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Enrichment, isolation and characterization of fungi tolerant to 1-ethyl-3-methylimidazolium acetate

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Abstract

Aims: This work aims to characterize microbial tolerance to 1-ethyl-3-methylimidazolium acetate ([C2mim][OAc]), ionic liquid that has emerged as a novel biomass pretreatment for lignocellulosic biomass.

Methods and Results: Enrichment experiments performed using inocula treated with [C2mim][OAc] under solid and liquid cultivation yielded fungal populationsdominated by Aspergilli. Ionic liquid-tolerant Aspergillus isolates from these enrichments were capable of growing in a radial plate growth assay in the presence of 10% [C2mim][OAc]. When a [C2mim][OAc]-tolerant *Aspergillus fumigatus* strain was grown in the presence of switchgrass, endoglucanases and xylanases were secreted that retained residual enzymatic activity in the presence of 20% [C2mim][OAc].

Conclusions: The results of the study suggest tolerance to ionic liquids is a general property of *Aspergilli*.

Significance and Impact of the Study: Tolerance to an industrially important ionic liquid was discovered in a fungal genera that is widely used in biotechnology, including biomass deconstruction.

Introduction:

Lignocellulosic biomass is a potential renewable feedstock that is available for conversion to biofuels that will replace a substantial amount of the petroleum-based transportation fuels (Gomez et al. 2008). Currently, most first-generation biofuels are derived from agricultural food sources such as corn grain and soybeans. Lignocellulosic biomass, which includes agricultural residues (corn stover, rice straw), agricultural by-products (corn fibre, rice hulls and sugarcane bagasse), woody biomass (poplar, pine) and dedicated energy crops (Miscanthus, switchgrass), has a low impact on human food supplies (Blanch et al. 2008). Pretreatment of lignocellulosic biomass is required for efficient saccharification to generate monomeric sugars for biofuel production. Established chemical pretreatment technologies, such as dilute acid and ammonia fibre expansion, greatly improve the yields of sugar from enzymatic hydrolysis, but new advances in pretreatment are needed for industrial production of cellulosic biofuels (Yang and Wyman 2008). Recently, ionic liquids (Fig. 1) have demonstrated excellent promise as solvents for biomass dissolution and cellulose recovery (Swatloski et al. 2002; Turner et al. 2004). The recovered cellulose is enzymatically hydrolyzed to glucose at much faster rates than observed for dilute acid pretreated biomass (Li et al. 2010).

Room temperature ionic liquid 1-ethyl-3-methylimidazolium acetate [C2mim][OAc] has proved to be particularly effective at dissolution of cellulose, hemicellulose and lignin from switchgrass, corn stover and eucalyptus (Singh et al. 2009). A combination of electrostatic and hydrogen-bonding interactions between ionic liquid and the plant polymers has been proposed as

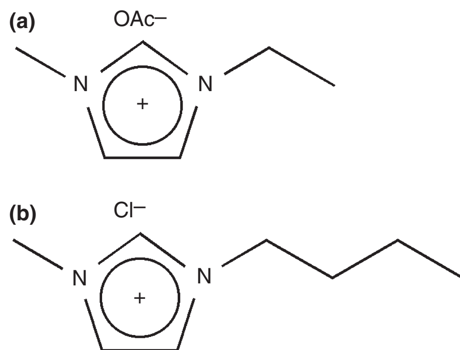

Figure 1 Ionic liquids discussed in this study: 1-ethyl-3-methylimidazolium acetate (a); 1-butyl-3-methylimidazolium chloride (b).

dissolution mechanism (Remsing *et al.* 2006; Sun *et al.* 2009). However, ionic liquids have been shown to inhibit enzymatic hydrolysis of cellulose (Turner *et al.* 2003, 2004; Docherty and Kulpa 2005). Therefore, microbes tolerant to ionic liquids may be valuable as sources of glycoside hydrolase enzymes that tolerate residual ionic liquid remaining in biomass. Ionic liquids have been reported to be toxic at low levels (<1% v/v) to both prokaryotes and eukaryotes; however, there are a few reports of high levels of tolerance by specific bacterial and fungal species (Docherty and Kulpa 2005; Petkovic *et al.* 2009). To identify ionic liquid-tolerant microbes that may be relevant to biomass deconstruction, we performed enrichments in the presence of [C2mim][OAc] in liquid cultures with inocula from lignocellulose-deconstructing environments and in solid-state cultures under simulated compost conditions. Here, we report that these enrichments are often dominated by *Aspergillus* populations and that multiple *Aspergillus* strains isolated from these lignocellulose-deconstructing environments grow in the presence of high levels of ionic liquids. Additionally, we report the activity of endoglucanases and xylanases secreted by one of these strains in the presence of ionic liquids.

