An Integrated Process of Ionic Liquid Pretreatment and Enzymatic Hydrolysis of Lignocellulosic Biomass with Immobilised Cellulase

Mihaela Ungurean,[a] Zsófia Csanádi,[b] László Gubicza,[b] and Francisc Péter [a,*]

An integrated process of lignocellulosic biomass conversion was set up involving pretreatment by an ionic liquid (IL) and hydrolysis of cellulose using cellulase immobilised by the sol-gel method, with recovery and reuse of both the IL and biocatalyst. As all investigated ILs, regardless of the nature of the anion and the cation, led to the loss of at least 50% of the hydrolytic activity of cellulase, the preferred solution involved reprecipitation of cellulose and lignin after the pretreatment, instead of performing the enzymatic hydrolysis in the same reaction system. The cellulose recovered after pretreatment with 1-ethyl-3-methylimidazolium acetate ([Emim][Ac]) and dimethylsulfoxide (DMSO) (1:1 ratio, v/v) was hydrolysed with almost double yield after 8 h of reaction time with the immobilised cellulase, compared to the reference microcrystalline cellulose. The dissolution capacity of the pretreatment mixture was maintained at satisfactory level during five reuse cycles. The immobilised cellulase was recycled in nine reaction cycles, preserving about 30% of the initial activity.

Keywords: Pretreatment; Ionic liquid; Poplar biomass; Cellulose hydrolysis; Immobilised cellulase

*Contact information: a: University Politehnica of Timişoara, Faculty of Industrial Chemistry and Environmental Engineering, C. Telbisz 6, 300001 Timişoara, Romania; b: University of Pannonia, Research Institute on Bioengineering, Membrane Technology and Energetics, Egyetem u. 10, H-8200 Veszprém, Hungary; *Corresponding author: francisc.peter@upt.ro*

INTRODUCTION

Bioethanol from cellulosic feedstock has emerged as an important biofuel to replace fossil fuels. Ethanol production from lignocellulosic biomass has several benefits, including utilisation of a renewable raw material, the possibility to valorise waste biomass, producing value-added co-products as well, and prevention of sulfur dioxide emissions that cause acid rain (Saxena *et al.* 2009).

Conversion of lignocellulosic biomass to fermentable pentoses and hexoses is a well-studied process (Brown and Brown 2013). However, the rigid crystalline structure of lignocellulosic materials hampers the access of cellulase to hydrolyse the polysaccharide polymer. Consequently, the use of a lignocellulosic pretreatment step in the saccharification process is compulsory to remove lignin and hemicelluloses, to lower cellulose crystallinity, and to increase the efficiency of cellulose hydrolysis (Zheng *et al.* 2009; Ioelovich and Morag 2012; Mood *et al.* 2013). The pretreatment step is probably the most extensively investigated subject in the topic of cellulosic bioethanol. Such a process should (i) be effective on a wide range of lignocellulosics; (ii) allow the recovery of most components; (iii) not cause the degradation of lignin, which inhibits cellulose hydrolysis; and (iv) be economical to operate (Zheng *et al.* 2009; Agbor *et al.* 2011). A

large number of physical and chemical pretreatment methods have been investigated and the available cellulases have become more efficient, but only one commercial-scale cellulosic ethanol facility (the Chemtex plant in Crescentino, Italy) is operational at present (Gusakov 2013).

Despite intense interest and extensive research, a generally accepted pretreatment method has not been developed yet. In this context, the investigation of new, non-conventional pretreatment methods is still attracting scientific interest. Ionic liquids (ILs) are a relatively new class of solvents that have several important properties that depend on the nature of the cation and the anion. They can dissolve a large number of biomacromolecules, including cellulose and lignin from wood (Sun *et al.* 2009). More than 40 ILs have been investigated for this purpose, concluding that viscosity, melting point, polarity, and hydrogen bond basicity of ILs are important properties in biomass dissolution (Maki-Arvela *et al.* 2010). The dissolution of biomass in an ionic liquid depends on the type of wood, anion or cation of the ionic liquid solvent, pretreatment time, temperature and wood-to-IL ratio (Maki-Arvela *et al.* 2010; Olivier-Bourbigou *et al.* 2010). However, cellulose dissolution in ILs can be restricted by degradation or acetylation of cellulose that some ILs generate (Karatzos *et al.* 2012). To prevent this, the appropriate IL, temperature, and duration of pretreatment must be carefully determined (Pinkert *et al.* 2011).

1-Ethyl-3-methylimidazolium acetate ([Emim][Ac]) is considered to be one of the promising ILs for the dissolution of cellulose without degradation; this IL is biodegradable and reasonably non-toxic and non-corrosive (Sun *et al.* 2009; Zavrel *et al.* 2009). Because of the strong basicity of the acetate anion, the inter- and intra-molecular hydrogen bonds in the wood can be efficiently disrupted. The low viscosity of [Emim][Ac] also helps the dissolution of the biomass, which results in a higher solvation power for cellulose and lignin than has been observed for 1-butyl-3-methylimidazolium chloride ([Bmim][Cl]) and other ionic liquids (Wang *et al.* 2010). [Emim][Ac] has been shown to dissolve 4% cellulose after 210 min and 4% lignin after 50 min at 90 °C (Casas *et al.* 2012). Lee *et al.* (2008) used [Emim][Ac] to decrease both the lignin content and cellulose crystallinity of maple wood flour. Removal of 40% of the original lignin content resulted in improved hydrolysis yield of wood flour cellulose (90%) (Lee *et al.* 2008). A polyoxometalate catalyst (*i.e.*, $[PV_2Mo_{10}O_{40}]^{5-}$) was employed to enhance the dissolution and delignification of biomass in [Emim][Ac] (Sun *et al.* 2011).

To overcome the technical difficulties caused by the high viscosity of the ILs, an organic co-solvent can be utilised. It has been demonstrated that mixtures composed of ionic liquids and polar solvents (*e.g.*, acetone, 1,4-dioxane, pyridine, dimethyl sulfoxide (DMSO), and *N*,*N*-dimethylacetamide) can also dissolve cellulose. Tian *et al.* (2011) pretreated microcrystalline cellulose with an organic electrolyte solution composed of 1-allyl-3-methylimidazolium chloride and dimethyl sulfoxide. A mixture of 90% (by mass) 1-butyl-3-methylimidazolium acesulfamate and 10% dimethyl sulfoxide was effective for the extraction of wood lignin (Pinkert *et al.* 2009). Solvation of both cellulose and xylose is possible using a solvent system composed of [Emim][Ac] and 1-methylimidazole (Bylin *et al.* 2014).

A major disadvantage of ILs used for biomass pretreatment is their relatively high cost. Therefore, the reuse of the IL solvent represents a key issue in decreasing the pretreatment cost. Ammonia-pretreated rice straw was mixed with 20-fold [Emim][Ac], and was incubated for 24 h at 130 °C, followed by reprecipitation of cellulose using ethanol as anti-solvent (Nguyen *et al.* 2010). The IL was recycled more than 20 times, but

a technology that needs two pretreatment steps, washing and drying after the ammonia pretreatment, as well as an anti-solvent recovery by distillation, seems not to be optimal. Another IL, [Emim][Ac], has been recycled four times for the IL pretreatment of maple wood flour without significantly lowering the hydrolysis efficiency (Lee *et al.* 2008). [Emim][Ac] is considered the most effective IL for pretreatment found to date, but is also the most expensive, as it costs 60 times more than [Bmim][Cl], a less efficient IL (Groff *et al.* 2013). [Bmim][Cl] was reused five times with high recovery yield for pretreatment of microcrystalline cellulose, the recovery being performed by vacuum distillation (Lozano *et al.* 2012). Despite the important research work assigned to this topic, a solution with economic and technologic feasibility, suitable for authentic lignin-containing materials, was not yet developed.

