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Enzymatic in situ saccharification of lignocellulose in a compatible ionic liquid-cellulase system

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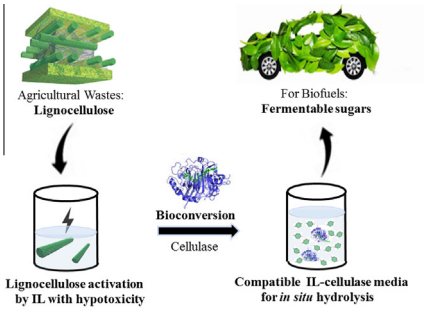
HIGHLIGHTS

- [Emim][DP] was effective to disrupt the shields formed by lignin inside rice straw.
- The cellulase from *Trichoderma aureoviride* showed high stability in [Emim][DP].
- The coupling of IL-activation and subsequent enzymatic hydrolysis process.
- An efficient IL-cellulase media for in situ enzymatic saccharification of biomass.

GRAPHICAL ABSTRACT



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ABSTRACT

Although ionic liquids (ILs) have emerged as a promising type of solvent for lignocellulose pretreatment, the enzymatic saccharification of regenerated lignocellulose must occur in a separate step due to the toxicity of ILs to cellulase. It is critical to develop a compatible IL-cellulase system in which the IL effectively activates the lignocellulosic biomass, while the enzyme remains highly stable. In this context, an exploration of ILs with high lignin-extraction capacity, for the first time showed 1-ethyl-3-methyl-imidazolium dimethylphosphate ([Emim][DP]) to be effective in disrupting the lignin-based shield within rice straw, thus enhancing the biodegradability of this plant material. The cellulase obtained from *Trichoderma aureoviride* showed high stability in this IL. After incubation in [Emim][DP] for 24 h, more than 40% of the lignin was successfully leached from the rice straw. The spectroscopic and morphological analyses showed that the synergistic effect of delignification and the partial dissolution of cellulose during the activation process significantly changed the crystalline molecular structure of rice straw. When the activated straw slurry was enzymatically hydrolyzed in IL diluted to 15% (w/v), a high yield of reducing sugars, 61%, was obtained. Thus, an efficient system coupled the activation and subsequent enzymatic hydrolysis of a native biomass in a one-pot procedure was successfully developed.

1. Introduction

Biofuels and chemicals produced using lignocellulosic feedstocks are important alternatives to conventional petroleum-derived products. However, lignocellulosic biomasses are recalcitrant to efficient enzymatic hydrolysis because of their structural complexity [1]. Lignocellulose comprises three main biopolymers, cellulose, hemicellulose, and lignin. Hemicellulose is relatively amorphous and can be readily degraded by glycosidases [2]. The cellulose within lignocellulose is highly crystalline, which protects it from biological degradation. Lignin, however, is a complex biopolymer composed of phenylpropanoid units, which serve as the "glue" that binds cellulose and hemicellulose, imparting rigidity and microbial resistance

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to lignocellulose [3]. Furthermore, due to its hydrophobicity, lignin prevents water from entering lignocellulose, thus restricting the efficacy of enzymatic hydrolysis [4]. Removing lignin is a prerequisite to counteract the recalcitrance of lignocellulose to digestion before the biomass can be further processed to produce biofuels.

Various types of lignocellulose pretreatments followed by a recovery process have been introduced to remove lignin and disrupt the crystallinity of the carbohydrate fraction to enhance their biodegradability. Nevertheless, the existing processes suffer from serious drawbacks, such as a high energy input and the generation of toxic byproducts [5]. More recently, ionic liquids (ILs) have emerged as a promising type of solvent for lignocellulose pretreatment through a "dissolution–regeneration process". The cellulose that was regenerated from the IL solution was found to be essentially amorphous and porous [6]. This approach has several advantages over conventional methods by being more environmentally friendly, and using solvents recognized as green chemicals [7]. However, in using this approach, enzymatic hydrolysis must be performed in a separate step after the regeneration process due to the toxicity of ILs to cellulase. Additionally, the excessive use of water and the necessary waste disposal associated with the process increase its overall cost and pose a challenge for scaling-up this technology.

To avoid an extensive clean-up step, the concept of "in situ saccharification" was previously posed. In this strategy, the IL-pretreatment and subsequent enzymatic hydrolysis are conducted in a one-pot procedure [8]. However, the inactivation of cellulase in the presence of most cellulose-dissolving ILs remains a serious obstacle to the use of this strategy. To implement this idea, it is necessary to find a type of IL that is not only efficient in increasing the porosity of cellulose, but is also enzyme-friendly. Unfortunately, recent investigations of ILs have focused particularly on those that are excellent for dissolving cellulose, which are likely to induce rapid enzyme deactivation, because those ILs are typically composed of anions (chloride [Cl^-], formate [$HCOO^-$], and acetate [CH_3COO^-]) that can form strong hydrogen bonds with cellulose microfibers, and protein molecules [9]. To overcome the above-described obstacle, an alternative method is to explore less polar ILs that are able to activate lignocellulose through delignification rather than dissolution of cellulose. Strong correlations between the digestibility of cellulose, lignin removal and the crystallinity index have been observed. Specifically, there is a good linear relationship (R^2 = 0.9618) between lignin removal and cellulose digestibility within a certain range [10]. It is noteworthy that the mechanism of lignin dissolution in ILs differs from that of cellulose. The cations in the lignin-dissolving ILs should exhibit the ability to interact with the π-systems of the aromatic units in lignin [11]. Furthermore, it is particularly fascinating that the anions of the IL useful for delignification are less polar than those required for cellulose dissolution [12], which makes the former type of IL more friendly to cellulase.

