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Trichoderma reesei derived cellulase activity in three N,N-dimethylethanolammonium akylcarboxylate ionic liquids

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Abstract

The activity and denaturation extent of cellulase from *Trichoderma reesei* (E.C. # 3.2.1.4) was investigated in three representative N,N-dimethylethanolammonium akylcarboxylate ionic liquids. Significant cellulase activity and absence of enzyme unfolding was found in all concentrations of N,N-dimethylethanolammonium acetate (DMEAA), including the pure liquid. Activities in 20% and 40% (v/v) solutions of DMEAA were equal to citrate buffer controls. Lower enzymatic activities and denaturation were observed in solutions of the corresponding formate and octanoate ionic liquids, although cellulose hydrolysis still proceeded at a substantial rate. The results provide the first proof-of-principle that cellulose can be enzymatically hydrolyzed in the presence of high ionic liquid concentrations.

Among the various raw biomaterials that will form part of the emerging biomass-based economy, cellulose will play a central role due to its large annual production via photosynthesis,[1] and its potential to be used for applications in the biofuels, fine chemicals, fibre, and polymer industries.[2] However, cellulose is resistant to the dissolution required for its subsequent functionalization.[3,4] Much of the efforts currently underway on converting cellulose into both fuels and platform molecules are concentrated on methods for converting crystalline cellulose into its monomeric glucose constituents.[5]

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To help meet the challenge of biomass conversion. ionic liquids (ILs) have attracted substantial research interest.[6] This attention is due primarily to the high stability and tunable properties of ILs towards the desired physical, chemical, and biochemical transformations of target compounds dissolved or suspended in these substances using thermal, photochemical, and/or catalytic routes.[7,8] In particular, there has been much work done on biocatalysis in ILs,[9,10] and a wide range of enzymatic transformations have been conducted in these versatile solvents.[11] While the hydrophobic effect that increases protein stabilization is absent in organic ILs, one advantage to biocatalysis in ILs versus aqueous buffers is the longer activity of enzymes in ILs, thought to be from the slow breaking and remaking of hydrogen bonds in the non-aqueous media. In some cases, the kinetic effects of reduced enzyme hydrogen bond oscillation in ILs may outweigh the thermodynamic instability caused by these solvents, leading to the retention or increase of enzymatic activity.[11]

Previous work has established that ionic liquids can effectively dissolve cellulose.[3,12-15] However, little is known about the molecular and macromolecular structures present in

the dissolved cellulose. The cellulose can subsequently be recovered in an amorphous form by addition of an “anti-solvent” to the ionic liquid/cellulose solution.[3,12-15] The regenerated amorphous cellulose is then more amenable towards enzymatic hydrolysis.[12,13] In a biorefinery, a process based on these findings would require three general steps that occur in sequential, multiple reactors: (1) dissolution of the incoming crystalline cellulose in pure IL (no IL has yet been found that can dissolve cellulose in the presence of substantial quantities of miscible protic or aprotic cosolvents such as water, methanol/ethanol, or acetone); (2) regeneration of solid amorphous cellulose by the addition of a miscible “anti-solvent” (e.g., water, alcohols), collection of the solid amorphous cellulose (e.g., filtration, centrifugation, etc.), separation of the IL from the antisolvent, and recycling the IL back to the start of the process train; and (3) enzymatic hydrolysis of the regenerated amorphous cellulose.

It would thus be of interest to find ILs which do not inhibit enzymatic amorphous cellulose hydrolysis (potentially avoiding the need for rigorous solvent/antisolvent separations), and which may sufficiently act upon the surface structure of crystalline cellulose to make it more amenable to direct enzymatic action without any requirement for conversion to the more reactive amorphous form. In this paper, we report studies regarding the activity of cellulase from *Trichoderma reesei* [16] in the following three representative “green” ionic liquids: N,N-dimethylethanolammonium formate (DMEAF), acetate (DMEAA), and octanoate (DMEAO).[17]

Cellulase unfolding was followed by methods based on fluorescence of the tryptophan and tyrosine groups within the enzyme.[18] Activity of the cellulase enzyme in each solution was determined by following liberation of an azo dye from cellulose azure.[19]

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Efforts were also made to quantify microcrystalline cellulose hydrolysis to glucose using standard bioassay and analytical methods based on liquid chromatography with refractive index (LC-RI) detection.[20] However, the ionic liquids coeluted with the target carbohydrate monomer analytes via LC-RI, preventing reliable identification and quantitation.

