Ionic liquid tolerant hyperthermophilic cellulases for biomass pretreatment and hydrolysis†

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One of the main barriers to the enzymatic hydrolysis of cellulose results from its highly crystalline structure. Pretreating biomass with ionic liquids (IL) increases enzyme accessibility and cellulose recovery through precipitation with an anti-solvent. For an industrially feasible pretreatment and hydrolysis process, it is necessary to develop cellulases that are stable and active in the presence of small amounts of ILs co-precipitated with recovered cellulose. However, a significant decrease in cellulase activity in the presence of trace amounts of ILs has been reported in the literature, necessitating extensive processing to remove residual ILs from the regenerated cellulose. Towards that end, we have investigated the stability of hyperthermophilic enzymes in the presence of the IL 1-ethyl-3-methylimidazolium acetate ([C2mim][OAc]) and compared it to the industrial benchmark *Trichoderma viride* (*T. viride*) cellulase. The endoglucanase from a hyperthermophilic bacterium, *Thermatoga maritima*, and a hyperthermophilic archaeon, *Pyrococcus horikoshii*, were over expressed in *E. coli* and purified to homogeneity. Under their optimum conditions, both hyperthermophilic enzymes showed significantly higher [C2mim][OAc] tolerance than *T. viride* cellulase. Using differential scanning calorimetry we determined the effect of [C2mim][OAc] on protein stability and our data indicates that higher concentrations of IL correlated with lowered protein stability. Both hyperthermophilic enzymes were active on [C2mim][OAc] pretreated Avicel and corn stover. Furthermore, these enzymes can be recovered with little loss in activity after exposure to 15% [C2mim][OAc] for 15 h. These results demonstrate the potential of using IL-tolerant extremophilic cellulases for hydrolysis of IL-pretreated lignocellulosic biomass, for biofuel production.

Introduction

The use and depletion of fossil fuels are major environmental and energy security issues for the twenty-first century.[1] Renewable biofuels produced from biomass are an important alternative to petroleum use for transportation. Lignocellulosic (LC) biomass is an abundant and, potentially, carbon-neutral energy resource for the production of biofuels and chemicals.[2] However, there are scientific and technological challenges that need to be overcome before LC biomass-derived biofuels can be a significant and economically competitive alternative to petroleum.

The production of ethanol from corn grain represents the most convenient and technically advanced option for biofuels in the United States today. However, the increased displacement of available corn supplies for fuel has adverse affects on food markets given the role of corn as a food commodity. LC biomass, on the other hand, is an abundant and potentially low cost resource that does not compete with human needs.[3] It includes agricultural residues (corn stover, wheat straw and rice straw), deciduous and coniferous woods, agricultural processing by-products (corn fiber, rice hulls and sugar cane bagasse) and energy crops like switch grass and miscanthus.[4]

Lignocellulosic biomass is made of three major components—cellulose (35–50%), hemicellulose (20–35%) and lignin (10–25%)—the relative concentrations of which vary according to the source.[5] For efficient utilization, the biomass has to undergo pretreatment before it can be enzymatically hydrolyzed to glucose and other constituent sugars. Cellulosic component of LC biomass is highly crystalline with polymeric cellulose chains held together by hydrogen bonds and van der Waals forces.[6] The polymeric chains are made of anhydroglucose units joined together by glycosidic bonds with degree of polymerization (DP) ranging from 1000–20 000 units.[7] Pretreatment is essential to reduce the DP of the cellulose and make it amorphous to facilitate access to the substrate for enzymatic cellulose hydrolysis.[8] Current strategies in pretreatment and recovery of cellulose from biomass have been primarily derived from pulp and paper industry, where the downstream process of converting cellulose to glucose is not a consideration and are incompatible

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with enzymatic hydrolysis; thereby requiring extensive cellulose processing for enzymatic hydrolysis and resulting in an increase in time and cost. It is therefore desirable and important to develop a biomass pretreatment process that is compatible with not only the enzymatic hydrolysis but also subsequent fermentation of glucose to biofuels.