Materials and methods

Chemicals

All chemicals were reagent grade and were obtained from Sigma (St Louis, MO, USA) unless otherwise noted and used as received.

Isolation of microbes with [C2mim][OAc] as sole carbon source

[C2mim][OAc] (1% v/v or 60 mmol l^{-1}) was added to 50 ml of IL media (0·3 g l^{-1} Na_2HPO_4, 0·4 g l^{-1} KH_2PO_4, 0·2 g l^{-1} NH_4Cl, 0·04 g l^{-1} $MgSO_4$, 0·02 g l^{-1} $CaCl_2$, 0·002 g l^{-1} $FeCl_3$; pH 7·0) in a 250-ml Erlenmeyer flask and then inoculated with *c.* 50 mg of green waste compost obtained from Grover Soil Solutions in Zamora, CA, USA (Allgaier *et al.* 2010). The flask was shaken at 150 rev min^{-1} for 4 days at 37°C, after which fungal growth was evident. Five millilitres of this enrichment was inoculated into a second 250-ml Erlenmeyer flask with 50 ml of IL media/1% [C2mim][OAc] and shaken for 4 days at 37°C. A second passage of the enrichment was performed under identical conditions, and then, 100 μl of the enrichment was spread onto 1·5% agar plates containing IL media/1% [C2mim][OAc] and the plates incubated at 37°C for days. After 2 days, fungal spores were sampled with an inoculating loop and streaked onto 1·5% agar/IL media/1% [C2mim][OAc] plates. These plates were incubated for 2 days, and then, the fungal spores were inoculated into potato dextrose broth for 3 days of growth. The absence of bacterial contamination was confirmed microscopically and the identity of the isolated fungal strain determined by DNA isolation and PCR amplification of the ITS1-5.8S-ITS2 region using primers 5′-TCCGTAGGTGAACCTGCGG-3′ (ITS1) and 5′-TCCTCCGCTTATTGATATGC-3′ (ITS4) (Gardes and Bruns 1993). An identical enrichment was performed at 30°C; however, only bacterial colonies were observed in the agar plates. Enrichments were performed under the same conditions (37 and 30°C) as described above with soil from a garden in Berkeley, CA, USA, and decaying wood collected from Mount Tamalpais State Park, Mill Valley, CA, USA, affording four additional fungal strains (see Results). All of these fungal strains were maintained on potato dextrose agar slants at 4°C.

Identification of fungi tolerant to [C2mim][OAc] from a compost culture collection

Fungi were isolated from green waste compost obtained from Grover Soil Solutions located in Zamora (DeAngelis *et al.* 2010). Three grams of compost was suspended in 30 ml of a 5-mmol l^{-1} NaCl solution in a 50-ml conical tube and vortexed for 3 min. Serial dilutions of the supernatant were spread on a variety of solid media (Luria–Bertini, potato dextrose, typtic soy broth) and cultured at 37°C for 1–3 days. Fungal isolates were identified by morphology and were restreaked three times on solid media to ensure the colonies were pure. Isolates were then tested for their ability to grow in LB, giving 19 fungi in the compost culture collection. Fungi tolerant to [C2mim][OAc] from this collection were identified by inoculating 5 μl of cultures grown overnight in LB media into 96-well plates containing 200 μl of LB media with

5% [C2mim][OAc], and growing the cultures for 5 days at 37°C.

Solid-state culturing of compost and switchgrass amended with [C2mim][OAc]

Milled switchgrass was wet with sterile distilled water to a targeted moisture content of 400% dry basis (80% wet basis) and allowed to equilibrate at 4°C overnight. Switchgrass was then mixed with the appropriate amount of [C2mim][OAc] to achieve 0, 2, 4, 6, 8 and 11% ionic liquid, based on wet weight. Immediately before loading bioreactors, green waste compost was mixed into each batch of switchgrass at a loading rate of 10% by dry weight.

Bioreactors and respiration measurements for solid-state cultures have been described previously (Reddy *et al.* 2009). Briefly, for each treatment, three bioreactors with a 0·2-l working volume were loaded with 10 g dry weight of the corresponding switchgrass/[C2mim][OAc]/compost mixture. Air was supplied to each bioreactor at 20 ml min^{-1} and incubations ran for 7 days. The incubator temperature was maintained at 30°C for 3 days, ramped to 55°C over 1 day and held at 55°C for 3 days. Temperature was monitored with a HOBO data logger (Onset Computer Corporation, Bourne, MA, USA).