Other than the pretreatment step, other drawbacks that hinder the industrial production of cellulosic bioethanol are the high cost of the hydrolysis enzymes, the difficulty in separating the enzymes from the solution, and the possible inactivation of the native cellulases during the process. Several authors have considered immobilising the cellulases onto solid supports to be the appropriate solution to make the enzymatic hydrolysis more competitive, due to improved enzyme stability under various denaturing conditions and the possibility of reuse of the biocatalyst (Dincer and Telefoncu 2007; Abd El-Ghaffar and Hashem 2010; Ogeda *et al.* 2012). Cellulase, immobilised onto a polymeric support (Amberlite XAD4) and coated with an IL, was successfully used for saccharification of cellulose dissolved in 1-butyl-3-methylimidazolium chloride (Lozano *et al.* 2011). Carrier-free immobilised cellulase, obtained by cross-linking with glutaraldehyde, was used in the presence of 2% 1-ethyl-3-methyl imidazolium diethyl phosphate for hydrolysis of microcrystalline cellulose, allowing five-time reuse of the biocatalyst (Jones and Vasudevan 2010). Among the different immobilisation methods, such as adsorption, covalent bonding, cross-linking with bifunctional reagents, and entrapment in polymer matrices, sol-gel encapsulation has been considered a promising route to immobilise cellulases. Cellulase that was immobilised by the sol-gel method using trimethoxymethylsilane and tetramethoxysilane precursors demonstrated enhanced temperature and pH stability, in addition to being recycled five times during the hydrolysis of microcrystalline cellulose (Ungurean *et al.* 2013).

Despite numerous reports concerning the IL pretreatment of lignocellulosic biomass and subsequent hydrolysis, the investigation of an integrated process involving all the steps of dissolution and subsequent regeneration of cellulose and lignin from woody biomass, as well as the utilisation of immobilised cellulase and the regeneration of the biocatalyst, has not yet been reported. Our study focused on the efficiency and recycling of the [Emim][Ac]/DMSO solvent system, the optimisation of the dissolution process, and the characterisation of the regenerated lignin and cellulose. The other major objective was the enzymatic hydrolysis of the cellulose regenerated after the IL pretreatment using a sol-gel immobilised cellulase, with multiple recycles of the enzyme. To our knowledge, this is the first report concerning such an integrated process cycle.

EXPERIMENTAL

Materials

The biomass material used in this study was poplar. Poplar samples were received as shavings and were ground into powder using an electric mill and then dried in an oven

at 100 °C. Microcrystalline cellulose utilised in the stability experiments with ionic liquids was acquired from Macherey Nagel (Germany), while the microcrystalline cellulose employed in the other studies was obtained from Sigma-Aldrich (Avicel PH101). Kraft lignin was obtained from Sigma-Aldrich, while Celluclast 1.5L from *Trichoderma reesei* CCN 03116 was purchased from Novozymes.

Silane precursors tetramethoxysilane (TMOS) 98% and methyltrimethoxysilane (MTMOS) 98% (Merck), as well as absolute ethanol (Merck), sodium fluoride (Sigma-Aldrich), 2-propanol (Merck), hexane (Merck), and hydrochloric acid (Sigma -Aldrich), were used for sol-gel immobilisation.

Glucose (Merck), 3,5-dinitrosalicylic acid (Merck), sodium sulphite (Fluka), sulphuric acid (Sigma-Aldrich), calcium carbonate (Loba Feinchemie), acetic acid (Sigma-Aldrich), sodium hydroxide (Chemapol, Czech Republic), and dimethylsulfoxide (Merck) were of analytical grade and were used as purchased.

The ionic liquids 1,3-dimethylimidazolium dimethyl phosphate ([Mmim][DMP]), 1-butyl-3-methylimidazolium chloride ([Bmim][Cl]), and 1-butyl-3-methylimidazolium tetrafluoroborate ([Bmim][BF4]) were obtained from Merck. Trihexyltetradecylphosphonium bromide ([P14,6,6,6][Br]), trihexyltetradecylphosphonium bis(trifluoromethylsulfonyl)imide ([P14,6,6,6][NTf2]), trihexyltetradecylphosphonium bis(2,4,4-trimethylpenthyl) phosphinate ([P14,6,6,6][M3PPh]), trihexyltetradecylphosphonium hexafluorophosphate ([P14,6,6,6][PF6]), 1-ethyl-3-methylimidazolium triflate ([EMIM][TfO]), 1-ethyl-3-methylimidazolium tosylate ([Emim][TOS]), tetrabutylphosphonium bromide [Bu4P][Br], 1-ethylpyridinium bromide ([EtPy][Br]), 1-butyl-3-methylimidazolium hexafluorophosphate ([Bmim][PF6]), and 1-hexyl-3-methylimidazolium hexafluorophosphate ([Hmim][PF6]) were purchased from IoLiTec (Germany). 1-Ethyl-3-methylimidazolium acetate ([Emim][Ac]) was a generous gift from BASF (Germany).

Methods

Activity of native and immobilised cellulase

Cellulase activity was assayed according to the original method reported by Ghose (1987), which has also been adapted for assaying immobilised enzyme activity and reported in our previous work (Ungurean *et al*. 2013). Activity was determined by incubating 5 µL of free enzyme, or 50 mg of immobilised enzyme, for 30 min with 2% cellulose in 1 mL of sodium acetate buffer (0.05 M, pH 4.8) at 50 °C. Blanks were prepared by incubating 2% cellulose in buffer or IL solution for 30 min. The resulting sugars were assayed spectrophotometrically at 575 nm (Jasco V-530 UV/VIS spectrophotometer) using the 3,5-dinitrosalicylic acid (DNS) method (Miller 1959). One unit (1 U) of activity was defined as the amount of enzyme that produced one µmole of glucose equivalent *per* min at the given reaction conditions. The determined initial activity of native cellulase (Celluclast 1.5L) was 53.7 U/mL.

Sol-gel immobilisation of cellulase

The sol-gel immobilisation method presented in our previous work was used (Ursoiu *et al*. 2012; Ungurean *et al*. 2013). The silane precursors tetramethoxysilane and methyltrimethoxysilane (total 6 mmoles) in a molar ratio of 3:1 were mixed with 0.2 mL of deionised water, 0.5 mL of ethanol, and 30 µL of 0.04 M hydrochloric acid in a 4-mL capped vial for 60 min (magnetic stirring at 200 revolution per minute (rpm) and room temperature). Cellulase solution (0.2 mL Celluclast 1.5 L in 0.8 mL of sodium acetate buffer, pH 4.8) and 50 µL of 1 M NaF aqueous solution were added and continuously

stirred at room temperature until gelation started. The resulting gel was kept in a refrigerator for 24 h to complete polymerisation. The bulk gel was washed to remove unreacted monomers and additives with isopropyl alcohol, then with sodium acetate buffer (0.05 M; pH 4.8), then with isopropyl alcohol again, and finally with hexane. The sol-gel encapsulated enzyme was dried in a vacuum oven at 25 °C for 8 h, crushed in a mortar, and kept in a closed vessel in the refrigerator.

Stability of cellulase in ionic liquids

The appropriate amount of the selected IL was mixed with cellulose suspension in acetate buffer (0.05 M; pH 4.8). The concentration of the cellulose in the mixture was 2% (w/v) in all experiments. The samples were heated to 50 °C, and 5 µL/mL of cellulase was added. The activity of cellulase was assayed after 30 min of incubation by the DNS method (Miller 1959), as described before. Reference samples were prepared under the same conditions but without enzyme.