The traditional "lignocellulose pretreatment" procedure generally includes a recovery process prior to the subsequent enzymatic hydrolytic process. In this study, we adopted the distinctive term "activation". Our aim was to select ILs that could activate lignocellulose, mainly through delignification, to enhance its biodegradability, but would not negatively affect the activity of cellulase. Focusing first on lignin extractability, a wide range of potential ILs was screened to determine their capacity to leach lignin from rice straw. Based on the results, the stability of *Trichoderma aureoviride* cellulase in the selected IL solvents was investigated to determine their toxicity. Finally, 1-ethyl-3-methylimidazolium dimethylphosphate ([Emim][DP]), which showed the advantages of both good lignin-extraction capacity and hypotoxicity, was selected. This IL also possessed a certain ability to dissolve cellulose. After activating rice straw using [Emim][DP] and diluting the solution to a certain IL concentration, the slurry could be effectively in situ hydrolyzed using the *T. aureoviride* cellulase. Similar phosphate-based ILs have been used for lignocellulose pretreatment; however, no details concerning their delignification capacities have been reported. To better understand the activation mechanism of [Emim][DP], the changes in the content and distribution of lignin and the degree of cellulose crystallinity within rice straw were evaluated using Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD) and scanning electron microscopy (SEM).

2. Materials and methods

2.1. Materials

The ILs (99% purity) were purchased from the ChengJie Company (Shanghai, China). Rice straw was collected in Huaian, China. Carboxymethyl-cellulose sodium (CMC-Na), and 3,5-dinitrosalicylic acid (DNS) were purchased from Sigma–Aldrich (USA). Kraft lignin was kindly donated by Huadong Lignin Co., Ltd. Microcrystalline cellulose (MC) and filter paper were purchased from Aoke (China). *T. aureoviride* HS (NCBI No. KJ610807) is an IL-tolerant strain that was isolated by our group from samples of chemically polluted microhabitats located near old chemical plants.

2.2. Solubilities of Kraft lignin and cellulose in various ILs

Kraft lignin (2.5 g) was added to glass vials containing IL (5 mL) to evaluate the solubility of lignin in the different ILs. The resulting suspension was stirred for 12 h at 110 °C. Then, an additional 1.5 g of Kraft lignin was introduced, and the solution was stirred for another 12 h. The supernatant was removed after centrifugation for 5 min at 11,000 rpm. Using the corresponding IL as the control, the supernatant was diluted using 0.1 mol L^{-1} NaOH, and the lignin content was determined from the absorbance at 280 nm according to the standard-curve method. To determine the solubility of cellulose, certain amounts of an IL and MC were added to glass vials equipped with a magnetic stirrer and placed in a heating oil bath at 110 °C. The mixture was stirred for 24 h, and the solubility of cellulose was estimated using both visual inspection and microscopy.

2.3. Ability of various ILs to extract lignin ability from rice straw

Twelve ILs, 1-butyl-3-methyl-imidazolium formate ([Bmim][HCOO]), 1-ethyl-3-methylimidazolium acetate ([Emim][CH_3COO]), 1-ethyl-3-methylimidazolium methyl-phosphonate ([Emim][(MeO)HPO_2]), 1-allyl-3-methylimidazolium chloride ([Amim][Cl]), 1-ethyl-3-methylimidazolium dimethylphosphate ([Emim][DP]), 1-ethyl-3-methylimidazolium phosphinate ([Emim][H_2PO_2]), tris (2-hydroxyethyl)-methylammonium methylsulfate (HEMA), 1-ethyl-3-methylimidazolium methylsulfate ([Emim][$MeSO_4$]), 1-butyl-3-methylimidazolium hexafluorophosphate ([Bmim][PF_6]), 1-butyl-3-methylimidazolium chloride ([Bmim][Cl]), 1-butyl-3-methylimidazolium tetrafluoroborate ([Bmim][BF_4]), and 1-butyl-3-methylimidazolium trifluoromethane-sulfonate ([Bmim][OTF]), were screened for their delignification capacity. Briefly, rice straw was incubated in the above-mentioned ILs at a biomass loading of 10 wt% with stirring at 110 °C for 24 h. After the incubation, the suspension was diluted using 0.1 mol L^{-1} NaOH solution and was centrifuged at 11,000g for 10 min. The lignin content of the supernatant was determined from the absorbance at 280 nm. To determine the total lignin content of the untreated rice straw, 0.05 g of rice straw was added to 2 mL of [Emim][CH_3COO] and was incubated at 110 °C for 24 h. The fully dissolved rice straw solution was