenerated cellulose (RC) resulting from precipitation with water–ethanol (see Table 1, entry 5) as substrate.

Besides the advantage of recovering IL, industrial interest for any such experimental approach will also be based on the suitability of the resulting cellulosic substrate for enzymatic degradation to produce glucose solutions. Fig. 2 shows the time course profiles for the total reducing sugars produced by the combined action of both cellulase and cellobiase on the resulting RC substrates obtained using the protocols described in Table 1. A control reaction was also carried out by using microcrystalline cellulose as substrate. As can be seen, all the RCs were suitable substrates for enzymatic hydrolysis, which was practically complete after 4 h. The positive effect of [Bmim][Cl] pretreatment on the disruption of the cellulose structure can be clearly observed by comparison with the reaction control, where the enzymatic hydrolysis of microcrystalline cellulose stopped at 58%. No further improvements in cellulose hydrolysis were observed even after a reaction time of 24 h. Furthermore, the suitability of these RCs, used as substrates for enzymatic hydrolysis, can also be observed in Fig. 3. By using the RC obtained with water–ethanol as antisolvent (see Table 1, entry 5), the enzymatic hydrolysis of this RC substrate showed excellent agreement between all the time course profiles of total reducing sugars, glucose and cellobiose into the reaction medium. Cellobiose was only observed at the beginning of the reaction course, when the RC concentration was high, after which the synergic action of cellulase and cellobiase hydrolysed cellobiose as fast as it was formed. The suitability of this RC substrate for enzymatic hydrolysis was also evident from the change in turbidity observed in the reaction medium (see inset pictures in Fig. 3), measured by transmittance at 660 nm, which was shifted from 0 to 70% during the 4 h the reaction. These results are clearly related by the weakening of the cellulose crystallinity and disruption of the fibre organization produced by the dissolution/precipitation pretreatment, which resulted in a more accessible substrate for efficient enzymatic hydrolysis.[11] However, full enzymatic hydrolysis of the RC substrate to its glucose monomeric units could only be explained by the added value made possible by the full recovery of the [Bmim][Cl] during the cellulose pretreatment process.

The negative effect of all ILs, that are able to dissolve cellulose, on the enzymatic activity of cellulase has been widely described.[7,8,10,12,13] For the case of both the [Bmim][Cl][7b] and 1,3-dimethylimidazolium dimethylphosphate (Mmim][DMP])[7c] ILs, it was reported that the cellulase activity decays continuously with increasing IL concentration in the reaction medium, resulting in a residual activity of up to 35–45% at 10% IL concentration. Furthermore, it was described how the cellulose hydrolysis degree was clearly reduced by the presence of residual IL (*e.g.*, 10% [Mmim][DMP]), giving a 40–70% hydrolysis yield at 24 h and did not increase with longer reaction times.[7c] The use of "compatible" IL–cellulase systems (*e.g.*, 15% [Emim][AcO]) for enzymatic hydrolysis of cellulosic biomass did not improve its conversion to glucose and cellobiose, giving only a 40–50% hydrolysis yield for the first 24 h.[8d] The presence of residual IL molecules bound to the cellulose polymer may have been involved in the low efficiency of cellulase in reaching full saccharification. Thus, the washing intensification steps carried out on regenerated cellulose (see Table 1) can be regarded as being the perfect substrate conditioning step for enzymatic hydrolysis, rather than an IL recovery process. The regenerated cellulose, that is to be used for enzymatic saccharification, should be fully free of residual IL content to reach full transformation to glucose monomeric units.