Total sugar, cellulose, and lignin content of poplar biomass

The total sugars content of poplar samples was assayed by quantitative saccharification upon acid hydrolysis, which was based upon the NREL Laboratory Analytical Procedure (Sluiter *et al*. 2008). The sample was treated with 72% (v/v) sulphuric acid at 30 °C for 1 h; afterwards, the acid concentration was reduced to 4% by diluting with distilled water. This mixture was then heated to 120 °C for 1 h. After neutralisation with calcium carbonate, the total reducing sugars were analysed by the DNS method. The total sugars content of the poplar wood was determined to be 78.5%.

The same analytical method was used to determine the cellulose content of poplar biomass (Sluiter *et al*. 2008). Following the acid hydrolysis and neutralisation, the sample was analysed by HPLC, using a Jasco HPLC chromatographic system (Jasco Analytical Instruments, Japan) equipped with a PU-2089 quaternary pump, RI-2031 Plus RI detector, AS-2055 Plus autosampler, and column thermostat. The analysis conditions were: Biorad Aminex HPX-87P column, mobile phase water, 80 °C, and flow rate 0.5 mL min^{-1}. The cellulose content was calculated on basis of the assayed glucose.

The initial lignin content (acid insoluble and acid soluble lignin) in the poplar biomass was assayed using NREL Laboratory Analytical Procedure, too (Sluiter *et al*. 2008). The acid soluble lignin content of poplar was 18.5%.

Pretreatment of poplar biomass sample

Poplar biomass samples (100 mg), or a reference mixture (80% cellulose and 20% lignin), were added to 4 mL of a dimethylsulfoxide and [Emim][Ac] mixture (1:1, v/v), heated to 90 °C, and magnetically stirred in an oil bath for 6 h. The hot mixture was filtered to separate any non-dissolved material. For the regeneration of the cellulose dissolved in DMSO/[Emim][Ac], 20 mL of distilled water was added to the liquor and stirred at 10 °C for 3 h. The precipitated cellulose was washed with hot water and separated by filtration. To remove residual ionic liquid and lignin, the regenerated cellulose was extracted with 0.1 M NaOH at 60 °C for 8 h.

The lignin dissolved in the DMSO/[Emim][Ac] mixture was assayed according to the method reported by Lee *et al*. (2008). A 0.1-g sample was removed and diluted with 0.9 mL of 0.1 N NaOH. The lignin content was measured with a UV-VIS spectrophotometer (Jasco-V-530, Japan) operating at 280 nm; a calibration curve was developed using kraft lignin. Then, the lignin was precipitated from the remaining

DMSO/IL mixture with excess water at low temperature. After precipitation of cellulose and lignin, the DMSO/[Emim][Ac] solvent was recovered by the evaporation of water and was reused in the next pretreatment cycle. The obtained cellulose and lignin were analysed by FT-IR (JASCO FT/IR 436 spectrophotometer).

Enzymatic hydrolysis of cellulose

Hydrolysis of 5 mg/mL cellulose in sodium acetate buffer (0.05 M, pH 4.8), catalysed by 5 μL/mL native cellulase Celluclast 1.5L (equivalent to 0.62 mg protein/mL), or 10 mg/mL immobilised cellulase (equivalent to 0.44 mg protein/mL), was carried out using an OmniStation modular reactor for parallel synthesis (Barnstead International, USA), operating at 200 rpm and 50 °C. Samples were taken from the reaction mixture at defined time intervals and were assayed for total reducing sugars, using the DNS method (Miller 1959), as previously described.

Reuse of sol-gel immobilised cellulase on pretreated cellulose substrate

Immobilised cellulose (100 mg) was added to 10 mL of sodium acetate buffer (0.05 M; pH 4.8), containing 5 mg/mL Avicel PH101 microcrystalline cellulose, previously pretreated with DMSO/[Emim][Ac], as previously described. The reaction mixture was incubated at 50 °C using the OmniStation modular reactor. After 8 h of hydrolysis, the immobilised enzyme was separated by decantation, washed with the same buffer solution, and reused in a new experiment at the same reaction conditions.

RESULTS AND DISCUSSION

In this study, an integrated approach was attempted to improve the conversion of lignocellulosic biomass into fermentable sugars. The flowchart of this process is presented in Fig. 1.

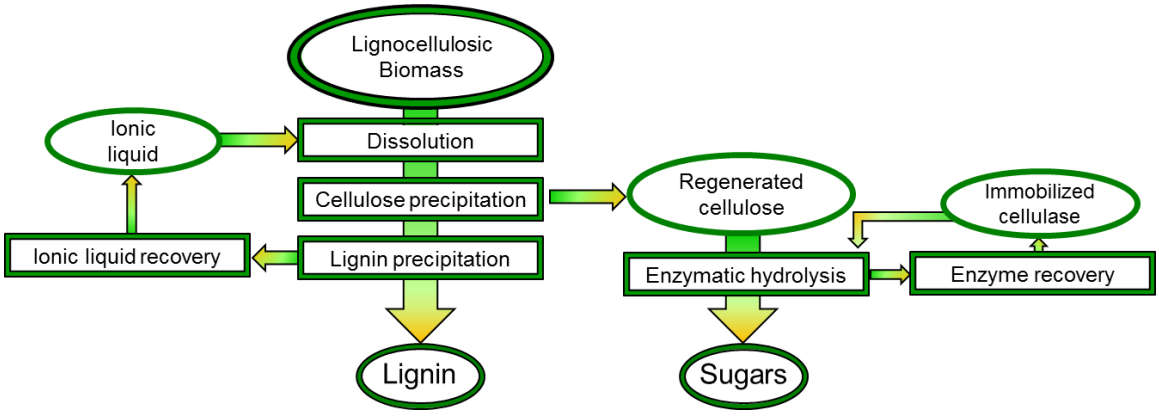

Fig. 1. Flowchart of the pretreatment and hydrolysis of lignocellulosic biomass, involving an ionic liquid and immobilised cellulase

Pretreatment by the dissolution of cellulose in an ionic liquid has been studied to reduce the crystallinity of cellulose and to increase its enzymatic digestibility. A mixture of an ionic liquid and an organic solvent was used to reduce the IL's viscosity, to facilitate the easy recovery of cellulose, and to recover of the ionic liquid for its reuse. This procedure also allowed for the partial recovery of the lignin, which can be further

valorised in a biorefinery process. The reprecipitated cellulose was hydrolysed by immobilised cellulases. Immobilisation of the enzyme enabled it to be recycled several times to achieve increased productivity and to reduce process costs.

Influence of Ionic Liquids on Cellulase Activity

The stability of cellulase in ILs was studied before investigating the individual stages of this process. If the cellulose activity can be preserved in ILs, then the biomass pretreatment process can be simplified without cellulose reprecipitation. Ionic liquids are non-conventional solvents with great potential to replace harmful organic solvents in several applications, such as reaction media for biocatalytic reactions (Moniruzzamana *et al*. 2010). Because numerous physical properties of ILs affect their possible utilisation for the dissolution of wood components and as a co-solvent in the cellulose hydrolysis process, we investigated the compatibility of several ILs with cellulase. Ionic liquids with a large trihexyltetradecylphosphonium cation were compared to others containing more extensively studied cations; the influence of different anions on the enzymatic activity was also studied. Following 30 min of incubation in various ILs at 50 °C, the enzymatic activity of Celluclast 1.5L was assayed and its stability was expressed as percentage of its original activity without IL incubation (Table 1).