Current pretreatment methods include mechanical reduction in biomass particulate size followed by dilute acid, flow-through ammonia fiber expansion, ammonia recycle percolation, lime, steam explosion, or organosolv (OS) pretreatment.[9] The drawbacks of these methods include severe reaction conditions (high temperature and/or high pressure and extremes of pH), and high processing costs. A new pretreatment approach using ionic liquids (ILs) has been shown to be effective in solubilizing crystalline cellulose.[10]

Room temperatures ILs are pure salts with melting points typically below 100 °C.[10,11] While the properties of ILs are very diverse, high thermal stability and non-volatility make this class of compounds an attractive alternative to organic solvents in several existing industrial processes.[12] ILs depending on their chemistry, can dissolve a wide range of polar and non-polar organic and polymeric compounds, including cellulose, and make the polysaccharide chains more accessible to enzymatic hydrolysis.[13] Of particular interest to the biofuels industry is the ability of several imidazolium based ionic liquids with weak conjugated-base anions to dissolve crystalline cellulose at moderate temperatures.[13,14] The cellulose is subsequently recovered in the amorphous form upon the addition of anti-precipitants like water[15–17] rendering it more accessible to enzymatic hydrolysis and subsequent rapid hydrolysis to glucose using cellulases.[18] The dissolution of lignocellulosic materials such as corn stalks, rice straw, bagasse, pine wood, and spruce wood in ILs followed by cellulose hydrolysis with acid or enzymes has also been recently reported in literature.[18–20]

Cost-effective enzymatic hydrolysis of cellulose to sugars would benefit from the development of enzymes that are effective under pretreatment conditions (which may be extreme pH or temperature). However, significant decreases in cellulase activity in the presence of trace amounts of ILs have been reported in literature.[21,22] In order to avoid extensive processing and clean up of the regenerated cellulose, the cellulases that are used must be able to perform optimally in up to 15% concentrations of IL that is co-precipitated with cellulose. The amount of leftover IL would however depend on the cost of recovery of ILs. Towards the goal of discovering cellulases that are stable and active in the presence of trace amounts of ILs, we investigated the stability of hyperthermophilic enzymes, broadly categorized with extremophilic enzymes, as a function of IL concentration. Since extremophilic organisms grow in extremes of pH, temperature and/or salinity *etc.*, these are a promising source of enzymes for industrial processes. The enzymes derived from hyperthermophilic organisms may be capable of tolerating ionic liquids since the native growth condition of these organisms is greater than 80 °C in a strongly reducing environment.[23]

A model extremophilic organism is the hyperthermophilic bacterium *Thermatoga maritima* (*Tma*), a member of the order thermotogales, which is an anaerobic heterotrophic hyperthermophile capable of fermenting both simple and complex sugars.[24] Roughly 7% of the predicted coding sequences in its genome encode enzymes that are involved in the metabolism of monosaccharides and polysaccharides. *Tma* Cel5A is a free-acting endoglucanase containing only a catalytic domain.[25] *Pyrococcus horikoshii* (*Pho*), a hyperthermophilic archaeon found in deep-sea hydrothermal vents, is an anaerobic heterotroph, which utilizes peptides as its main carbon source at temperatures approaching 100 °C but has been shown to contain an endoglucanase (EG) that is functional at an optimum temperature of 97 °C.

Since cellulases from *Trichoderma* constitute an established cellulase hydrolysis process for benchmarking, here we present data comparing the efficiency of hydrolysis of *Tma* Cel5A and *Pho* EG with the *Trichoderma viride* cellulase. *Tma* Cel5A and *Pho* EG were purified to homogeneity and enzymatic hydrolysis activity was measured in the presence of varying concentrations of [C2mim][OAc]. Both enzymes showed significant specific activity at 15% (v/v) [C2mim][OAc] with specific activities decreasing at higher concentrations. Decreased specific activities at higher concentrations showed a direct correlation to enzyme stability as indicated by decreases in unfolding temperatures by DSC measurements. Both enzymes were very stable at high temperatures for extended periods of time and were tolerant to 2 M NaCl and KCl. These enzymes are also active on pretreated biomass. Long half-lives and a near 100% recovery of activity from ILs make them promising targets for further investigation for use in large-scale saccharification reactions employing this pretreatment technology.

Results

Hyperthermophilic enzymes and specific activity assays

Previous studies by Chhabra *et al.*[26] showed that the *Tma* Cel5A from *Tma* is intracellularly expressed in *T. maritima* and has an optimum temperature (T_{opt}) of 80 °C. The EG from *Pho* has a even higher T_{opt} of 97 °C and specific activity of 8.5 U mg^{-1} (1 U = 1 μmole of reducing sugar produced min^{-1} using CMC as substrate) at pH 5.6 and 85 °C.[27] Thus, both of these enzymes were attractive targets for our work. The enzymes were expressed in *E. coli* in autoinduction media and purified by nickel affinity chromatography to homogeneity as detected by SDS-PAGE. Maximal amounts of soluble *Tma* Cel5A were obtained following induction at 37 °C, yielding 30 mg L^{-1}. The yield of *Pho* EG was around 2 mg L^{-1}, under similar conditions except that an additional anion exchange chromatography step was added to obtain purer protein. Both cellulases were active on carboxymethyl cellulose (CMC). In our experiments, the specific activities of *Tma* Cel5A and *Pho* EG were 30 U mg^{-1} and 1.9 U mg^{-1}, respectively (Table 1). The commercially available *T. viride* cellulase, has a specific activity of 10–12 U mg^{-1} at 37 °C with CMC as the substrate, depending on the batch of enzyme used.