Despite the great interest of recovering all the IL used for conditioning cellulose for subsequent enzyme catalysis, its reuse in

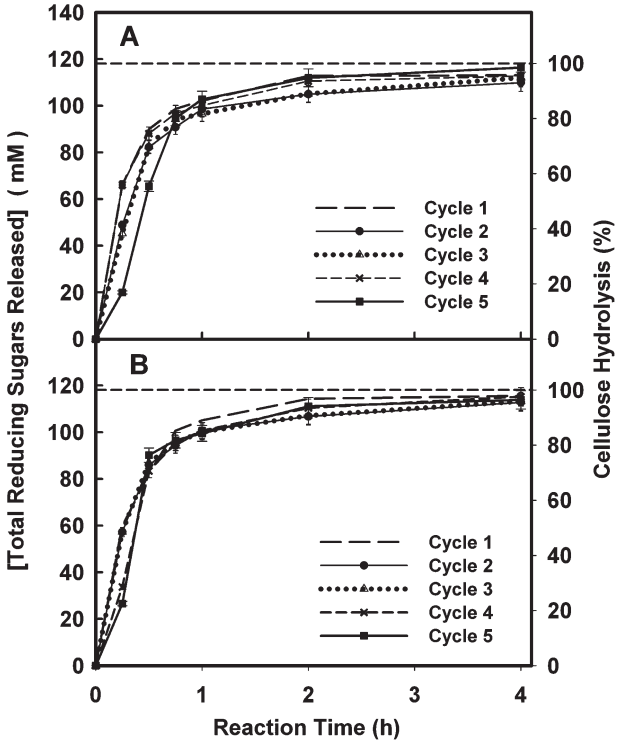

Fig. 4 Time-course profiles of both cellulose hydrolysis degree, and total reducing sugars released by the combined action of cellulase and cellobiase on regenerated cellulose (RC). The RCs were obtained through consecutive cycles of recovery and reuse of the [Bmim][Cl] used to dissolve cellulose, and by using water at 80 °C (A), or 1 : 1 (mol : mol) ethanol–water at 60 °C (B), as antisolvents during the cellulose regeneration step.

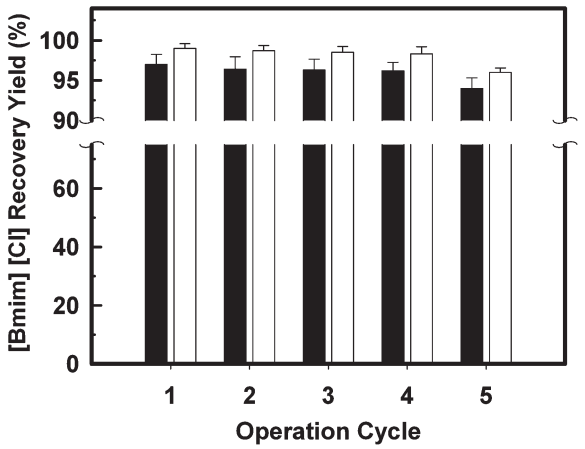

Fig. 5 Recovery yield of [Bmim][Cl] during consecutive operation cycles for cellulose dissolution/regeneration by using either water (black bars), or 1 : 1 (mol : mol) ethanol–water (white bars), as the antisolvent agent in the precipitation step.

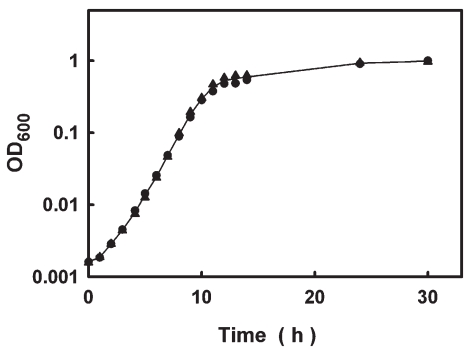

Fig. 6 Aerobic growth of *S. cerevisiae* in YPD medium containing 2% (w/v) glucose solution at pH 6.5 and at 30 °C. The glucose was obtained by enzymatic hydrolysis of free-IL RC (▲), and from commercial suppliers (●).