All ILs had an inhibitory effect on cellulose activity; however there were significant differences noticed amongst the constituent anions and cations. The capability of several ionic liquids to dissolve cellulose and other wood components is well known (Fort *et al.* 2007), and has been comprehensively reviewed by Mäki-Arvela *et al.* (2010). However, unlike other enzymes, cellulases are generally inactivated in the presence of cellulose-dissolving ionic liquids (Engel *et al*. 2010), as it was also demonstrated by Turner *et al.* (2003) for [Bmim][Cl] and cellulase derived from *Trichoderma reesei*.

Table 1. Relative Activity of Celluclast 1.5 L after 30 min of Incubation in Different ILs at 50 °C, Expressed as Percentage of its Activity without IL

Ionic Liquids		Relative Activity (%)
Cations	**Anions**	
[EtPy]	[Br]	47.2
[Bu4P]		36.7
[P14,6,6,6]		35.3
[Hmim]	[PF6]	0
[Bmim]		0
[P14,6,6,6]		0
[P14,6,6,6]	[Br]	35.3
	[M3PPh]	20.7
	[NTf2]	25.5
[Emim]	[OTs]	42.0
	[TfO]	29.2
	[Ac]	20.1

Our experiments showed that cellulase was totally inactivated with ILs containing the hexafluorophosphate anion. Cellulase activity was higher with the 1-ethyl-pyridinium cation (47%) when compared to larger alkyl phosphonium cations (*ca*. 36%) in the IL homolog series containing the bromide anion. In the case of ILs with the same imidazolium cation (Emim), the enzyme activity decreased in the following order of

anions: [OTs] > [TfO] > [Ac]. This observation demonstrated that sulphonate anions were less inactivating than acetate. Among the ILs containing the alkyl phosphonium cation [P14,6,6,6], the bromide anion caused less inactivation of the cellulase than larger cations, like as trimethylphenylphosphinate ([M3PPh]) or bis(trifluoromethylsulfonyl)imide ([NTf2]). The stability of cellulose in ILs is a complex result of different influences: structure and hydrophobicity of the IL; water stripping capacity around the active center; and increase of mass transfer resistance during the hydrolytic cellulase reaction. Although the number of ILs studied was too limited to recognise a direct structural dependence, the importance of the observations reported here emerges in connection with the cellulose-dissolving ability of ILs.

Unfortunately, the ability of ILs to dissolve cellulose is not correlated with the preservation of the enzymatic activity. The miscibility of the IL with water plays an important role in both cellulose-dissolving capacity and preservation of enzyme activity. Solubilities of the ILs in water are more strongly dependent upon the anion than the cation chain length size (Freire *et al.* 2007). The enzymes usually show high stability in water-immiscible ILs, whereas water-miscible ILs, such as 1-butyl-3-methyl imidazolium chloride or 1-allyl-3-methyl imidazolium chloride, are able to dissolve cellulose; unfortunately, these water-miscible ILs are reported to cause strong inactivation of cellulases by protein unfolding (Lozano *et al.* 2011).

A possible solution to reduce the negative effect of ILs on cellulase could be to use aqueous solutions that contain lower concentrations of ILs. To investigate the influence of IL concentration on cellulase activity, four water-miscible ILs, [Bmim][Cl], [Mmim][DMP], [Emim][Ac], and [Bmim][BF4], were examined. The reactions were carried out with 2% cellulose suspension, at 50 °C, pH 4.8 (0.05 mM sodium acetate buffer), at various IL concentrations (between 10% and 100%).

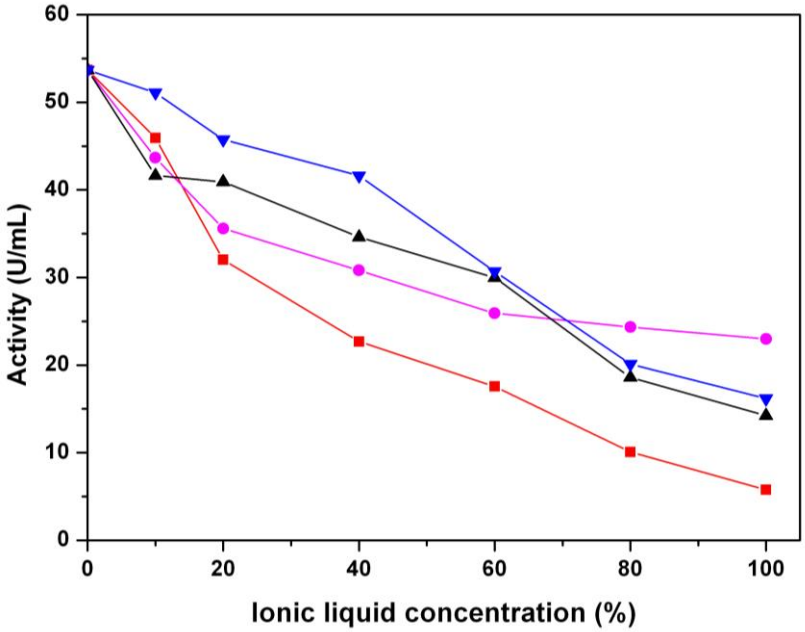

Fig. 2. Influence of IL concentration on the activity of cellulase: [Bmim][Cl] (-■-), [Mmim][DMP] (-●-), [Emim][Ac] (-▲-), and [Bmim][BF4] (-▼-)

Increasing the concentration of the ILs decreased cellulase activity, regardless of the IL used (Fig. 2). Among the tested ILs, [Mmim][DMP] impacted the cellulase activity the least at high concentrations, whereas high concentrations of [Bmim][Cl] had the greatest inhibitory effect on cellulase activity. Other than [Bmim][Cl], the differences between the ILs were not significant, particularly in the concentration range of 50 to 80%; however, in these cases, approximately half of the enzymatic activity was lost.

Pretreatment of Poplar with DMSO/[Emim]Ac with Regeneration of Cellulose and Lignin

Pretreatment of lignocellulosic biomass using an IL is restricted by its recovery and reuse. [Emim][Ac] has a good capacity to dissolve lignocellulosic biomass; however, it is difficult to recover, primarily due to its high viscosity. Although several strategies are suitable for this purpose, it remains a difficult task. As found in this study, Celluclast 1.5 L preserved more than 50% of its activity in the presence of limited IL amounts. For this reason, hydrolysis could have been performed directly following the pretreatment step, without cellulose reprecipitation. However, recovery of fermentable sugars from the resulting liqueur and reuse of the IL cannot be easily accomplished. *In situ* enzymatic saccharification of pure cellulose, leading to 80% yields, was carried out using aqueous 1-ethyl-3-methyl imidazolium dimethylphosphate as pre-treating IL, but the IL was not reused and the lignin was not recovered (He *et al.* 2011). An interesting proposal was the formation of complexes of the sugars with phenylboronic or naphthalene-2-boronic acid (Brennan *et al*. 2010). These complexes can be isolated by solvent extraction, but is not likely that such a process can be economically feasible. Moreover, it is not acceptable to lose almost 50% of the enzymatic activity, as happens in the [Emim][Ac] medium. Therefore, we selected an alternative procedure for cellulose reprecipitation and for recovering the IL after the biomass pretreatment step. The difficulties caused by the high viscosity of the IL can be overcome with the addition of an organic co-solvent, which is miscible with the IL. Such a solvent also must have a high boiling point for it to be fully recovered with the IL after cellulose regeneration. Among several organic solvents tested, dimethyl sulfoxide (DMSO) met these criteria. The effectiveness of dipolar aprotic co-solvents, such as DMSO, for dissolution of cellulose have already been reported in derivatisation reactions for cellulose (Fort *et al.* 2006; Gericke *et al.* 2012). The viscosity of cellulose/IL solutions significantly decreased with the amount of co-solvent added (Gericke *et al.* 2012). Therefore, pretreatment of poplar biomass was carried out in a DMSO/[Emim][Ac] co-solvent system. He *et al*. have also used DMSO in addition to IL and water, but in very small amounts (about 1%) (He *et al.* 2011). In this study, the role of DMSO was to facilitate the recovery of the IL; therefore its relative content in the pretreatment mixture was much higher. A mixture of 80% pure microcrystalline cellulose (Avicel PH101) and 20% kraft lignin was used as a reference sample, to emphasize the more difficult recovery of cellulose and lignin owed to the presence of other components in the real sample.