Specific activity in the presence of [C2mim][OAc]

The purified recombinant enzymes were assayed in the presence of [C2mim][OAc] and the specific activity results were compared with the cellulase from *T. viride*. [C2mim][OAc] was selected as the IL of choice. The assays were preformed under optimum pH and temperatures for *T. maritima* and *T. viride* enzymes, as

Table 1 Biochemical properties of hyperthermophilic enzymes: optimum temperature and pH, specific activity, and stability in presence of [C2mim][OAc]. The % specific activities (last column) are reported as percentages of residual specific activity, taking the specific activity of IL free enzyme as 100% activity

	Properties					
Enzymes	T_{opt}/°C	pH_{opt}	$T_{1/2}$/h in 0% IL	$T_{1/2}$/h in 15% IL	Specific activity[a]	% Activity recovered after incubation in 15% IL for 15 h
T. viride cellulase[b]	37	4.5	9	0	11 ± 2	0
Tma Cel5A[b]	80	4.8	20 ± 2	19 ± 3	30 ± 2	44
Pho EG[c]	>95	6.4	24 ± 4	23 ± 4	1.9 ± 0.3	79

[a] Units is μmoles of reducing sugars min^{-1} mg^{-1} with carboxymethylcellulose as substrate. [b] Assays were done at T_{opt}. [c] Assays were done at 80 °C.

shown in Table 1. The *Pho* EG was assayed at 80 °C where its specific activity is around 10% less than at 95 °C (data not shown). As seen in Fig. 1, the cellulase from *T. viride* rapidly lost activity with increasing concentration of [C2mim][OAc]. In the presence of 5% (v/v) [C2mim][OAc] the enzyme lost 60% of its activity while it was undetectable in the presence of 10% (v/v) [C2mim][OAc]. Both the recombinant hyperthermophilic enzymes behaved very differently. *Tma* Cel5A in the presence of 5% [C2mim][OAc] loses 24% of its specific activity. At 15% IL (v/v), *Tma* Cel5A retains 50% of its specific activity. The highest residual activities were observed with *Pho* EG which retains 100% activity in 20% IL (v/v) (Fig. 1). Pre-incubation overnight (15 h) in 15% [C2mim][OAc] resulted in an almost complete loss of activity for the *T. viride* cellulases, as expected (Table 1) while the *Tma* Cel5A and *Pho* EG retained 44% and 70% respectively of their activity. The hyperthermophilic enzymes also exhibited longer half-lives, around 20–24 h, compared to the 9 h for the fungal *T. viride* cellulase, at their optimal pH and temperatures (Table 1).

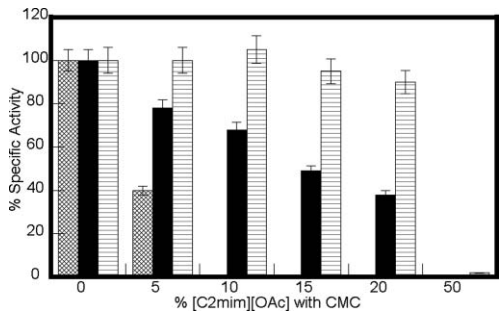

Fig. 1 Enzymatic hydrolysis of CMC by cellulase from *T. viride* (cross line), *Tma* Cel5A (solid) *and Pho* EG (horizontal line), in the presence of various [C2mim][OAc]: 2% CMC in and 0–50% of [C2mim][OAc] (v/v) were incubated at 37 °C (*T. viride*), 80 °C (*Tma* EG, *Pho* EG). The specific activities were calculated as μmoles of reducing sugars formed per min per mg of enzyme. The specific activities are reported as percentages of residual specific activity, taking the specific activity of IL free enzyme as 100% activity. Error bars indicate the standard deviation.