Carbon dioxide concentration was measured on the influent and effluent air of the reactors using an infrared CO_2 sensor (Vaisala, Woburn, MA, USA), and flow was measured with a thermal mass flow meter (Aalborg, Orangeburg, NY, USA). Carbon dioxide and flow data were recorded every 20 min using a data acquisition system (Reddy *et al.* 2009). Carbon dioxide evolution rates (CER) were calculated from mass balances on the reactors according to the following equation:

$$\mathrm{CER} = F(\mathrm{CO}_{2,\mathrm{OUT}} - \mathrm{CO}_{2,\mathrm{IN}}) \quad (1)$$

where F is the air flow rate (mg air day^{-1} gdw^{-1}), $CO_{2,OUT}$ and $CO_{2,IN}$ are the concentrations of carbon dioxide in the effluent and influent air, respectively (mg CO_2 mg air^{-1}). Numerical integration of CER results to find cumulative carbon dioxide evolution (cCER) was performed using KaleidaGraph ver. 4.0 (Synergy Software, Reading, PA, USA).

Fungal hyphae were sampled from bioreactors with visible fungal mats, which included treatments with 2, 4 and 6% [C2mim][OAc] (Fig. 3). DNA was extracted from the hyphae with cetyl trimethylammonium bromide and bead beating, as described previously (Allgaier *et al.* 2010). DNA was amplified with ITS1/ITS4 primers (described above) and cloned into *Escherichia coli* using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA). Sequenced inserts were assembled, trimmed and aligned by a Blast search with Geneious 4.8.5 (http://www.geneious.com).

Radial growth assay in the presence of [C2mim][OAc]

Aspergillus strains JF1 (*A. fumigatus*), JF4 (*A. ustus*) and UCDF1 (*A. oryzae*) were grown on potato dextrose agar slants, and 5 ml of sterile water added to each to generate a spore suspension (10^6–10^7 spores ml^{-1}). Fifty microlitres of the spore suspension was pipetted onto 1·5% agar containing IL media and varying amounts of [C2mim][OAc] and [C4mim][Cl] as described in the Results section. Plates inoculated with spores from strains JF4 and UCDF1 were incubated at 30°C for 4 days, and plates inoculated with spores from strain JF1 were incubated at 37°C for 4 days. All plate assays were performed in triplicate.

Enzymatic activity in supernatants of strain JF1 grown on biomass substrates

Spores of strain *A. fumigatus* JF1 were inoculated into 50 ml of potato dextrose broth and grown at 37°C for 3 days. After 3 days of growth, 5 ml of spores was inoculated into 250-ml Erlenmeyer flasks containing 50 ml of IL media and 1% w/v of biomass substrates (Avicel, birchwood xylan, corn switchgrass, corn stover) as described in the Results section. These cultures were shaken for 3 days at 37°C and the cultures filtered through MiraCloth™ to separate culture supernatants and fungal mycelia. Endoglucanase and xylanase activities were measured using carboxymethylcellulose and birchwood xylan, respectively, as substrates. Reactions were incubated for 30 min at 50°C in 100 mmol l^{-1} NaOAc pH 5·0 buffer and sugar release measured by DNS assay (Xiao *et al.* 2005; Datta *et al.* 2010). To assess the stability of the endoglucanases and xylanases in the presence of [C2mim][OAc], the culture supernatant was diluted 1 : 2 in 100 mmol l^{-1} NaOAc pH 5·0 with 0 and 10% [C2mim][OAc] and incubated for 12 h at 50°C. Aliquots were removed at 0, 6 and 12 h and assayed for activity as described earlier. All assays were performed in triplicate.

Results

Microbial isolates enriched with [C2mim][OAc] as sole carbon source

Initial studies focused on liquid culture enrichment of microbes from lignocellulose-deconstructing environments using [C2mim][OAc] as the sole carbon source. Inoculation of minimal media containing 1% (v/v) [C2mim][OAc] with green waste compost at 37°C

produced fungal growth in the enrichment culture. After successive transfers in liquid media, the fungal-dominated culture was plated onto agar containing 1% [C2mim][OAc]. A fungal isolate with typical *Aspergillus* morphology was obtained, and the amplicon obtained using primers designed to amplify the fungal ITS1-5.8S-ITS2 sequence was identical to the sequences obtained for strains of *A. fumigatus* (Table 1). Fungal isolates of identical morphology were also obtained from enrichments performed under the same conditions with garden soil and decaying wood at 37°C; PCR amplification of the same region also demonstrated that these isolates had sequences nearly identical to characterized strains of *A. fumigatus*. Complementary enrichments were performed with the same media and inocula at 30°C. In contrast to the 37°C isolations, a strain with an identical sequence to *A. fumigatus* was only isolated from the 30°C garden soil enrichment. Enrichment with decaying wood as inoculum afforded a microbial isolate with a morphology and an amplified sequence (99% identical) consistent with its description as *Aspergillus ustus*. In contrast, an enrichment with green waste compost at 30°C afforded bacterial colonies. No microbial growth was observed in companion enrichments with 1-butyl-4-methylimidazolium chloride ([C4min][Cl]), another commonly used ionic liquid in biomass pretreatment, as the sole carbon source (Remsing *et al.* 2006; Fort *et al.* 2007).