Increasing pretreatment time and temperature obviously led to improved cellulose dissolution, but it also enhanced its thermal degradation. In a preliminary experiment, 50 mg of Avicel PH101 microcrystalline cellulose was added to 1 mL of [Emim][Ac] and incubated at 90 °C with stirring. The cellulose was completely dissolved after 30 min. Increasing the incubation time led to a brownish discoloration of the sample, and after 10 h of reaction, the sample turned dark brown in color. Following 6 h of incubation at 90 °C, 97% of the initial cellulose amount was recovered from the ionic liquid solution by

precipitation with water, as compared to only 80% cellulose recovered after 8 h of incubation. Therefore, the pretreatment temperature and operation time were set as 90 °C and 6 h, respectively. An equal volume mixture of [Emim][Ac] and DMSO was chosen for the dissolution of cellulose and lignin. At the end of the pretreatment process, the non-dissolved material was separated by filtration. Because accumulation of lignin in the pretreatment solution was not desirable, the lignin that still remained dissolved in the DMSO/[Emim][Ac] mixture after the recovery of cellulose was partially precipitated with excess water at low temperature. The lignin dissolved in each cycle was calculated as the difference between the total lignin content and the lignin remained in the solution after the previous cycle. The DMSO/[Emim][Ac] solution was easily recovered and reused after each recycle.

Table 2. Recovery of Cellulose and Lignin Following Pretreatment at 90 °C for 6 h in an Equal Volume Mixture of DMSO and [Emim][Ac] after Repeated Recycles

Number of recycles	Regenerated cellulose[a] (%)		Dissolved lignin content[b] (%)		Regenerated lignin[c] (%)	
	Reference sample	Poplar	Reference sample	Poplar	Reference sample	Poplar
0	98.9	56.3	94.5	22.9	32.4	25.9
1	95.0	52.4	95.9	18.0	36.0	23.4
2	85.4	36.9	87.3	5.6	30.9	29.9
3	70.7	29.7	86.0	3.2	31.2	15.5
4	63.8	29.4	82.3	3.0	29.3	20.5

[a]Regenerated cellulose (%) is reported with respect to the initial cellulose content in the reference mixture and poplar, respectively
[b]Dissolved lignin content (%) is reported with respect to the total lignin present in the sample
[c]Regenerated lignin (%) is reported with respect to total dissolved lignin

The results of the pretreatment experiments (Table 2) showed that in the initial cycle (recycle number = 0) near complete recovery of Avicel PH101 cellulose (cellulose reference sample) and kraft lignin (dissolved lignin reference sample) was achieved, whereas only 56.3% of the cellulose and 22.9% of the lignin were recovered from the poplar sample. Tian *et al.* (2011) reported that microcrystalline cellulose was completely dissolved in a co-solvent of 1-allyl-3-methylimidazolium chloride ([Amim][Cl]) and DMSO where the [Amim][Cl] comprised 0.3 or higher molar fraction of the organic electrolyte solution. At the first recycle of the DMSO/[Emim][Ac] solution (Table 2), the effectiveness slightly decreased. Following the fourth recycle of the DMSO/[Emim][Ac] solution, only 63.8% cellulose from the reference mixture sample and 29.3% from the poplar sample were recovered. These results were better than the 28% cellulosic material recovered from poplar biomass dissolved in [Bmim][Cl]/DMSO after a single 6-h cycle at 100 ºC (Fort *et al.* 2006). The recovered lignin amount from both the reference sample (*i.e.*, kraft lignin) and the poplar sample decreased after each reuse of the pretreatment mixture. Following the fourth recycle of the DMSO/[Emim][Ac] pretreatment mixture, approximately 29% lignin was recovered from the reference sample versus 20% lignin recovered from the poplar sample.

Utilisation of the DMSO/[Emim][Ac] mixture as a pretreatment agent has several advantages: lower co-solvent viscosity, easier mixing, easier separation of non-dissolved material, and greater reuse of co-solvent solution. However, the initially colorless DMSO/[Emim][Ac] mixture became more intensely colored and more viscous after each

recycle, which was due to the accumulation of lignin and different degradation products. This accumulation in the recycled co-solvent reduced its ability to dissolve new biomass material after repeated cycles. Sun *et al.* (2009) observed improved dissolving capacity at longer pretreatment times, in addition to the partial degradation of the dissolved cellulose and the [Emim][Ac] ionic liquid. An additional cleaning step of the pretreatment mixture would be necessary if this potential process is scaled-up to a commercial operation.

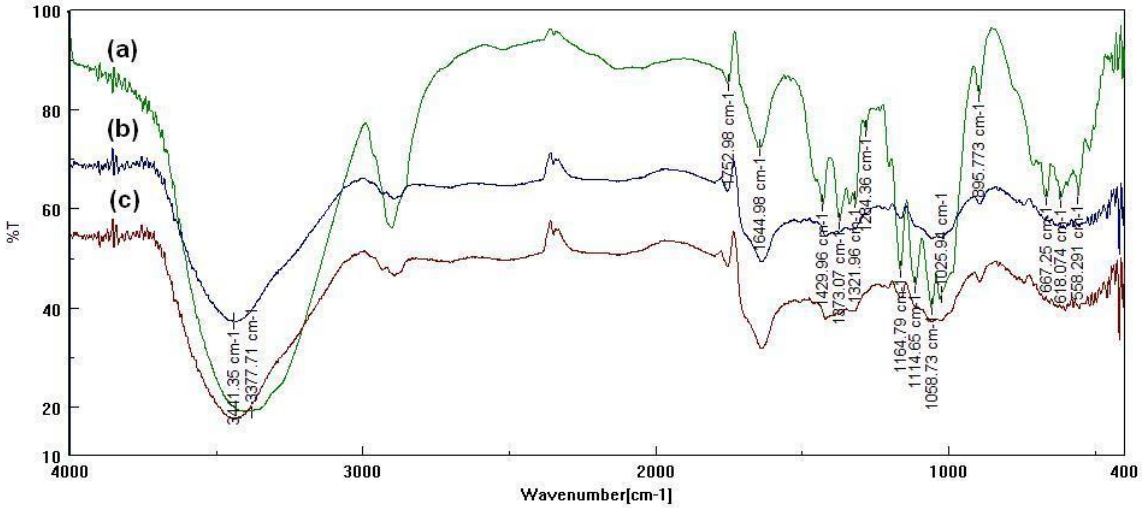

Fig. 3. FT-IR spectra of the IL pretreated celluloses: (a) native Avicel PH101 cellulose (untreated reference); (b) cellulose recovered from the reference mixture; and (c) cellulose recovered from poplar biomass