Effect on enzyme stability

To understand the effect of ILs on protein stability, differential scanning calorimetry studies were done on the *T. viride* cellulase and compared to *Tma* Cel5A and Pho EG. The unfolding temperatures of the enzymes are listed in Table 2 and the thermograms for *Tma* Cel5A and *Pho* EG are shown in the ESI, Figure 1A and 1B.† The decrease in unfolding temperatures for *T. viride* cellulase between 0 and 5% [C2mim][OAc] is 7.2 °C

Table 2 Unfolding temperatures from differential scanning calorimetry thermograms of *Tma* Cel5A (5 mg mL^{-1}), *T. viride* cellulase (2.2 mg mL^{-1}) and *Pho* EG (1.8 mg mL^{-1}). The decrease in unfolding temperature of *Tma* Cel5A, *T. viride* cellulase and *Pho* EG is compared to the decrease in percent specific activity

Enzyme	% [C2mim][OAc] (v/v)	Unfolding T/°C	Decrease in % specific activity	Decrease in unfolding T/°C
T. viride cellulase	0	64.2	—	—
	5	57.0	60	7.2
	10	52.4	100	11.8
	15	49.4	100	14.8
	20	47.5	100	16.7
T. maritima endoglucanase (*Tma* Cel5A)	0	92.0	—	—
	5	89.8	23	2.2
	10	89.3	35	2.7
	15	88.5	52	3.5
	20	87.2	58	4.8
	50	67.0	98	25.0
P. horikoshii Endoglucanase (*Pho* EG)	0	102.3	—	—
	5	99.3	0	3
	10	97.5	0	4.8
	15	94.9	5	7.4
	20	91.8	10	10.5
	50	66.1	100	36.2

compared to a 2.2 °C drop in *Tma* Cel5A and a 3 °C drop in *Pho* EG (Table 2). This difference gets more pronounced at 10% [C2mim][OAc] (11.8 °C *vs.* 2.7 °C) when the *T. viride* cellulase is completely inactive. In the case of *Tma* Cel5A, there is a 25 °C drop in unfolding temperature at 50% [C2mim][OAc] (v/v) when the specific activity of *Tma* Cel5A is undetectable using the DNS assay. Similarly there is a 36.2 °C drop in unfolding temperature of Pho EG when the specific activity becomes undetectable. To understand the basis of tolerance to ILs we also looked at the effect of high concentrations of salt on the specific activity of the hyperthermophilic enzymes. To investigate whether the IL tolerance of the *Tma* Cel5A can be correlated with ionic strength of the buffer, *Tma* Cel5A was assayed in the presence of high salt concentration. As seen in the ESI, Figure 2A and 2B,† *Tma* Cel5A and *Pho* EG retained 65% and 85% of its activity respectively, in the presence of 2 M NaCl and 2 M KCl.

Effect on pretreated biomass

Corn stover refers to stalks, leaves and cobs that remain in the fields after the corn kernel harvest and is the largest quantity of biomass residue in the United States[28] and as such is also a LC-biomass source for producing cellulosic ethanol in the United States.[29] [C2mim][OAc] pretreated corn stover was used as a substrate to verify enzymatic efficiency on IL-pretreated biomass and a comparison made with [C2mim][OAc] pretreated Avicel. Avicel is a commercially available microcrystalline cellulose and a good model substrate for analysis. Similar assays were done in the presence of [C2mim][OAc] to simulate real biomass processing and saccharification scenarios. The assay products for the reactions in the presence of IL were detected by high performance anion exchange chromatography (HPAEC). The enzymatic hydrolysis reaction was carried out for 8 h for the *T. viride* cellulase and 15 h for the hyperthermophilic enzymes, taking into account the half-lives of these enzymes. To compare the amounts of total sugars formed between different reactions we chose to calculate the sum of glucose, xylose and cellobiose and report as μmoles of sugars formed per min per mg of enzyme used (Table 3). A comparison of assay results between pretreated and untreated substrate indicates a 2–6 fold increase in hydrolysis products after pretreatment irrespective of the enzyme used, thereby confirming that IL pretreatment can increase the efficiency of hydrolysis of cellulose recovered from biomass. The fungal enzyme was more active on untreated substrates compared to the hyperthermophilic enzymes probably due to the presence of cellobiohydrolases and xylanases in the enzyme mix (Table 3). The catalytic efficiency of the fungal enzyme however, decreases with increasing IL concentration in the reaction mix. After normalizing for the reaction time and enzyme amounts, the amount of sugars formed is undetectable at 15% [C2mim][OAc]. The catalytic efficiencies of the hyperthermophilic enzymes with insoluble substrates and in the presence of [C2mim][OAc] follow a similar trend as seen with the soluble substrate CMC. The yield of sugars from *Tma* Cel5A hydrolysis of pretreated Avicel in the presence of 15% IL decreases by 43% compared to 0% IL, while the decrease is only 10% in the case of *Pho* EG on the same substrate under similar conditions (Table 3). Cellobiose was the major product in the case of *Pho* EG, while a mixture of sugars were obtained for Cel5A.

