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# Simultaneous pretreatment and enzymatic saccharification of (ligno) celluloses in aqueous-ionic liquid media: A compromise

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ABSTRACT

In view of decreasing the amount of IL to achieve efficient simultaneous pretreatment and saccharification, a comprehensive study was undertaken. Different types of lignocellulosic biomasses were investigated in various enzymatic aqueous-IL systems including 1-ethyl-3-methylimidazolium acetate ([C2mim][OAc]) or 1-ethyl-3-methylimidazolium methylphosphonate ([C2mim][MeO(H)PO2]). To better understand how 10% (v/v) of IL in the reaction medium led to the highest yields of glucose without fractionation from sawdust, distinct cellulosic models were then used as substrates. Kinetic studies demonstrated that both ILs affect synergistic action of cellulolytic enzymes depending on both constitutive anion and cellulosic substrate. Concentrations above 10% v/v of ILs deactivated cellulase even on highly digestible model substrates. β and α Kamlet-Taft parameters constituted more physicochemical pertinent indicators than apparent pH value to investigate effects of IL on cellulase performances. Fine description of these effects was proposed onto individual EG, CBH and BG. [C2mim][MeO(H)$PO_2$] was demonstrated a better compatible IL for enzymes up to a concentration of 30% (v/v). The efficiency of simultaneous pretreatment and saccharification was governed by a compromise between better substrate accessibility and enzyme deactivation.



## 1. Introduction

Lignocellulosic biomasses (LCBs) such as dedicated crops, agricultural and wood residues represent promising renewable bioresources for the production of chemicals and biofuels [1]. A promising way to release glucose monomers from constitutive cellulose is the cellulase-catalyzed hydrolysis usually carried out in buffered aqueous media. Some advantages are the use of mild conditions (low temperature process and no fermentation inhibitors), the specificity of reaction and thus, the decrease in by-products production [2]. Cellulase are glycoside hydrolases which act in synergy to hydrolyze β-1,4 glycosidic bonds of the cellulose chains. This enzyme mixture is composed of (i) endoglucanases (EG, EC 3.2.1.4) which would preferentially act on amorphous regions thus randomly generating new cellulose chains extremities and oligo/polysaccharides; (ii) cellobiohydrolases (CBH, EC 3.2.1.91) which hydrolyze free chains extremities in a processive manner, resulting mainly in cellobiose release and (iii) β-glucosidases (BG, EC 3.2.1.21) which cleave cellobiose into glucose [3,4]. EG and CBH act mainly on the insoluble cellulosic substrate after adsorption while BG acts preferentially on soluble substrate constituting a synergistic system with both heterogeneous and homogenous catalyses [4]. However, enzymatic activity is constrained by the recalcitrant complex structure of lignocellulose, requiring thus a preliminary pretreatment step [5].

Pretreatment of LCB by hydrophilic ionic liquids (ILs) prior to cellulase-catalyzed hydrolysis are now recognized as attractive to decrease the recalcitrance of cellulosic and lignocellulosic substrates to cellulase [6–12]. These solvents are defined as organic salts with interesting physico-chemical properties such as thermal stability, low vapor pressure, and ability to solubilize both carbohydrate and lignin polymers under mild conditions for imidazolium-based ILs with low toxicity for some of them [13–16]. Moreover, recent studies demonstrated: (i) the feasibility to recycle ILs without loss of pretreatment efficiency [9,17,18], (ii) the compatibility between ILs and microorganisms leading to consider integrated processes for biorefinery [19,20].

The dissolution of pure cellulose in ILs would be induced by the formation of electron donor – electron acceptor complexes between constitutive hydroxyl groups of cellulose and IL [21]. These interactions may lead to the disruption of its inter- and intra-hydrogen bond network during the pretreatment and allow

*Abbreviations:* LCB(s), lignocellulosic biomass(es); IL, ionic liquid; [C2mim][OAc], 1-ethyl-3-methylimidazolium acetate; [C2mim][MeO(H)$PO_2$], 1-ethyl-3-methylimidazolium methylphosphonate; EG, endoglucanases; CBH, cellobiohydrolases; BG, β-glucosidases.

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**Table 1**
Chemical compositions of lignocellulosic substrates [a].

| Lignocellulosic biomass | Cellulose | Lignin | Xylose | Arabinose | Extractives |
|---|---|---|---|---|---|
| Spruce sawdust | 55.4 ± 1.3 | 28.7 ± 1.0 | 4.2 ± 0.4 | 1.4 ± 0.2 | 3.3 ± 1.0 |
| Oak sawdust | 44.7 ± 0.3 | 26.7 ± 0.8 | 14.8 ± 1.3 | 1.2 ± 0.1 | 4.6 ± 1.1 |
| *Miscanthus* x *giganteus* | 44.8 ± 0.2 | 23.8 ± 2.7 | 22.3 ± 1.7 | 3.4 ± 0.9 | ND |

[a] Compositions are expressed as percentage of the residues. ND: not determined.

increasing enzymatic digestibility [17,22]. When applied to LCB the disorganization occurred similarly between the components leading again to a more accessible cellulosic fraction [9,10,23]. Although some studies reported compatible IL-cellulase systems, high concentration of ILs in the hydrolysis medium induced total deactivation of cellulases [24–28]. For these reasons, pretreatment by IL of LCB is usually separated from its enzymatic saccharification by regeneration and washing steps with polar solvent [29,30]. This multi-steps strategy may lead to technical and economical drawback for the transposition at larger scale in integrated biorefinery process [31,32]. In this context, *in situ* enzymatic saccharification in aqueous-IL media could thus represent an alternative pathway for avoiding regeneration step of pretreated substrate and decreasing the amounts of ILs and polar solvents required. Kamiya et al. introduced firstly this concept for pure cellulose as substrate in aqueous-IL medium with the cellulase from *Trichoderma reesei* [33]. To date, literature data presented mainly feasibility studies with model cellulosic substrates or LCBs in various aqueous-IL media and proposed for example optimization approaches in including enzyme protection from IL by immobilization [24,25,27,30,34–36].