the further dissolution/precipitation of cellulose is a key factor for the economic sustainability of the proposed technology for biomass processing to produce biofuels. In this way, the IL recovered by using either water or an equimolar water–ethanol mixture, as antisolvent (see Table 1, entries 3 and 5, respectively) were applied in iterative cycles of RC production. The suitability of the resulting amorphous celluloses to be hydrolysed by the combined action of cellulase and cellobiase can be observed in Fig. 4, where the time course profiles for total reducing sugars are depicted. As can be seen, the reuse and recycling of [Bmim][Cl] for 5 cycles in the production of RC provided an excellent substrate for enzymatic hydrolysis, because the maximum conversion yield (higher than 96% for all cases) was reached at 4 h of reaction. The ability of [Bmim][Cl] to dissolve cellulose remained unchanged for 5 cycles, despite slight darkening, which could be removed by using charcoal. Fig. 5 shows the [Bmim][Cl] recovery yield obtained after each cellulose dissolution/precipitation cycle using either water or equimolar water–ethanol mixture as antisolvent. As can be seen, the suitability of the proposed methodology is clearly demonstrated by the excellent recovery yield (higher than 93%) obtained in all cases. The slight decrease observed as the number of cycles increases can be attributed to handling errors during manipulation of the large number of washing liquid fractions. Furthermore, the ^{1}H-NMR analysis of the recovered [Bmim][Cl] did not shows any sign of the decomposition and/or contaminating side products (see ESI†) with respect to the standard IL, which clearly demonstrates the excellent suitability of the proposed protocol.

The suitability of the glucose solution resulting from the enzymatic hydrolysis of RC was also tested as a carbon source for growing *Saccharomyces cerevisiae*. A YPD medium for growing microbes was prepared by adding the corresponding amount of peptone and yeast extract to a 2% glucose solution in 5 mM citrate buffer pH 4.8, previously obtained by the enzymatic hydrolysis of IL-free RC (see Experimental section), being compared with a control YPD medium containing commercial glucose. The citrate buffer concentration was ten-times lower than the usual concentration used for enzymatic activity assays to minimize its role as an alternative carbon source for growing yeast. In both YPD cases, the pH of the medium was adjusted to 6.5 before inoculation. Fig. 6 shows the aerobic growth of *S. cerevisiae* in both YPD media at 30 °C. As can be seen, both time-course profiles of microbial growth were identical, which clearly demonstrates the excellent suitability of the glucose solution obtained from the enzymatic reaction media for RC hydrolysis.

These results can be directly attributed to the absence of residual IL in the RC substrate, the final glucose solution showing identical suitability to the commercial one for microbial transformation. The negative effect of the residual content of IL in *S. cerevisiae* growing media has been reported as a primary source of inhibition on downstream microbial growth and ethanol production.[16] It should be noted at this point, how the reported approaches for IL separation from monosaccharides did not lead to full recovery of the IL. Thus, in agreement with the first principle of green chemistry,[22] dealing with prevention, it is better (and easier) to recover ILs from RC than to treat or clean up the resulting glucose solution from the RC hydrolysis step.

In conclusion, this work has shown how the suitability of IL technology in biomass processing to provide useful substrates for enzymatic hydrolysis is clearly enhanced by the IL recovery. By combining green molecular antisolvents, like water or ethanol, and ultrasound, efficient protocols to fully recover the IL from cellulose solutions can be designed. Moreover, the IL can be purified by simple distillation, before being applied in further biomass processing. In addition to the economic and environmental benefits provided by the full recovery of IL, the excellent suitability of the resulting amorphous cellulosic substrate for full transformation into directly fermentable glucose should be emphasized. Fundamental studies on enzyme reactors need to be carried out to establish clear criteria for specifically pairing the most appropriate enzyme mixture with the corresponding pretreated biomass substrate in continuous operation. Once again, the IL technology appears as an enhancer of enzyme technology for developing green chemical bioprocesses.

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Notes and references

1 For recent reviews on biomass processing to produce biofuels: (*a*) R. E. H. Sims, E. Mabee, J. N. Saddler and M. Taylor, *Bioresour. Technol.*, 2010, **101**, 68–75; (*b*) V. B. Agbor, N. Cicek, R. Sparling, A. Berlin and D. B. Levin, *Biotechnol. Adv.*, 2011, **29**, 675–685; (*c*) M. Rose and R. Palkovits, *ChemSusChem*, 2012, **5**, 167–176; (*d*) N. Sarkar, S. K. Ghosh, S. Bannerjee and K. Aikat, *Renewable Energy*, 2012, **37**, 19–27.

