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Communication

Cellulases in Ionic Liquids—The Long Term Stability of *Aspergillus* sp. Cellulase

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Abstract: It is a well known fact that some ionic liquids (ILs) have the potential to dissolve crystalline, water-insoluble cellulose that could be used as a source of glucose and subsequently derived molecules. Nevertheless, in the presence of high IL concentrations, cellulase activity and, even more so, cellulase stability are still challenging difficulties that need to be overcome. Therefore four fungal cellulase preparations were assayed in the presence of 30% (*v*/*v*) of five different ILs. Thereby the cellulase from *Aspergillus* sp. (Sigma-Aldrich) was not only remarkably active (up to 28 U/mg in 30% (*v*/*v*) IL ([BMMIM]Cl)), but furthermore stayed active during several weeks in the presence of 60% (*v*/*v*) IL (up to 50% relative activity after 9 weeks).

Keywords: ionic liquid; cellulase; *Aspergillus*

1. Introduction

The enzymatic conversion of cellulose into glucose, and subsequently biofuels, could provide a renewable and sustainable energy source. A major difficulty that needs to be mastered in this degradation process is the insolubility of crystalline cellulose in water. Several ionic liquids (ILs) have been shown to dissolve water-insoluble cellulose and/or wood. Among these is 1-butyl-3-methylimidazolium chloride ([BMIM]Cl) [1,2]. Hence, ILs could be used as a reaction medium for the enzymatic hydrolysis of cellulose, if cellulases were shown to be active and stable in the presence of high IL concentrations. To date only a limited number of cellulases have been described as being

active in the presence of ILs. The majority of cellulases, including a preparation from *Trichoderma reesei*, appear to be inactive in the presence of IL concentrations above 20% (*v*/*v*) [3,4]. In contrast, some thermostable cellulases have shown high activity in the presence of about 30% (*v*/*v*) IL [5,6]. Nevertheless, the stability of cellulolytic activity in the presence of high IL concentrations during relevant time periods remains to be demonstrated. Fungal enzymes are often more stable due to glycosylation, and have been shown to be persistent in the presence of proteases, high temperatures, and surfactants [7–9].

2. Results and Discussion

The cellulases derived from *Trichoderma reesei* and *T. viride* were almost inactive in the presence of 30% (*v*/*v*) of all tested ILs (Figure 1), whereby 30% (*v*/*v*) IL was chosen, as it turned out to be a critical concentration in previous studies [3]. The *Aspergillus niger* preparation was only noticeably active in the presence of [BMIM]Cl, but the cellulase derived from *Aspergillus* sp. showed high activities in the presence of 30% (*v*/*v*) of all tested ILs, and was altogether the most active and IL-tolerant enzyme preparation (Figure 1). For this reason, this extract was chosen for the evaluation of its long term stability. Correspondingly, the extract was co-incubated with the respective ILs (60% (*v*/*v*)) at room temperature, and the cellulolytic activity was measured after up to 63 days, in the presence of 30% (*v*/*v*) IL at 37 °C. During this time the enzyme showed stable activities in the presence of [BMIM]Cl, [BMMIM]Cl, [EMIM] [ATF], and [EMIM] [OTF], after a decrease to about 50% relative activity in the first days. For example, 17 U/mg (50% relative activity) were observed after 63 days in the presence of [EMIM] [OTF] (Figure 2) and 12 U/mg (29% relative activity) in the presence of [BMIM]Cl, an IL that has been shown to dissolve crystalline cellulose. To our knowledge, in comparison with bacterial as well as fungal cellulases, the observed high activities over a long time period represent by far the highest and longest stability of cellulolytic activity in the presence of high IL concentrations that have been described. Reasons for this high activity and stability might be that the cellulase extract of *Aspergillus* sp. is a mixture of different cellulases that work concertedly. Furthermore it has been shown that IL tolerance can be correlated to thermostability and halotolerance [5]. Interestingly, different *Aspergillus* species have been shown to be halotolerant [10].

Figure 1. Specific activity of cellulase extracts of *A. niger*, *A.* sp., *T. reesei* and *T. viride* in the presence and absence of 30% (*v*/*v*) IL at 37 °C after 30 min.