Discussion

Hyperthermophilic proteins exhibit a remarkable balance between heat stability and functionality. This makes such proteins suitable for industrial applications like enzymatic hydrolysis of biomass for biofuels production. These enzymes generally owe their stability to a higher number of ion pairs and the polarity of exposed surfaces.[30] We started our present studies with two endoglucanase from hyperthermophilic organisms (one characterized representative each from bacterial and archaeal), to look into the effect of ILs with an eye on developing a large scale bioprocess.

Current processing conditions do not allow an *in situ* pretreatment and saccharification using an ionic liquid solvent, since ILs has been shown to affect the activity of cellulases. Turner *et al.*[31] reported the inhibition of *T. reesei* cellulases by the IL 1-butyl-3-methylimidazolium chloride. A recent report by Pottkämper *et al.*[32] reported the IL tolerance of a salt-tolerant cellulase from an uncultured microorganism[33] and discovered that a GH5 family cellulase that was active at IL concentrations of up to 30% (v/v). However the specific activity of these cellulases was very low, in the range of 0.014 μmoles min^{-1} mg^{-1} with the assay performed with cell extracts.[32]

We wanted to conduct an extensive study to look into the effect of ILs on hyperthermophilic cellulases with an eye on developing a large scale bioprocess with the hypothesis that hyperthermophilic enzymes would better tolerate ILs. Both *Tma* Cel5A and *Pho* EG were found to retain between 44% and 79% respectively of their activity in the presence of 15% [C2mim][OAc] unlike the *T. viride* cellulase with greater than 85% recovery of activity after exposure to 15% [C2mim][OAc] for 15 h. To verify enzymatic activity on biomass, we compared the efficiency of the recombinant cellulases to those from *T. viride* cellulase using both corn stover and Avicel as substrates and compared the results with assays on IL pretreated Avicel and corn stover. The pretreated substrates were more efficiently hydrolyzed compared to the untreated substrates. None of our recombinant proteins contain carbohydrate-binding domains, yet we detect the formation of sugars in the presence of untreated Avicel or corn stover. Further studies are needed to differentiate between the possibility of hydrolysis of amorphous cellulose in these samples or the possible effect of hyperthermophilic enzymes without carbohydrate-binding domains on insoluble substrates. However, as would be expected from reactions with insoluble substrates, enzymatic efficiency was less compared to results obtained using CMC. These results also show the importance of evaluating enzymatic efficiency with "real-world" insoluble substrates as opposed to synthetic soluble substrates that are easier to manipulate but cannot be used for translating analysis to actual substrates. The hyperthermophilic enzymes displayed higher activity compared to *T. viride* cellulase in the presence of [C2mim][OAc] (Table 3) for IL-pretreated cellulose, verifying our earlier results using a soluble substrate. The CMCase activity results of the three cellulases are not indicative of the activity on non-pretreated biomass where the *T. viride* outperforms the extremophilic cellulases. However since IL pretreatment increases the amount of sugars released, it would thus be preferred to pretreat the biomass prior to enzymatic hydrolysis, thus underscoring the need for IL-tolerant cellulase.

Table 3 Activity assay on insoluble substrates in the absence of [C2mim][OAc] and presence of [C2mim][OAc]. The hydrolysis reactions were performed under pH and temperatures for each enzyme as listed in the Methods section. The reactions were run over a 14 h period with 5–10 μg of enzyme with 6% (w/v) enzyme loading in optimum buffer for each enzyme and run in duplicate. The reaction products were measured on a HPLC and HPAEC. The amounts of sugars detected are shown in μmoles and normalized with regards to amount of enzyme and time of reaction. Different reactions under each enzyme were run under exactly similar conditions