2 L. O. Sukharnikov, B. J. Cantwell, M. Podar and I. B. Zhulin, *Trends Biotechnol.*, 2011, **29**, 473–479.

3 S. Zhu, Y. Wu, Q. Chen, Z. Yu, C. Wang, S. Jin, Y. Ding and G. Wu, *Green Chem.*, 2006, **8**, 325–327; P. Alvira, E. Tomás-Pejó, M. Ballesteros and M. J. Negro, *Bioresour. Technol.*, 2010, **101**, 4851–4861; A. R. F. C. Ferreira, A. B. Figueiredo, D. V. Evtuguin and J. A. Saraiva, *Green Chem.*, 2011, **13**, 2764–2767.

4 R. P. Swatloski, S. K. Spear, J. D. Holbrey and R. D. Rogers, *J. Am. Chem. Soc.*, 2002, **124**, 4974–4975.

5 (*a*) D. A. Fort, R. C. Remsing, R. P. Swatloski, P. Moyna, G. Moyna and R. D. Rogers, *Green Chem.*, 2007, **9**, 63–69; (*b*) D. M. Alonso, J. Q. Bond and J. A. Dumesic, *Green Chem.*, 2010, **12**, 1493–1513; (*c*) S. Bose, D. W. Armstrong and J. W. Petrich, *J. Phys. Chem. B*, 2010, **114**, 8221–8227; (*d*) M. E. Zakrzewska, E. Bogel-Lukasik and R. Bogel-Lukasik, *Energy Fuels*, 2010, **24**, 737–745.

6 (*a*) P. Lozano, *Green Chem.*, 2010, **12**, 555–569; (*b*) P. Dominguez de María and Z. Maugeri, *Curr. Opin. Chem. Biol.*, 2011, **15**, 220–225; (*c*) P. Lozano, E. Garcia-Verdugo, S. V. Luis, M. Pucheault and M. Vaultier, *Curr. Org. Synth.*, 2011, **8**, 810–823; (*d*) R. A. Sheldon, *Chem. Soc. Rev.*, 2012, **41**, 1437–1451.

7 (*a*) M. B. Turner, S. K. Spear, J. G. Huddleston, J. D. Holbrey and R. D. Rogers, *Green Chem.*, 2003, **5**, 443–447; (*b*) A. C. Salvador, M. C. Santos and J. A. Saraiva, *Green Chem.*, 2010, **12**, 632–635; (*c*) P. Engel, R. Mladenov, H. Wulfhorst, G. Jager and A. C. Spiess, *Green Chem.*, 2010, **12**, 1959–1966.

8 (*a*) N. Kamiya, Y. Matsushita, M. Hanaki, K. Nakashima, M. Narita, M. Goto and H. Takahashi, *Biotechnol. Lett.*, 2008, **30**, 1037–1040; (*b*) H. Zhao, G. A. Baker, A. Song, O. Olubajo, T. Crittle and D. Peters, *Green Chem.*, 2008, **10**, 696–705; (*c*) J. Vitz, T. Erdmenger, C. Haensch and U. S. Shubert, *Green Chem.*, 2009, **11**, 4117–4424; (*d*) Y. Wang, M. Radosevich, D. Hayes and N. Labbe, *Biotechnol. Bioeng.*, 2011, **108**, 1042–1048; (*e*) M. F. Thomas, L. L. Li, J. M. Handley-Pendleton, D. van der Lelie, J. J. Dunn and J. F. Wishart, *Bioresour. Technol.*, 2011, **102**, 11200–11203.

9 P. Lozano, B. Bernal, J. M. Bernal, M. Pucheault and M. Vaultier, *Green Chem.*, 2011, **13**, 1406–1410.

10 T. Zhang, S. Datta, J. Eichler, N. Ivanova, S. D. Axen, C. A. Kerfeld, F. Chen, N. Kyrpides, P. Hugenholtz, J. F. Cheng, K. L. Sale, B. Simmons and E. Rubin, *Green Chem.*, 2011, **13**, 2083–2090.