Significantly different cellulase unfolding profiles (inferred from fluorescence studies) were observed for the three ionic liquids over the concentration range from exclusively citrate buffer to pure ionic liquid (Fig. 1). DMEAF did not influence cellulase fluorescence up to 40% (v/v) solutions, above which the fluorescence intensity decreased linearly to about 30% that of the citrate buffer control at both 80% and 100% (v/v) IL content. Cellulase displayed relative fluorescence insensitivity towards the presence of DMEAA, suggesting that this ionic liquid does not substantially denature the enzyme in any concentration. In the presence of DMEAA, relative fluorescence intensity decreased to about 80% that of the citrate control at 40% and 60% (v/v) content, but recovered to near unit (93-94%) intensity at 80% and 100% ionic liquid volumetric concentrations. For DMEAO, cellulase fluorescence decreased linearly to near zero (8%) between 0% and 40% (v/v) content. Higher DMEAO volumetric concentrations between 60% and 100% led to a subsequent linear increase in fluorescence to about 50% that of the control.

The lack of apparent cellulose denaturation in the presence of DMEAA over all concentration ranges, and in the presence of moderate (<50%) DMEAF concentrations, and the unusual profile of cellulase fluorescence in the presence of increasing DMEAO concentrations, led us to conduct spectrophotometric cellulase degradation assays using

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release of azo dye from cellulose azure as a proxy over a 3-day period. Time series of the various volumetric trials in each of the three ionic liquids indicate that the majority of cellulose hydrolysis occurred during the initial 24 h period, with relatively stable dye concentrations over the subsequent 2-days (Fig. 2).

For both DMEAF and DMEAO, the citrate buffer controls (with no ionic liquid added) exhibited the highest levels of cellulase activity. In contrast, both 20% and 40% (v/v) solutions of DMEAA showed equivalent activity as the citrate buffer over the course of the trials ($p>0.05$ using the two-way Tukey-Kramer pairwise comparisons test). At the end of the reaction period, the order of cellulase activity in each IL solution (0% (v/v) is pure 0.05 M citrate buffer; 100% (v/v) is pure IL) was as follows: DMEAF, 0%>20%>40%>100%>60%>80%; DMEAA, 20%≈0%≈40%>60%>80%>100%; DMEAO, 0%>20%>100%>80%≈60%>40% (differences tested with the two-way Tukey-Kramer pairwise comparisons test at $\alpha=0.05$).

The observed hydrolysis activity behaviour of DMEAO, with a minima at intermediate IL-buffer concentrations, was consistent with fluorescence studies. As well, it has been widely reported in the literature that many enzymes tolerate solvents when nearly anhydrous or in dilute aqueous solution, but become deactivated at intermediate concentrations.[21] It is thought this pattern results because the hydrophobic effect decreases in the presence of a solvent (including ILs), leading to a decrease in the stability margin of the enzyme until deactivation occurs at a certain concentration.[11]

The effect of ILs on enzyme activity is governed by bulk solvent properties (e.g., polarity, acidity/basicity) and the contributions of the individual ions.[10] This is especially

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true in binary aqueous:IL systems, where the ILs can dissociate into their constituent cations and anions. The kosmotropicity of salts such as ILs strongly influences the activity and stability of enzymes.[22] Kosmotropes strongly hydrate ions that accelerate water restructuring, while chaotropes suppress water restructuring by weak hydration.[23] Overall in aqueous solutions, strongly kosmotropic anions stabilize, and strongly kosmotropic cations destabilize, enzymes. Thus, enzymes are generally stabilized by kosmotropic anions and chaotropic (anti-kosmotropic) cations.[24] Quaternary ammonium cations (such as the N,N-dimethylethanolammonium series considered herein) are known to be strong chaotropes,[25] and have higher chaotropicity than imidazole derivatives,[26] likely due to the larger cation size of the ammonium series.