The regenerated cellulose was characterised by FT-IR spectrophotometry (Fig. 3). The FT-IR spectra of the recovered cellulose showed only small differences compared to the standard cellulose. These differences can be partially attributed to the decrease of cellulose crystallinity after the dissolution in the DMSO/[Emim][Ac] co-solvent. Decrease of crystallinity of cellulose as a consequence of dissolution and regeneration in the IL was previously demonstrated for microcrystalline cellulose regenerated from [Emim]Ac (Casas *et al.* 2012). Figure 3 shows that several of the absorption bands broadened, such as the in-plane HO-C bond of alcohol groups at 1429 cm^{-1}, the in-plane OH deformation at 1373 cm^{-1}, and the CH_2 vibration band at 1321 cm^{-1}, in the spectra of the regenerated celluloses of the reference and the poplar sample. The absorption band at 3377 cm^{-1}, which was assigned to intra-molecular hydrogen bonds in the untreated standard cellulose, was replaced by a narrower band in the spectra of the regenerated celluloses at 3441 cm^{-1}. Other differences between the regenerated celluloses and the untreated standard cellulose were observed at 1164 cm^{-1} (asymmetric C-O-C stretching), 1114 cm^{-1} (glucose ring stretching), and 1058 cm^{-1} (C-O stretching). These spectral modifications of the regenerated cellulose were in accordance with previous reports (Casas *et al.* 2012). It looks plausible that, as a consequence of pretreatment with the IL, some of the inter- and intramolecular hydrogen bonds were disrupted, decreasing the crystallinity of cellulose and leading to a more disordered structure. This conclusion is sustained by other reports, as Avicel cellulose regenerated from several chloride- and acetate-based ILs (without organic solvent) was 58 to 75% less crystalline than the untreated one, based on lateral order index calculations (Zhao *et al.* 2009) .

Influence of IL Pretreatment on Enzymatic Hydrolysis of Cellulose by Native and Sol-Gel Immobilised Cellulase

The regenerated cellulose, obtained as described before from the standard cellulose-lignin mixture and from poplar samples, was subjected to enzymatic hydrolysis using native and sol-gel immobilised cellulase. Efficient immobilisation of the cellulase using the sol-gel method was reported in our previous paper (Ungurean *et al*. 2013); however, the utilisation of such a biocatalyst for the hydrolysis of IL pretreated poplar wood has not yet investigated. The enzymatic reactions were monitored by measuring the total reducing sugars produced at different intervals over 24 h (Fig. 4). The degree of hydrolysis was calculated by considering that 1 g of cellulose yields 1.19 g of glucose when fully hydrolysed.

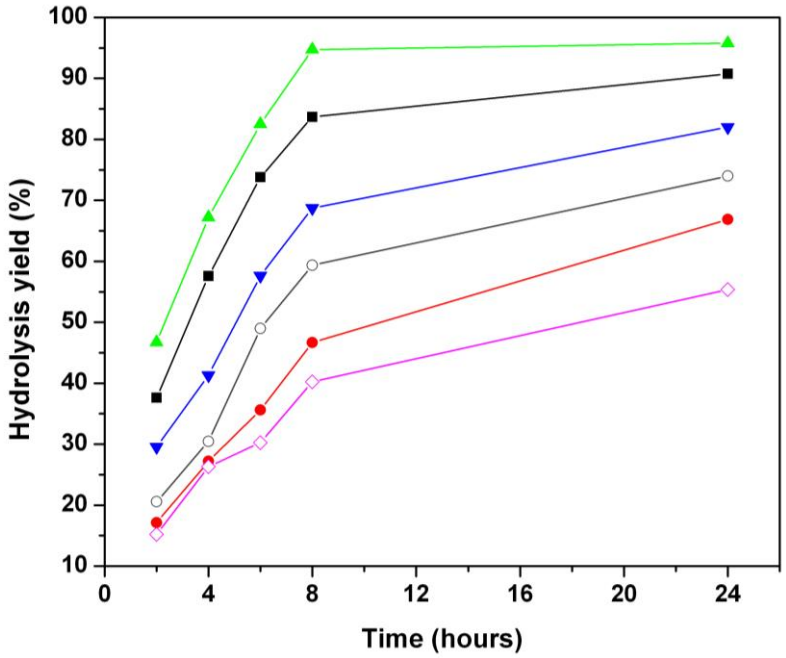

Fig. 4. Time course of enzymatic hydrolysis of 5 mg/mL of cellulose, catalysed by native and immobilised cellulases. Symbols: -▲- pretreated standard (Avicel) cellulose, native cellulase; -■- pretreated standard cellulose, immobilised cellulase; -▼- microcrystalline standard cellulose, native cellulase; -●- microcrystalline standard cellulose, immobilised cellulase; -○- cellulose from poplar biomass, native cellulase; and -◊- cellulose from poplar biomass, immobilised cellulase

Throughout the studied period, the amount of released sugars increased, regardless of the type of cellulose used. The hydrolysis yield was significantly increased after the IL ([Emim][Ac]) pretreatment, due to an increased accessibility of the cellulose surface, which enabled the cellulase to be more efficient. The cellulase hydrolysis yield from the pretreated Avicel cellulose increased by 26% when compared to the yield from the untreated microcrystalline cellulose (94% *vs*. 69% at 8 h, respectively). Utilisation of the immobilised cellulase resulted in lower hydrolysis yields than the native cellulase; however, the increase of the reaction rate following the IL pretreatment was remarkable. The hydrolysis yield increased by 38% when subjected to the same 8-h hydrolysis time, which indicated that it almost doubled for the pretreated cellulose (84% *vs*. 46%). As expected, the hydrolysis yields of a real lignocellulosic substrate (*i.e*., pretreated poplar) were lower compared to the pure microcrystalline cellulose; however, these results were significant and very promising.

The most important objective of our study was to demonstrate the possibility of the hydrolysis of pretreated poplar biomass using cellulase immobilised onto a sol-gel. The obtained yields, approximately 40% at an 8-h reaction time and 50% at a 24-h reaction time, certify that further optimisation of this process (*e.g.*, enzyme-substrate ratio, temperature, and pH) will allow almost quantitative hydrolysis of the pretreated biomass. The lower hydrolysis rate, when compared to the native enzyme, can be explained by the increased mass transfer resistance of a high molecular weight substrate, such as cellulase, into the pores of the silica sol-gel matrix. However, this resistance is compensated by the better operational stability and multiple reuse of the immobilised cellulase.

Hydrolysis of IL Pretreated Cellulose by Sol-Gel Immobilised Cellulose in Multiple Reaction Cycles

One of the main advantages of immobilised enzymes is its multiple reuses, thus increasing its productivity. In this study, sol-gel immobilised cellulase was repeatedly used for the hydrolysis of cellulose after its recovery from IL pretreatment. Following every reaction cycle, the immobilised enzyme was easily separated, washed, and subjected to a new hydrolysis cycle with the same amount of pretreated substrate. As shown in Fig. 4, the pretreated cellulose was more efficiently hydrolysed compared to the microcrystalline cellulose. This increased hydrolysis capability had a positive influence on the operational stability of the immobilised enzyme after multiple reuses. The results, expressed in Fig. 5 as the relative activities *versus* the initial run (Cycle No. 1), showed the preservation of approximately 30% of the enzymatic activity after eight reuses (Cycle No. 9).