Fungal tolerance to [C2mim][OAc] was also tested by establishing an isolate collection of fungi from the same green waste compost sample described earlier. From enrichments on various media, 19 fungal isolates were obtained. These isolates were cultured in 96-well plates in LB media in the presence of varying amounts of [C2mim][OAc]. Of these fungal isolates, only one grew in >1% [C2mim][OAc]. Its ITS1-5.8S-ITS2 amplicon was identical to the *A. fumigatus* isolates previously obtained by enrichment with [C2mim][OAc] (Table 1).

Solid-state cultures in the presence of [C2mim][OAc]

The enrichment of *Aspergilli* in the presence of [C2mim][OAc] was further demonstrated by performing solid-state incubations with compost as inoculum and switchgrass as substrate. Microbial community analysis of a simulated composting incubation with the same compost inoculum and switchgrass as the sole carbon source cultivated a diverse microbial community dominated by bacteria (Allgaier *et al.* 2010). A peak in respiration was observed after the temperature was ramped to 55°C, demonstrating the presence of thermophiles in the inoculum (Allgaier *et al.* 2010). Under these identical conditions, respiration profiles were significantly altered with [C2mim][OAc] amendment. Lag time increased and cCER decreased with increasing ionic liquid concentration (Fig. 2). For treatments with >0% [C2mim][OAc], there was no increase in activity during the thermophilic phase of the incubation, demonstrating only mesophilic activity in the presence of ionic liquid. Above 8% [C2mim][OAc], there was no measurable microbial activity. Treatments with 2, 4 and 6% [C2mim][OAc] had fungal mats on the surface of the switchgrass (Fig. 3). The density of the mat increased with increasing ionic liquid between 2 and 4%. Thirty-three clones were obtained by PCR amplification of the ITS1-5.8S-ITS2 region of DNA extracted from these fungal mats. Sequences of the majority of these clones were closely related to *Aspergillus* strains (*A. niger*, *A. oryzae*, *A. flavus*) confirming the resistance of *Aspergilli* to high levels of [C2mim][OAc] (Table 2). *Lichtheimia ramosa*, a saprotrophic fungi (Alastruey-Izquierdo *et al.* 2010), was also present in the bioreactor

Table 1 Ionic liquid-tolerant fungal isolates from lignocellulose-deconstructing environments

Strain	Inoculum	Enrichment conditions	Fungal identification*,†
JF1	Green waste compost	1% [C2mim][OAc]/IL media, 37°C	*Aspergillus fumigatus* (HQ882183)
JF4	Garden soil	1% [C2mim][OAc]/IL media, 30°C	*Aspergillus ustus* (HQ882184)
JF5	Decaying wood	1% [C2mim][OAc]/IL media, 30°C	*A. fumigatus* (HQ882185)
JF6	Garden soil	1% [C2mim][OAc]/IL media, 37°C	*A. fumigatus* (HQ882186)
JF7	Decaying wood	1% [C2mim][OAc]/IL media, 37°C	*A. fumigatus* (HQ882187)
JF11	Green waste compost	LB media, 37°C	*A. fumigatus* (HQ882188)
UCDF1	Green waste compost	Solid state/PD agar, 30°C	*Aspergillus flavus/oryzae*‡,§ (HQ882189)
UCDF3	Green waste compost	Solid state/PD agar, 30°C	*A. flavus/oryzae*§ (HQ882190)
UCDF5	Solid-state bioreactor	Solid state/PD agar, 30°C	*Lichtheimia ramosa* (HQ882191)

*Fungal identification was based on ≥99% identity of ITS1-5.8S-ITS2 sequence to known strains.

†Nucleotide accession numbers for isolates deposited in GenBank are in parenthesis.

‡Identification was validated by amplification and sequencing of β-tubulin gene (100% identity). For UCDF1, the β-tubulin sequence was 100% identical to the *A. oryzae* RIB40 (Machida *et al.* 2005).

§*A. flavus* and *A. oryzae* cannot be distinguished by sequencing of the ITS1-5.8S-ITS2 region (Kumeda and Asao 1996).