Our finding of high cellulase activity in all concentrations of DMEAA, and substantial enzymatic activity in modest concentrations of DMEAF and DMEAO, is in contrast to the strong deactivation of cellulase when exposed to even small quantities of the widely employed $[C_4MIM]Cl$ ionic liquid for cellulose dissolution and regeneration.[27] In this previous work, cellulase denaturation was linked to the IL's chloride anion. This structure-activity relationship is expected based on the known high kosmotropicity of the $[C_4MIM]^+$ cation and high chaotropicity of the chloride anion. However, we note that the predictive nature of physico-chemical descriptors towards enzyme activity in ILs remains controversial, and some authors dispute any rationalization of enzyme activity based on kosmo-/chao-tropicity, polarity, viscosity, hydrogen bonding ability, or hydrophobic characteristics.[11] Indeed, our observation of lower cellulase activity and higher levels of denaturation in DMEAF than DMEAA is consistent with this perceived lack of general predictivity. The formate anion in DMEAF should be more kosmotropic than the acetate anion in DMEAA, and both solvents contain the same chaotropic N,N-

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dimethylethanolammonium cation. As is discussed below, the lower than expected activity could be due to competitive interactions between the solvent and enzyme for access to the substrate surface, or blocking of the enzyme active site by solvent. The higher than expected denaturation (based on kosmotrophicity arguments) in DMEAF may result from solvent removal of a required full hydration shell [11] around the cellulase, and subsequent replacement by a shell of small formate ions that results in enzyme unfolding.

The relative fluorescence intensities of cellulase and the observed extents of azo dye release from cellulose hydrolysis in each ionic liquid solution were also compared (Fig. 3). With the exception of the 20% through 80% (v/v) solutions of DMEAF and the 80% and 100% (v/v) solutions of DMEAA, fluorescence intensity was a reasonable predictor of cellulase activity. The results suggest that in some cases, the enzyme may remain active, but solvent effects may prevent the cellulase from accessing the cellulose interface. The presence of voids in the ILs, able to contain substrates, results in these media behaving more like polymeric matrices than molecular solvents.[7] Competitve hydrogen bonding [5] between the ILs and cellulose surface at high IL concentrations may also restrict cellulase access to the substrate. Similarly, the IL may be a suitable fit for the enzyme active site, blocking the substrate from interacting. Previous work has also reported lower than expected enzymatic activities in ILs due to the high viscosity of these pure solvents, which can lead to internal diffusion limitations and increased difficulty for substates and enzyme coupling.[26] On the other hand, the high viscosity of ILs is also thought to maintain higher enzymatic activity and thermal stability than in molecular organic solvents, due to slowing the migration of protein domains from an active to an inactive conformation.[11]

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Low solubilities of simple sugars in pure ILs have also been observed (ranging from 5 to 62 mg/mL depending on the sugar),[5] which can potentially reduce the driving force for cellulose hydrolysis in neat solutions. However, we did not observe any difficulties in solubilizing up to 50 mg/mL glucose (higher than our 20 mg/mL cellulose azure starting concentrations) in DMEAF, DMEAA, or DMEAO. Consequently, the observed lower than predicted activity (based on fluorescence measurements of denaturation) of cellulase in high concentrations of DMEAA (80% and 100% (v/v), and all buffer-IL combinations for DMEAF, is likely due to the high viscosity of the solvents, and competitive hydrogen-bonding behaviour between the ILs and the cellulose surface.

In conclusion, we present here the first report of high cellulase activities in concentrated solutions of three representative N,N-dimethylethanolammonium akylcarboxylate ionic liquids. The findings indicate the potential to conduct cellulose hydrolysis in the presence of selected members of this solvent class, and may open up the potential for coupled IL modification of cellulose surface properties with conjoint enzymatic cellulase hydrolysis to glucose. These results may then be fermented to bioethanol or derivatized/transformed into high-value chemicals and platform molecules.

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16. Cellulase from *Trichoderma reesei* ATCC 26921 was purchased from Sigma-Aldrich as a 700 unit/g aqueous solution (d=1.2 g/mL at 25°C).

17. ILs were of synthetic grade from Bioniqs (York, UK) and were dried at 70°C in a vacuum oven (100 torr) for 4 hours before use without additional purification. All ILs were completely miscible in water and 0.05 M citrate buffer over the concentration ranges studied.