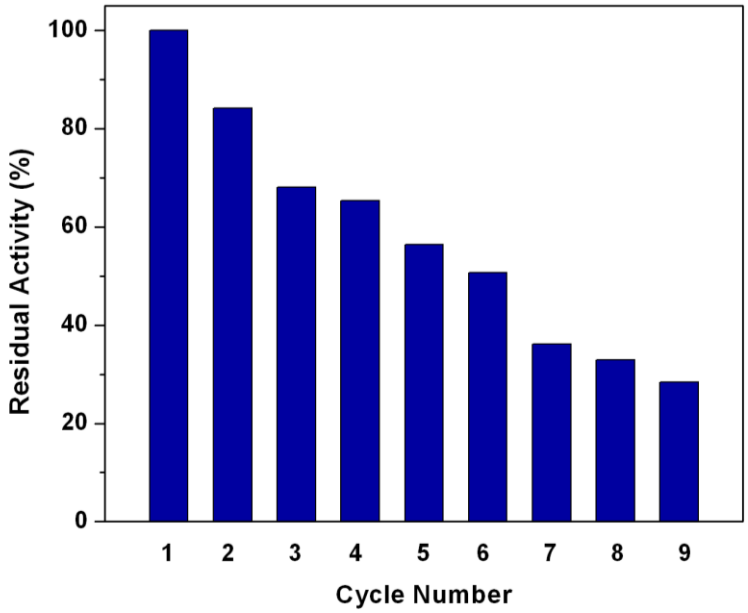

Fig. 5. Repeated reuses of the cellulase immobilised by sol-gel method for the hydrolysis of IL pretreated Avicel cellulose (5 mg/mL), at pH 4.8, 50 °C, and 8-h reaction time. Residual activities after each recycle were calculated relative to the initial run (*i.e.*, Cycle No. 1)

The decrease of the residual activity of immobilised cellulases after each reuse has also been observed by other investigators (Dincer and Telefoncu 2007; Li *et al.* 2007), and can be explained by the limited stability of cellulase in aqueous solution.

Using cross-linked cellulase as biocatalyst, the immobilized enzyme was reused five times, the glucose yield decreasing to 42% of the first run value (Jones and Vaseduvan 2010). The present results represent an improvement compared to our previous report as well, when we observed 20% residual activity after 6 hydrolysis cycles, for the hydrolysis of untreated Avicel substrate catalysed by sol-gel immobilised cellulase (Ungurean *et al.* 2013). The increase of reuse cycle numbers means increased enzyme productivity, *e.g.* a higher amount of fermentable sugars produced by a certain amount of enzyme, suggesting possible large-scale applications of this biocatalyst.

CONCLUSIONS

1. Pretreatment of poplar biomass using [Emim][Ac] ionic liquid and DMSO as co-solvent (1:1 ratio, v/v) was successfully demonstrated, which allowed the recovery of both cellulose and lignin in a form that facilitates their subsequent processing.
2. Cellulose with a lower crystallinity and unmodified lignin were easily precipitated from the solution, while the pretreatment mixture was recovered and reused several times. However, the dissolution capacity of the pretreatment solvent decreased after each reuse because of the accumulation of lignocellulosic fragments in the recycled solution.
3. All ILs tested in this study partially inactivated the cellulase enzyme; using these ILs as the reaction medium for the cellulose hydrolysis step was not considered the best option. Instead, the separation of the regenerated cellulose prior to the enzymatic hydrolysis was applied.
4. The sol-gel immobilised cellulase displayed improved operational stability in the aqueous solution when compared to the native enzyme. Cellulase immobilised by the sol-gel method proved to be an efficient biocatalyst for the conversion of IL pretreated cellulose to glucose. The enzymatic hydrolysis rate of the regenerated cellulose was much higher when compared to the microcrystalline cellulose.
5. Cellulase immobilised by the sol-gel was reused eight times on the pretreated cellulose substrate while preserving approximately 30% of its activity. Future experiments will be carried out to optimise both the stability and reusability of this sol-gel immobilised cellulase on different wood biomass samples.

ACKNOWLEDGMENTS

This work was partially supported by the strategic grant POSDRU 2009, project ID 50783, of the Ministry of Labour, Family and Social Protection, co-financed by the European Social Fund - Investing in People. We acknowledge the support of the Hungarian State and the European Union under the TÁMOP-4.2.2.A-11/1/KONV-2012-0071 and TÁMOP-4.2.1-08/1-2008-0007 projects.

REFERENCES CITED

Abd El-Ghaffar, M. A., and Hashem, M. S. (2010). "Chitosan and its amino acids condensation adducts as reactive natural polymer supports for cellulase immobilization," *Carbohyd. Polym.* 81(3), 507-516.

Agbor, V. B., Cicek, N., Sparling, R., Berlin, A., and Levin, D. B. (2011). "Biomass pretreatment: Fundamentals toward application," *Biotechnol. Adv.* 29(6), 675-685.

Brennan, T. C. R., Datta, S., Blanch, H. W., Simmons, B. A., and Holmes, B. M. (2010). "Recovery of sugars from ionic liquid biomass liquor by solvent extraction," *BioEnergy Res.* 3(2), 123-133.

Brown, T. R., and Brown, R. C. (2013). "A review of cellulosic biofuel commercial-scale projects in the United States," *Biofuel. Bioprod. Bioref.* 7(3), 235-245.

Bylin, S., Olsson, C., Westman, G., and Theliander, H. (2014). "Solvation of cellulose and xylan in the MIM/EMIMAc ionic liquid solvent system: Parameters for small-scale solvation," *BioResources* 9(1), 1038-1054.

Casas, A., Palomar, J., Alonso, M. V., Oliet, M., Omar, S., and Rodriguez, F. (2012). "Comparison of lignin and cellulose solubilities in ionic liquids by COSMO-RS analysis and experimental validation," *Ind. Crops Prod.* 37(1), 155-163.

Dincer, A., and Telefoncu, A. (2007). "Improving the stability of cellulase by immobilization on modified polyvinyl alcohol coated chitosan beads," *J. Mol. Catal. B. - Enzym.* 45(1-2), 10-14.

Engel, P., Mladenov, R., Wulfhorst, H., Jager, G., and Spiess, A. C. (2010). "Point by point analysis: How ionic liquid affects the enzymatic hydrolysis of native and modified cellulose," *Green Chem.* 12(11), 1959-1966.

Fort, D. A., Remsing, R. C., Swatloski, R. P., Moyna, P., Moyna, G., and Rogers, R. D. (2007). "Can ionic liquids dissolve wood? Processing and analysis of lignocellulosic materials with 1-*n*-butyl-3-methylimidazolium chloride," *Green Chem.* 9(1), 63-69.

Freire, M. G., Santos, L. M. N. B. F., Fernandes, A. M., Coutinho, J. A. P., and Marrucho, I. M. (2007). "An overview of the mutual solubilities of water–imidazolium-based ionic liquids systems," *Fluid Phase Equilibr.* 261(1-2), 449-454.

Gericke, M., Fardim, P., and Heinze, T. (2012). "Ionic liquids - Promising but challenging solvents for homogeneous derivatization of cellulose," *Molecules* 17(6), 7458-7502.

Ghose, T. K. (1987). "Measurements of cellulase activities," *Pure Appl. Chem.* 59(2), 257-268.

Groff, D., George, A., Sun, N., Sathitsuksanoh, N., Bokinsky, G., Simmons, B.A., Holmes, B. M., and Keasling, J. D. (2013). "Acid enhanced ionic liquid pretreatment of biomass," *Green Chem.* 15(5), 1264-1267.

Gusakov, A.V. (2013). "Cellulases and hemicellulases in the 21st century race for cellulosic ethanol," *Biofuels* 4(6), 567-569.

He, Y. C., Ma, C. L., Xia, D. Q., Ding, L., Li, L. Z., and Mu, X. (2011). "Enzymatic saccharification of cellulose in aqueous-ionic liquid 1-ethyl-3-methylimidazolium dimethylphosphate-DMSO media," *Chem. Papers* 65(6), 792–-797.