% [C2mim][OAc]	Enzyme	Substrate	Sugars produced [a]/μmoles min^{-1} mg^{-1}
0	*T. viride* cellulase	Avicel	3 ± 0.5
		pAvicel	5 ± 0.9
		Corn stover	2.8 ± 0.8
		pCorn stover	6 ± 0.9
	Tma Cel5A	Avicel	1.1 ± 0.1
		pAvicel	6.6 ± 0.8
		Corn stover	3 ± 0.9
		pCorn stover	9 ± 1
	Pho EG	Avicel	0.54 ± 0.1
		pAvicel	3.2 ± 0.2
		Corn stover	0.58 ± 0.1
		pCorn stover	2.14 ± 0.1
5	*T. viride* cellulase	Avicel	2.1 ± 0.6
		pAvicel	4 ± 0.9
		Corn stover	2.3 ± 0.5
		pCorn stover	3.6 ± 0.6
	Tma Cel5A	Avicel	1.0 ± 0.2
		pAvicel	6 ± 0.5
		Corn stover	2 ± 0.3
		pCorn stover	8 ± 0.9
	Pho EG	Avicel	0.5 ± 0.1
		pAvicel	3.3 ± 0.2
		Corn stover	0.59 ± 0.1
		pCorn stover	2.2 ± 0.14
10	*T. viride* cellulase	Avicel	0.2 ± 0.1
		pAvicel	1 ± 0.1
		Corn stover	0.6 ± 0.3
		pCorn stover	1.1 ± 0.3
	Tma Cel5A	Avicel	0.9 ± 0.1
		pAvicel	5.7 ± 1
		Corn stover	2.4 ± 0.8
		pCorn stover	7 ± 1
	Pho EG	Avicel	0.52 ± 0.1
		pAvicel	3.1 ± 0.2
		Corn stover	0.57 ± 0.1
		pCorn stover	2.0 ± 0.2
15	*T. viride* celulase	Avicel	<0.01
		pAvicel	<0.01
		Corn stover	<0.01
		pCorn stover	<0.01
	Tma Cel5A	Avicel	0.6 ± 0.1
		pAvicel	4 ± 0.5
		Corn stover	2.3 ± 0.5
		pCorn stover	5.9 ± 1
	Pho EG	Avicel	0.6 ± 0.08
		pAvicel	2.9 ± 0.06
		Corn stover	0.59 ± 0.1
		pCorn stover	1.9 ± 0.1

[a] The sum of glucose, cellobiose and xylose obtained, normalized by enzyme amount and time. [b] The reactions were spiked with 0 to 15% (v/v) [C2mim][OAc], everything else being the same for all substrates for an enzyme. [c] The sum of glucose, cellobiose and xylose obtained, normalized by enzyme amount and time.

Future studies would focus on using optimum sized particles, solids loading during the saccharification reaction as well as enzyme loadings.

The DSC experiments indicate that at higher concentrations of [C2mim][OAc] the enzymes unfold and are less stable. Thus, the loss of activity could be accounted by the denaturation of the enzyme or a disruption of the tertiary structure of the protein. It has been speculated that halotolerant enzymes are more stable in ILs in comparison to non-halotolerant enzymes, because of the inherent ability of such organisms to live in high salt concentration environments.[11] However, we found that the recombinant proteins *Tma* Cel5A and *Pho* EG from non-halotolerant organisms maintained around 80% of their activity in 1.5 M sodium or potassium chloride (ESI, Fig. 2).† This is in contrast to general findings that non-halotolerant cellulases show reduced activity at high salt concentrations.[21] This implies a different mechanism of denaturation depending on the identity of the cation and anion of the IL. Further studies are needed to better understand the differences in behaviour of *Tma* Cel5A and *Pho* EG at higher concentrations of ionic liquids to get a better understanding of the mechanisms of deactivation, and to investigate if other IL's used in biomass pretreatment would better stabilize the cellulases.

The recombinant enzymes studied here are members of the GH5 family, supporting the speculation that the higher resistance to ILs is due to a common structural motif.[32] Based on saturation mutagenesis studies, J. Pottkämper *et al.*[32] have suggested that motifs within the N-terminal part of the protein and within carbohydrate-binding domain are responsible for IL tolerance. We speculate that the hyperthermophilic properties of these enzymes may lead to stability of the enzymes in ionic liquids. Related studies with IL tolerance of other cellulases from hyperthermophilic organisms support a similar trend (S. Datta, J. I. Park and R. Sapra—unpublished results).

Conclusions

While there are a number of reports in literature on using ILs to deconstruct cellulosic biomass, the present work reports on the effects of ILs on purified and active recombinant cellulases. Our work suggests that hyperthermophilic cellulases can tolerate around 15% (v/v) of ILs, which reflects the IL content that may be present from a large-scale commercial IL pretreatment process. Fungal enzymes studied did not possess this tolerance to ILs. Further work is needed to discover, evolve and engineer cellulases with longer half-lives that are more stable and active in higher concentrations of IL, towards the ultimate goal of cheaper and efficient saccharification of biomass.