18. Cellulose unfolding experiments were performed on a 96-well Spectra Max Gemini EM (Molecular Devices Corp.; Sunnyvale, CA, USA) spectrofluorimeter. Spectra were acquired in the fluorescence top read mode with excitation at 284 nm and emission monitored between 325 to 500 nm (2 nm steps) using a 325 nm cutoff filter. The instrument was autocalibrated, the photomultiplier set at automatic, and 6 reads/well were obtained in a standard opaque 96-well plate at 45°C. Plate wells contained 300 µL

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of solution varying from 0% to 100% ionic liquid (v/v) in 0.05 citrate buffer and 0.21 mg of cellulase. All runs were background corrected for solvent fluorescence using the corresponding ionic liquid/buffer solution without added cellulase. Substantial solvent fluorescence was observed for the N,N-dimethylethanolammonium octanoate solutions, particularly at 40% (v/v) ionic liquid in 0.05 M citrate buffer. Maximum fluorescence intensities were observed between 335 and 345 nm depending on solution composition. Cellulase fluorescence underwent a hypsochromic shift from 345 nm to 337 nm upon addition of 20% (v/v) octanoate ionic liquid, after which no significant change in the $\lambda_{em,max}$ was observed. Both the formate and acetate ionic liquids displayed similar $\lambda_{em,max}$ profiles, decreasing from 345 nm at 0% composition to 339 nm at 40% and 60% (v/v) content, and increasing to 345 nm at 100% ionic liquid.

19. Cellulose azure was purchased from Sigma-Aldrich and used as received following the method of H.N. Fernley, Biochem. J., 1963, 87, 90-95. Triplicate trials were performed in 3 mL ReactiVials (Pierce Chemical Co.; Rockford, IL, USA) with teflon caps. Each ReactiVial contained 10 mg of cellulose azure, 30 units of cellulase, and varying combinations (from 0% to 100% v/v) of 0.05 M citrate buffer (pH 4.8) and each ionic liquid. Sodium citrate buffer was prepared by dissolved sodium citrate monohydrate in Millipore-grade water and adjusting the pH to 4.8 using 1 M NaOH. The reaction mixtures were sealed, vortexed for 30 s, and incubated at 50°C in a shaker-bath (110 rpm). At each sampling time, vials were vortexed for 30 s, allowed to sit at room temperature for 15 min to ensure settling of unreacted cellulose, and samples (100 µL) collected, diluted to 1 mL with Millipore grade water. UV-Vis spectra were collected on a Varian Cary 50 Bio spectrometer over the range from 200 to 800 nm. Release of the azo dye was quantified by determining the absorbance at the wavelength of maximum

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absorption in the range from 572 to 580 nm (bathochromic shifting in the λ_{max} was observed with increasing ionic liquid content). To account for abiotic hydrolysis of the azo dye, each trial contained a corresponding control without cellulase addition. At each time step, the absorbance of the control was subtracted from the absorbance of the cellulase trial to discriminate enzymatically-mediated dye release.

20. Triplicate trials were performed in 3 mL ReactiVials (Pierce Chemical Co.; Rockford, IL, USA) with teflon caps following the standard methods (scaled down from 10 mL to 2 mL) outlined by the US NREL: L. Brown and R. Torget, Enzymatic Saccharification of Lignocellulosic Biomass LAP-009, United States Department of Energy, Washington, DC, USA, 1996. Each ReactiVial contained microcrystalline cellulose, cellulase, and varying combinations (from 0% to 100% v/v) of 0.05 M citrate buffer (pH 4.8) and each ionic liquid to a total volume of 2 mL. The reaction mixtures were sealed, vortexed for 30 s, and incubated at 50°C in a shaker-bath (110 rpm). At each sampling time (0, 3, 5, and 7 days), vials were vortexed for 30 s, allowed to sit at room temperature for 15 min to ensure settling of unreacted cellulose, and 50 μL of the reaction solution was diluted to 0.5 mL in 0.05 M citrate buffer, vortexed for 30 sec, and analyzed for monomeric sugars by standard methods: A. Sluiter, Determination of Structural Carbohydrates and Lignin in Biomass, United States Department of Energy, Washington, DC, USA, 2006. Broad, shifting, and dominating refractive index peaks were observed for each of the ionic liquid solutions that obscured the monomeric sugar peaks eluting between 11 and 15 min.