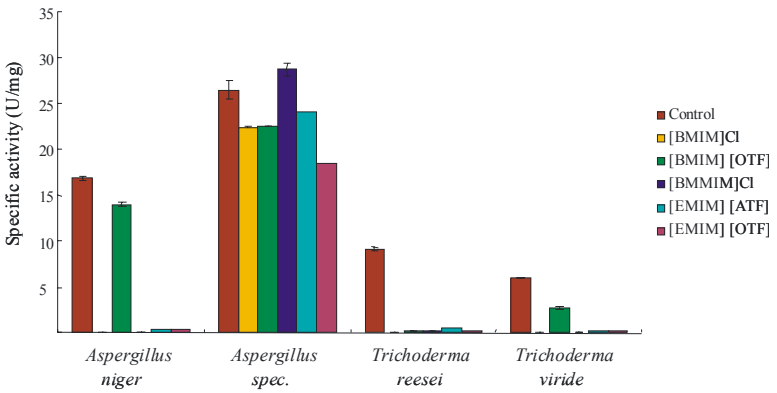
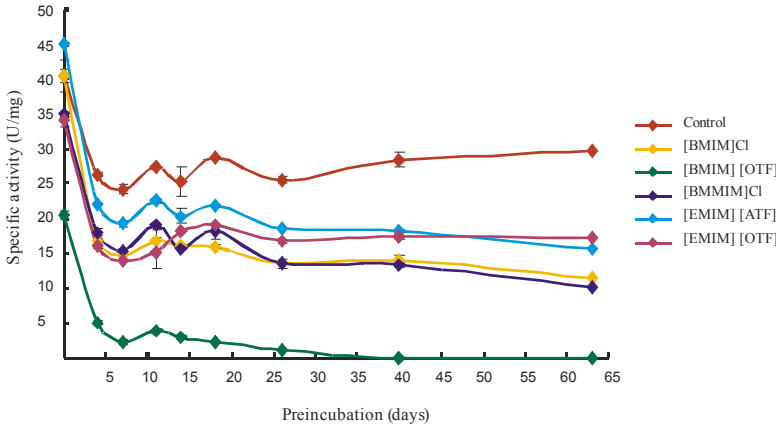


Figure 2. Stability of specific activity of cellulase extracts of *A. niger*, *A.* sp., *T. reesei* and *T. viride* after incubation in the presence and absence of 60% (*v*/*v*) ILs at room temperature; activity was measured in 30% IL (*v*/*v*) at 37 °C after 30 min.



3. Experimental Section

In the current study, four commercial cellulase preparations derived from fungi (*Aspergillus niger*, Sigma-Aldrich; *Aspergillus* sp., Sigma-Aldrich; *Trichoderma reesei*, Sigma-Aldrich; *Trichoderma viride*, Fluka) were tested, as these turned out to be most active in previous work with different *Trichoderma*, *Candida*, *Aspergillus*, and *Fusarium* extracts (data not shown). The cellulases may contain traces of dextrins, glucose, propylene glycol, and/or sorbitol. The extracts were not further purified and diluted appropriately in TRIS buffer (50 mM, pH 7), so that the impurities in the assay would be negligible. The cellulolytic activity and stability was assayed in the presence of the following ILs: 1-butyl-3-methylimidazolium chloride ([BMIM]Cl), 1-butyl-3-methylimidazolium trifluoromethanesulfonate ([BMIM] [OTF]), 1-butyl-2,3-dimethylimidazolium chloride ([BMMIM]Cl), 1-ethyl-3-methylimidazolium trifluoroacetate ([EMIM] [ATF]), and 1-ethyl-3-methylimidazolium trifluoromethanesulfonate ([EMIM] [OTF]). Cellulolytic activity against carboxymethylcellulose (CMC) was measured at 37 °C with the dinitrosalicylic acid (DNSA) method as described previously [11], using 1 µg protein in each 500 µL reaction. Units are given as µmol/min.

4. Conclusions

The observed high activity and stability of *Aspergillus* sp. cellulase in the presence of high IL concentrations raises hope that an industrial process employing cellulose for the generation of glucose and subsequent molecules via enzymatic hydrolysis in ionic liquids is possible. Future plans for achieving this goal would be tests on crystalline cellulose and IL treated natural cellulosic substrates like switchgrass or wood. The increase of IL content in the reactions would also be a future prospect.

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Conflict of Interest

The authors declare no conflict of interest.

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