The aim of this work is to present a complementary vision on the behavior of cellulase and its isolated monocomponents in non-conventional media with different substrate sources. In this context we propose to investigate the cellulase-catalyzed saccharification of different lignocellulosic and cellulosic model substrates through an approach combining a chemical and structural characterization of substrates and enzyme kinetic study in non-conventional media. Two distinct hydrophilic ILs were chosen: 1-ethyl-3-methylimidazolium acetate ([C2mim][OAc]) and 1-ethyl-3-methylimidazolium methylphosphonate ([C2mim][MeO(H)$PO_2$]) due to their efficiency in separated IL pretreatment of LCB [10,37,38]. To our knowledge, [C2mim][MeO(H)$PO_2$] was never studied for simultaneous IL pretreatment and enzymatic saccharification of lignocellulosic biomasses. First, *in situ* enzymatic saccharification in aqueous-IL media of three representative LCBs including softwood and hardwood residues (industrial sawdusts from spruce and oak) and dedicated crops (*Miscanthus* x *giganteus*) catalyzed by the cellulase from *Trichoderma reesei* was investigated. We performed then kinetic study of cellulase-catalyzed saccharification in presence of ILs on two model cellulosic substrates: (i) α-cellulose, a well-defined biomimetic model of wood cellulose and (ii) long fibers of cellulose from cotton, distinct by their respective enzymatic digestibility after separated IL pretreatment [22]. To ascertain effect of the IL between the substrates and the enzymes, the direct impact of each IL was assessed by distinguishing the pretreatment step from enzymatic saccharification. Thus, highly digestible celluloses, *ie* IL pretreated celluloses, were used as substrate for enzymatic sacharification in aqueous-IL media. Finally, influences of ILs-interactions onto three isolated cellulolytic enzymes (EG, CBH and BG) were examined.

## 2. Materials and methods

### 2.1. Raw materials and chemical composition

Two distinct model celluloses from Sigma-Aldrich (Steinheim, Germany) were selected: 1) α-cellulose extracted from wood by alkaline treatment (containing insoluble fibrous residues and both xylan and mannan residual units) and 2) cotton cellulose exhibiting a high purity degree (sizes ranging from 125 to 400 μm *i.e.* long fibers of cellulose). Industrial oak (*Quercus petra)* and spruce *(Picea abies*) residues were provided by the forest industry SARL Husson Paul (Bathelémont, France). *Miscanthus* x *giganteus* was supplied by UMR FARE (UMR 614 INRA URCA, France). LCBs were milled with a planetary ball miller (Retsch PM 400) for 20 min at 300 rpm to achieve a size of less than 0.8 mm, then freeze-dried. Chemical composition of the three representative biomasses were determined according the procedures described in our previous works [9,10] (Table 1). SEM analyses of each LCB were presented in Supplementary data 1.

### 2.2. Chemicals and enzymes

Ionic liquids, 1-ethyl-3-methylimidazolium acetate ([C2mim][OAc], >98%) and 1-ethyl-3-methylimidazolium methylphosphonate ([C2mim][MeO(H)$PO_2$], >98%) were purchased from Solvionic SA (Verniolle, France). ILs were stored under vacuum in a desiccator to limit water absorption. Some physicochemical properties of these ILs are reported in Supplementary data S2.

Cellulase from *Trichoderma reesei* (EC 3.2.1.4) were supplied by Sigma-Aldrich (Steinheim, Germany) and present a specific activity of 5 U/mg; one unit liberates 1.0 μmole of glucose from cellulose in one hour at pH 5.0 at 37 °C (2 h of incubation). The Cellobiohydrolases I (CBH I, EC 3.2.1.4) and endoglucanases II (EG II, EC 3.2.1.91) from *Trichoderma* sp. were furnished by Megazyme (Wicklow, Ireland) and exhibit a specific activity of 68 U/mg and 0.05 U/mg, respectively. The β-Glucosidases from *Aspergillus niger* (BG, EC 3.2.1.21) was purchased from Fluka Sigma-Aldrich (Steinheim, Germany) with a specific activity of 66.6 U/g; one unit liberates 1.0 μmole of glucose from salicin in one minute at pH 5.0 at 37 °C.

### 2.3. Enzymatic saccharification of (ligno)celluloses in aqueous-IL media

Freeze-dried model cellulose or LCBs were suspended in various ILs volumes in Eppendorf tubes; the whole incubated at 110 °C (thermostated oil bath) under vigorous stirring for 40 min, and then cooled down in an ice bath. For the investigation of purified BG activity, cellobiose was used as substrate in the same reactive conditions. Following incubation, acetate buffer (50 mM, pH 4.8; qsp 900 μL) was then added in order to mimic a regeneration step (40 °C, 30 min in Eppendorf Thermomixer 5436) and enzymatic saccharification was directly initiated by addition of 100 μL of cellulase preparation. Final concentrations of substrates and ILs were 2% (w/v) and displayed in a range from 0 to 100% (v/v), respectively. *T. reesei* cellulase preparation and purified CBH I, EG II and BG present a final specific activity of 5.0; 0.005; 5.0 and 0.7 U/mL, respectively. Different assay controls were also carried out: aqueous buffer/substrate; IL (0–100% v/v)/substrate and aqueous buffer/substrate/thermally deactivated enzymes resulting in no reducing sugars release. The hydrolysis reaction was monitored over time by quantifying the reducing sugar concentration using DNS assay or HPAEC-PAD. Each reaction was repeated three