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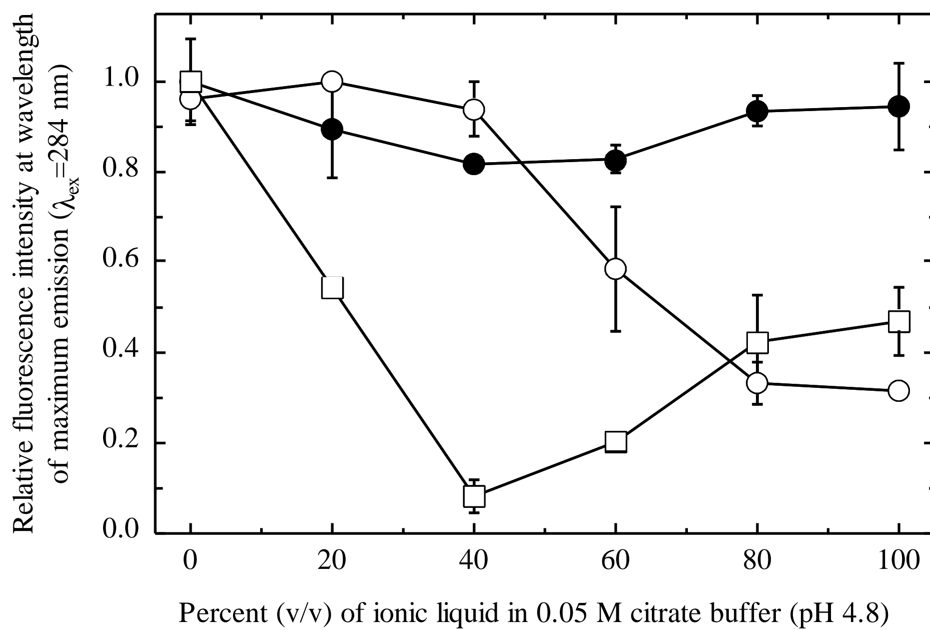

Fig. 1 Relative fluorescence intensities of cellulase unfolding in 0.05 M citrate buffer solutions of (○) N,N-dimethylethanolammonium formate, (●) N,N-dimethylethanolammonium acetate, and (□) N,N-dimethylethanolammonium octanoate. Error bars are standard deviations about the mean of triplicate trials.

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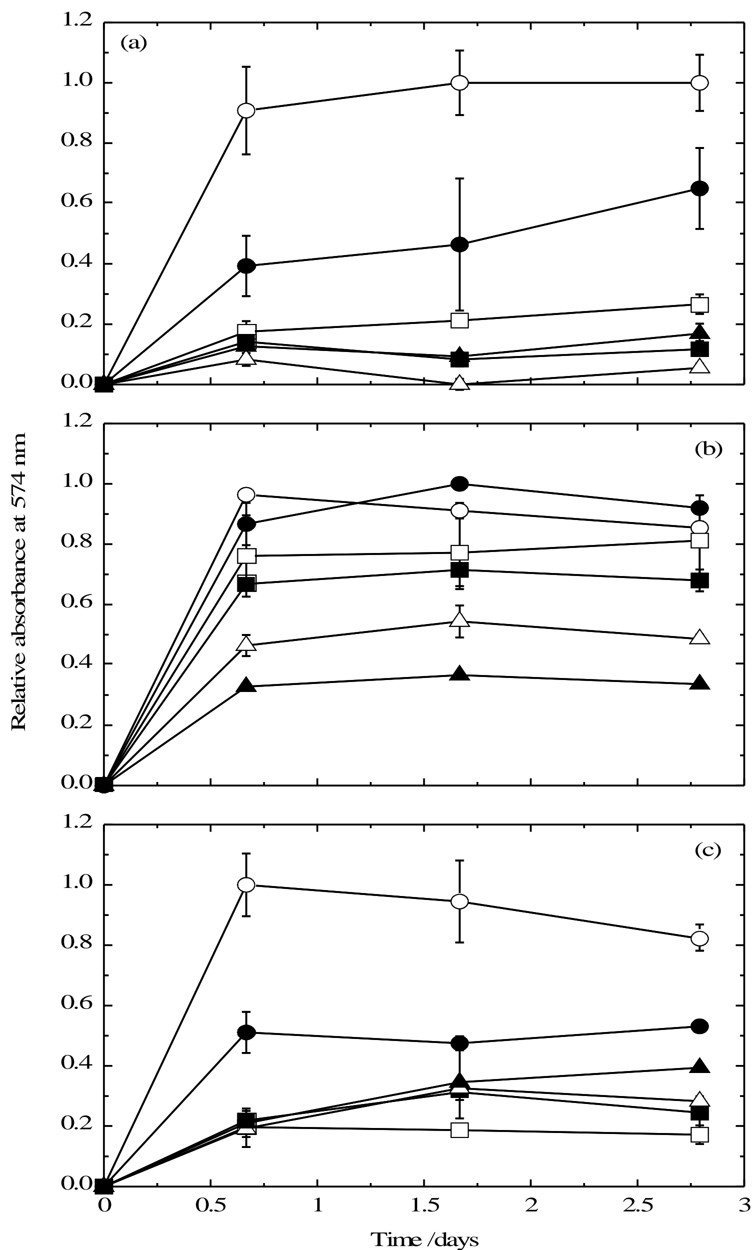