Experimental

Chemicals

All chemicals used were reagent grade. *Trichoderma viride* cellulase (Sigma Cat # C9422), sodium acetate, 3,5-dinitrosalicylic acid (DNS), sodium hydroxide, sodium potassium tartrate and carboxymethylcellulose (CMC) was obtained from Sigma (St. Louis, MO). 1-ethyl-3-methylimidazolium acetate ([C2mim][OAc]) was obtained from BASF, USA.

PCR and cloning of endoglucanase from *T. maritima* and *P. horikoshii*

The open reading frame of the endoglucanase from *Tma* Cel5A (NP_229549), and *Pho* EG (UniProt ID: O58925) was synthesized and codon optimized for protein expression in *E. coli* (GenScript Corporation, Piscataway, NJ). *Tma* Cel5A was cloned by ligation independent cloning method into the expression vector, pCDF2 LIC/Ek (Novagen/EMD Chemicals, Gibbstown, NJ), using the kit supplied by the manufacturer. The *Pho* EG amplicon was gel-purified, and then cloned into pDONR221 (Invitrogen, Carlsbad, CA) *via* BP recombination reaction to create an entry vector. The *Pho* EG gene was then inserted into pET DEST42 vector (Invitrogen, Carlsbad, CA) as a fusion to a C-terminal V5 epitope and His(×6) tags by LR recombination reaction to create pET DEST42-PhoEG plasmid. The DNA sequence of entry and expression vectors for *Pho* EG and expression vector containing *Tma* Cel5A was confirmed by DNA sequencing.

Protein expression and purification

Escherichia coli BL21(DE3) star transformed with the plasmid construct was grown in Luria–Bertani (LB) medium containing streptomycin (50 μg mL^{-1}) at 37 °C, induced with 0.5 mM IPTG (at $A_{600\,nm} = 0.5–0.7$), and then grown overnight at 30 °C. *Tma* Cel5A with a C-terminal His tag was purified from cells (10 g wet weight) suspended in 100 mL of 10 mM potassium phosphate buffer (pH 7.2) and containing 0.15 mg mL^{-1} lysozyme and one tablet of complete EDTA-free protease inhibitor (Roche). After sonication, NaCl (150 mM) and imidazole (10 mM) were added to achieve the specified final concentrations and loaded onto a Ni-nitrilotriacetic acid column (GE Healthcare, Piscataway, NJ) equilibrated with phosphate buffer containing 150 mM NaCl, and 10 mM imidazole. The column was washed extensively, and proteins were eluted with a 10–400 mM imidazole gradient. The protein eluted between 100 and 350 mM imidazole and were pooled and dialyzed against 100 mM Acetate buffer (pH 4.8). The *Pho* EG was purified under similar conditions with an additional purification step by Q Sepharose High Performance column (GE Healthcare, Piscataway, NJ) using manufacturer recommended protocol and dialyzed against 100 mM MES buffer (pH 6.4). The protein concentrations were measured by Bradford Assay for both recombinant and commercial enzymes used in this study. The recombinant protein purity was visually assessed using SDS-PAGE.

Avicel and corn stover pretreatment by [C2mim][OAc]

[C2mim][OAc] (1 kg, as received, < 0.2% moisture specified) was heated to 130 °C in a 1.5 L glass reaction vessel with mechanical stirring and 8% w/w (corn stover, 4.8% moisture) or 10% w/w (Avicel PH-101, 3% moisture). After around 3 h when the majority (corn stover) or all (Avicel) had dissolved, the dissolved corn stover or Avicel was allowed to cool to below 80 °C and was added to 2 L of 95% ethanol with rapid agitation to induce the precipitation of dissolved materials. The resulting slurry was filtered under pressure through polypropylene filter cloth and the solids redispersed in 2 L of additional ethanol. The filtration and redispersion steps were repeated twice to remove

most residual ionic liquid and the filter cake dried under vacuum at 40 °C to yield a free-flowing powder product. Residual free ionic liquid remaining in the dried biomass is estimated to be less than 4% (w/w).