Ioelovich, M., and Morag, M. (2012). "Study of enzymatic hydrolysis of mild pretreated lignocellulosic substrates," *BioResources* 7(1) 1040-1052.

Jones, P. O., and Vasudevan, P. T. (2010). "Cellulose hydrolysis by immobilized *Trichoderma reesei* cellulase," *Biotechnol Lett.* 32(1), 103-106.

Karatzos, S. K., Edye, L. A., and Wellard, R. M. (2012). "The undesirable acetylation of cellulose by the acetate ion of 1-ethyl-3-methylimidazolium acetate," *Cellulose* 19(1), 307-312.

Lee, S. H., Doherty, T. V., Linhardt, R. J., and Dordick, J. S. (2008). "Ionic liquid-mediated selective extraction of lignin from wood leading to enhanced enzymatic cellulose hydrolysis," *Biotechnol. Bioeng.* 102(5), 1368-1375.

Li, C., Yoshimoto, M., Fukunaga, K., and Nakao, K. (2007). "Characterization and immobilization of liposome-bound cellulase for hydrolysis of insoluble cellulose," *Bioresource Technol.* 98(7), 1366-1372.

Lozano, P., Bernal, B., Bernal, J. M., Pucheault, M., and Vaultier, M. (2011). "Stabilizing immobilized cellulase by ionic liquids for saccharification of cellulose solutions in 1-butyl-3-methylimidazolium chloride," *Green Chem.* 13(6), 1406-1411.

Lozano P., Bernal B., Recio I., and Belleville M.-P. (2012). "A cyclic process for full enzymatic saccharification of pretreated cellulose with full recovery and reuse of the ionic liquid 1-butyl-3-methylimidazolium chloride," *Green Chem.* 14(9), 2631-2637.

Maki-Arvela, P., Anugwom, I., Virtanen, P., Sjoholm, R., and Mikkola, J. P. (2010). "Dissolution of lignocellulosic materials and its constituents using ionic liquids - A review," *Ind. Crops Prod.* 32(3), 175-201.

Miller, G. L. (1959). "Use of dinitrosalicylic acid reagent determination of reducing sugar," *Anal. Chem.* 31(3), 426-428.

Moniruzzaman, M., Nakashima, K., Kamiya, N., and Goto, M. (2010). "Recent advances of enzymatic reactions in ionic liquids," *Biochem. Eng. J.* 48(3), 295-314.

Mood, S. H., Golfeshan, A. H., Tabatabaei, M., Jouzani, G. S., Najafi, G. H., Gholami, M., and Ardjmand, M. (2013). "Lignocellulosic biomass to bioethanol. A comprehensive review with a focus on pretreatment," *Renew. Sust. Energ. Rev.* 27(11), 77-93.

Nguyen, T.-A. D., Kim, K.-R., Han, S. J., Cho, H. Y., Kim, J. W., Park, S. M., Park, J. C., and Sim, S. J. (2010). "Pretreatment of rice straw with ammonia and ionic liquid for lignocellulose conversion to fermentable sugars," *Bioresource Technol.* 101(19), 7432-7438.

Ogeda, T. L., Silva, I. B., Fidale, L. C., El Seoud, O. A., and Petri, D. F. S. (2012). "Effect of cellulose physical characteristics, especially the water sorption value, on the efficiency of its hydrolysis catalyzed by free or immobilized cellulase," *J. Biotechnol.* 157(1), 246-252.

Olivier-Bourbigou, H., Magna, L., and Morvan, D. (2010). "Ionic liquids and catalysis: Recent progress from knowledge to applications," *Appl. Catal. A: General* 373(1-2), 1-56.

Pinkert, A., Goeke, D. F., Marsh, K. N., and Pang, S. (2011). "Extracting wood lignin without dissolving or degrading cellulose: Investigations on the use of food additive-derived ionic liquids," *Green Chem.* 13(11), 3124-3136.

Pinkert, A., Marsh, K. N., Pang, S., and Stainger, M. P. (2009). "Ionic liquids and their interaction with cellulose," *Chem. Rev.* 109(12), 6712-6728.

Saxena, R. C., Adhikari, D. K., and Goyal, H. B. (2009). "Biomass-based energy fuel though biochemical routes: A review," *Renew. Sust. Energ. Rev.*13(1), 167-178.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., and Crocker, D. (2008). "Determination of structural carbohydrates and lignin in biomass, laboratory analytical procedure (LAP)," Technical Report NREL/TP-510-42618, National

Renewable Energy Laboratory (NREL), U.S. Dept. of Energy, Golden, CO (http://www.nrel.gov/biomass/pdfs/42618.pdf).

Sun, N., Rahman, M., Qin, Y., Maxim, M. L., Rodríguez, H., and Rogers, R. D. (2009). "Complete dissolution and partial delignification of wood in the ionic liquid 1-ethyl-3-methylimidazolium acetate," *Green Chem.* 11(5), 646-655.

Sun, N., Jiang, X., Maxim, M. L., Metlen, A., and Rogers, R. D. (2011). "Use of polyoxometalate catalysts in ionic liquids to enhance the dissolution and delignification of woody biomass," *ChemSusChem* 4(1), 65-73.

Tian, X., Fang, Z., Jiang, D., and Sun, X. (2011). "Pretreatment of microcrystalline cellulose in organic electrolyte solutions for enzymatic hydrolysis," *Biotechnol. Biofuels* 4(11), 53-67.

Turner, M. B., Spear, S. K, Huddleston, J. G., Holbrey, J. D., and Rogers, R. D. (2003). "Ionic liquid salt-induced inactivation and unfolding of cellulase from *Trichoderma reesei*," *Green Chem.* 5(4), 443-447.

Ungurean, M, Paul, C., and Peter, F. (2013). "Cellulase immobilized by sol-gel entrapment for efficient hydrolysis of cellulose," *Bioproc. Biosyst. Eng.* 36(10), 1327-1338.

Ursoiu, A., Paul, C., Kurtán, T., and Péter, F. (2012). "Sol-gel entrapped *Candida antarctica* lipase B - A biocatalyst with excellent stability for kinetic resolution of secondary alcohols," *Molecules* 17(11), 13045-13061.

Wang, J., Zheng, Y., and Zhang, S. (2010). "The application of ionic liquids in dissolution and separation of lignocellulose, in clean energy systems and experiences," in: *Clean Energy Systems and Experiences*, K. Eguchi (ed.), InTech, Rijeka, Croatia, Chap. 4 (http://www.intechopen.com/books/clean-energy-systems-and-experiences/the-application-of-ionic-liquids-in-dissolution-and-separation-of-lignocellulose).

Zavrel, M., Bross, D., Funke, M., Büchs, J., and Spiess, A. C. (2009). "High-throughput screening for ionic liquids dissolving (ligno-) cellulose," *Bioresource Technol.* 100(9), 2580-2587.

Zhao, H., Jones, C. L., Baker, G. A., Xia, S., Olubajo, O., and Person, V. N. (2009). "Regenerating cellulose from ionic liquids for an accelerated enzyme hydrolysis," *J. Biotechnol.* 139(1), 47-54.

Zheng, Y., Pan, Z., and Zhang, R. (2009). "Overview of biomass pretreatment for cellulosic ethanol production," *Int. J. Agric. Biol. Eng.* 2(3), 51-68.

Article submitted: March 26, 2014; Peer review completed: June 15, 2014; Revised version received: August 8, 2014; Accepted: August 9, 2014; Published: August 21, 2014.