11 P. M. Grande and P. Dominguez de María, *Bioresour. Technol.*, 2012, **104**, 799–802.

12 (*a*) A. P. Dadi, S. Varanasi and C. A. Schall, *Biotechnol. Bioeng.*, 2006, **95**, 904–910; (*b*) H. Zhao, C. L. Jones, G. A. Baker, S. Xia, O. Olubajo and V. N. Person, *J. Biotechnol.*, 2009, **139**, 47–54; (*c*) I. H. Samayan and C. A. Schall, *Bioresour. Technol.*, 2010, **101**, 3561–3566; (*d*) E. Husson, S. Buchoux, C. Avondo, D. Cailleu, K. Djellab, I. Gosselin, O. Wattraint and C. Sarazin, *Bioresour. Technol.*, 2011, **102**, 7335–7342; (*e*) F. Hong, X. Guo, S. Zhang, S. F. Han, G. Yang and L. J. Jonsson, *Bioresour. Technol.*, 2012, **104**, 503–508.

13 (*a*) F. Yang, L. Li, Q. Li, W. Tan, W. Liu and M. Xian, *Carbohydr. Res.*, 2010, **81**, 311–316; (*b*) K. Ninomiya, K. Kamide, K. Takahashi and N. Shimizu, *Bioresour. Technol.*, 2012, **103**, 259–265.

14 S. Bose, C. A. Barnes and J. W. Petrich, *Biotechnol. Bioeng.*, 2012, **109**, 434–443.

15 C. W. Cho, T. P. T. Pham, Y. C. Jeon, K. Vijayaraghavan, W. S. Choe and Y. S. Yun, *Chemosphere*, 2007, **69**, 1003–1007; J. S. Torrecilla, J. Garcia, E. Rojo and F. Rodriguez, *J. Hazard. Mater.*, 2009, **164**, 182–194.

16 (*a*) K. Shill, S. Padmanabhan, Q. Xin, J. M. Prausnitz, D. S. Clark and H. W. Blanch, *Biotechnol. Bioeng.*, 2011, **108**, 511–520; (*b*) D. Feng, L. Li, F. Wang, W. Tan, G. Zhao, H. Zou, M. Xian and Y. Zhang, *Appl. Microbiol. Biotechnol.*, 2011, **91**, 399–405.

17 (*a*) M. Ouellet, S. Datta, D. C. Dibble, P. R. Tamrakar, P. I. Benke, C. L. Li, S. Singh, K. L. Sale, P. D. Adams, J. D. Keasling, B. A. Simmons, B. M. Holmes and A. Mukhopadhyay, *Green Chem.*, 2011, **13**, 2743–2749; (*b*) K. Nakashima, K. Yamaguchi, N. Taniguchi, S. Arai, R. Yamada, S. Katahira, N. Ishida, H. Takahashi, C. Ogino and A. Kondo, *Green Chem.*, 2011, **13**, 2948–2953.

18 P. Stepnowski, J. Nichthauser, W. Mrozik and B. Buszewski, *Anal. Bioanal. Chem.*, 2006, **385**, 1483–1491.

19 E. Albers and C. Larsson, *J. Ind. Microbiol. Biotechnol.*, 2009, **68**, 401–405.

20 B. Lindman, G. Karlstrom and L. Stigsson, *J. Mol. Liq.*, 2010, **156**, 76–81.

21 (*a*) M. Imai, K. Ikari and I. Suzuki, *Biochem. Eng. J.*, 2004, **17**, 79–83; (*b*) F. Yang, L. Li, Q. Li, W. Tan, W. Liu and M. Xiam, *Carbohydr. Polym.*, 2010, **81**, 311–316; (*c*) J. P. Mikkola, A. Kirilin, J. C. Tuuf, A. Pranovich, B. Holmbom, L. M. Kustov, D. Y. Murzin and T. Salmi, *Green Chem.*, 2007, **9**, 1229–1237.

22 P. T. Anastas And J. C. Warner, *Green Chemistry: Theory and Practice*, Oxford University Press, New York, 1998, p. 30.