Enzyme activity assays on soluble and insoluble polysaccharides

Enzymatic activity was measured on soluble polysaccharide substrates, CMC, using the 3,5-dinitrosalicylic acid (DNS) reducing sugar assay.[34] Briefly, 2% CMC (w/v) in 120 μL acetate buffer, (100 mM, pH 4.5 and pH 4.8 for *T. viride* and *Tma*, respectively, and 100 mM MES buffer, pH 6.4 for *P. hori* EG) was incubated at 80 °C for 30 min for *Tma* Cel5A and *Pho* enzymes and 37 °C and 10 min for *T. viride*. The solution was cooled down to 4 °C and 80 μL of DNS solution was added. The reactants were incubated at 95 °C for 5 min and cooled down to room temperature before the absorbance was read at 540 nm. The reducing sugar concentration in the sample was calculated from its absorbance using the standard curve of D-glucose and cellobiose. All experiments were run in triplicate. For hydrolysis reactions in the presence of [C2mim][OAc], control reactions with CMC in the presence of IL but without no enzyme was subtracted from each measurement.

The solid substrate (Avicel and corn stover) hydrolysis reactions were conducted at 80 °C for 15 h for the recombinant enzymes and at 37 °C for 8 h in the case of *T. viride* cellulase at the optimum pH for each enzyme. The reaction products were monitored using an Agilent 1200 HPLC equipped with Varian 380-LC Evaporative Light Scattering Detector. The total reaction volumes were 500 μL and shaken at 900 rpm at temperature controlled shakers. Enzyme loadings were 0.4 mg per g of glucan and each data point was measured in triplicate. Separation was achieved using a Varian/Polymer labs Hi-Plex Pb carbohydrate analytical column (300 × 7.7 mm) with a guard column (50 × 7.7 mm) at 85 °C (Polymer Laboratories, Varian Inc., Shropshire, UK). The mobile phase was deionized water with a flow rate of 0.6 mL min^{-1}. Hydrolysis reactions in the presence of [C2mim][OAc] were monitored by either by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) on a Dionex DX600 equipped with a Dionex Carbopac PA-20 analytical column (3 × 150 mm) and a Carbopac PA-20 guard column (3 × 30 mm) (Dionex, Sunnyvale, CA). Eluent flow rate was 0.4 mL min^{-1} and the temperature was 30 °C. A gradient consisting of a 12 min elution with 14 mM NaOH followed by a 5 min ramp to 450 mM NaOH for 20 min, then a return to the original NaOH concentration of 14 mM for 10 min prior to the next injection. Product concentrations were determined by the integrations of the appropriate peaks from the HPLC or HPAEC chromatograms.

Estimation of temperature dependence and thermostability

T. viride enzyme was assayed at 37 °C and pH 4.5 as suggested by the manufacturer. The pH dependence of the recombinant enzymes was determined by measuring specific activities of the enzyme on CMC in a series of pH values between 4 and 5 with 100 mM sodium acetate buffer, and between 5 and 8 with sodium phosphate buffer. The optimal temperature of *Tma* Cel5A was determined by measuring specific activity of the enzyme on CMC in 100 mM sodium acetate buffer (pH 4.8) at various temperatures. Similar experiments in 50 mM MES buffer, pH 6.4 was done for *Pho* EG. Thermostability was determined by incubating the enzyme at 80 °C in 100 mM acetate buffer, pH 4.8 for Cel5A and 50 mM MES buffer, pH 6.4 for *Pho* EG and for 24 h prior to determining specific activity.

Differential scanning calorimetry

Unfolding temperatures of *Tma* Cel5A (5 mg mL^{-1}) in the absence and presence of 5–50% (v/v) of [C2mim][OAc] were determined with a multi-cell differential scanning calorimeter (Calorimetry Sciences Corporation, Lindon, UT, USA). The [C2mim][OAc] containing samples were dialyzed overnight against 100 mM sodium acetate buffer (pH 4.8) in the presence of similar amounts [C2mim][OAc] as in sample. The dialyzed enzyme was scanned between 25 and 100 °C using a scan rate of 0.5 °C min^{-1}. The enzyme scans were corrected with a buffer–buffer baseline. The *T. viride* cellulase (2 mg mL^{-1}) was run under similar conditions in the presence of 0–20% (v/v) [C2mim][OAc]. Unfolding temperatures of *Pho* EG (2.6 mg mL^{-1}) were determined similarly to the Tma but on a NanoDSC, Differential Scanning Calorimeter (TA Instruments—Waters LLC, New Castle, DE). The protein sample was dialyzed overnight against the presence of similar amounts of IL as in sample in the pH_{opt} buffer. A scan rate of 2 °C min^{-1} was used and the data analyzed using the NanoAnalyze software supplied by the manufacturer.

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