Fig. 2 Cellulase activity in (a) N,N-dimethylethanolammonium formate, (b) N,N-dimethylethanolammonium acetate, and (c) N,N-dimethylethanolammonium octanoate at (○) 0%, (●) 20%, (□) 40%, (■) 60%, (△) 80%, and (▲) 100% volumetric composition of ionic liquid in 0.05 M citrate buffer. Error bars are standard deviations about the mean of triplicate trials.

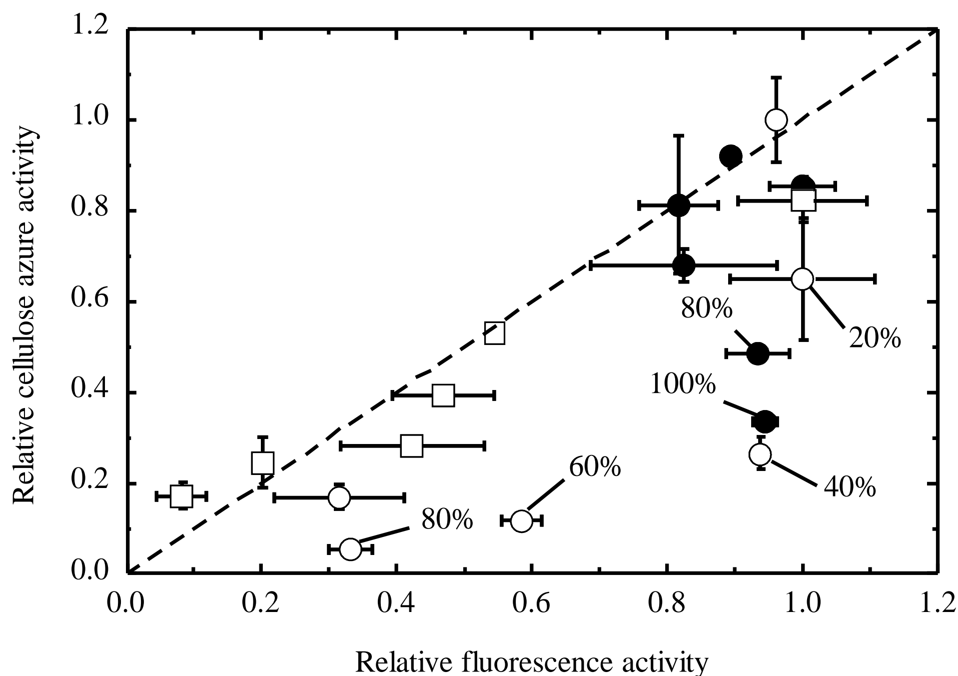

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Fig. 3 Relationship between predicted cellulase activies as measured by fluorescence and observed activity against cellulose azure for (○) N,N-dimethylethanolammonium formate, (●) N,N-dimethylethanolammonium acetate, and (□) N,N-dimethylethanolammonium octanoate. Error bars are standard deviations about the mean of triplicate trials for each variable. A 1:1 line (dashed) is shown to aid in comparison. Labels indicate percent volumetric composition of ionic liquid in citrate buffer where shown.