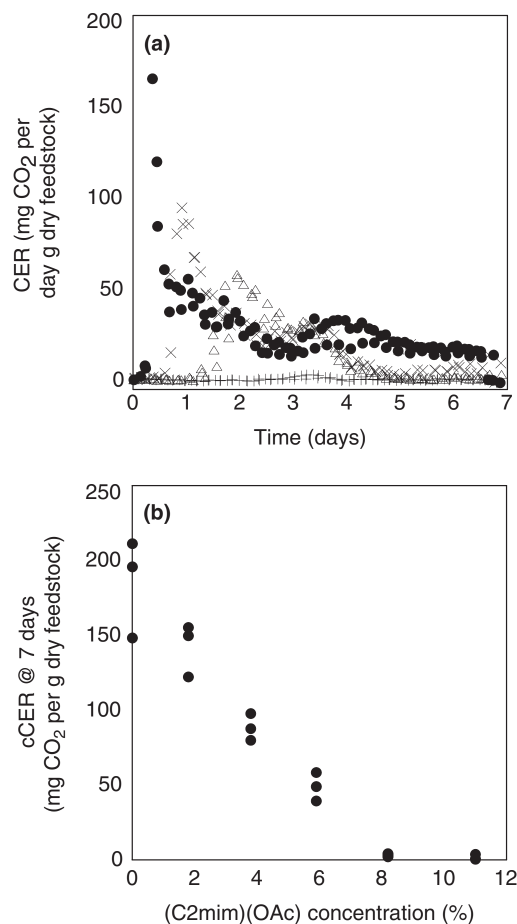

Figure 2 Respiration profiles for solid-state cultures in the presence of [C2mim][OAc]: (a) carbon dioxide evolution rate (CER) over 7 days; (b) cumulative carbon dioxide evolution (cCER) after 7 days. All cultivations were performed in triplicate. (●, 0 %; ×, 2 %; △, 4 %; and +, 8 %).

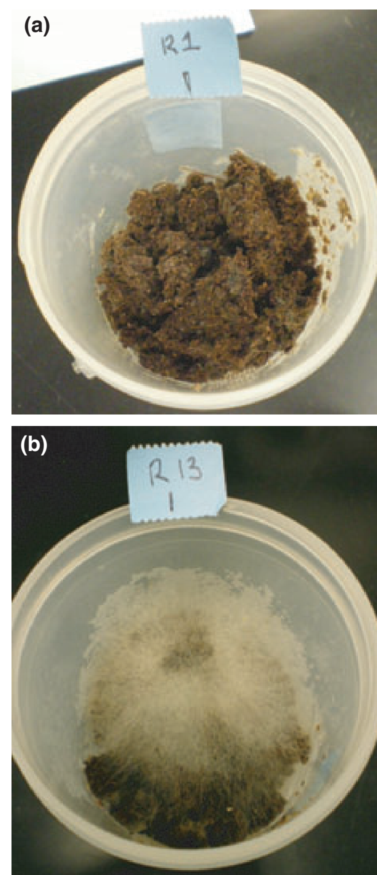

Figure 3 Pictures of solid-state fermentation reactors containing compost and switchgrass after 7 days incubation as described in Materials and methods in the presence of: (a) 0% [C2mim][OAc]; (b) 4% [C2mim][OAc].

with 4% [C2mim][OAc] amendment and was isolated from the same bioreactor (Table 2). Isolates closely related to *A. flavus* and *A. oryzae* (UCDF1 and UCDF3) were obtained from the 2% bioreactor.

Table 2 Identification of PCR products from solid-state fermentation of switchgrass inoculated with green waste compost in the presence of [C2mim][OAc]

[C2mim][OAc] amendment	No. of clones	% Pairwise identity	Fungal identification
2%	10	100	*Aspergillus flavus/oryzae*
4%	1	99·8	*Aspergillus niger*
	2	100	*Lichtheimia ramosa*
6%	20	100	*A. flavus/oryzae*

Demonstration of IL tolerance of *Aspergillus* strains

To determine the extent of ionic liquid tolerance in the isolated *Aspergilli*, three strains were chosen: strain JF1, JF4 and UCDF1, which represented the range of *Aspergillus* species enriched in the presence of [C2mim][OAc] (Table 3). These three strains were incubated on agar plates containing varying levels of [C2mim][OAc] and radial growth measured (D'Annibale *et al.* 2006). In all three cases, radial growth was inhibited at increasing concentrations of [C2mim][OAc]; however, growth was evident at 10% [C2mim][OAc], consistent with a very high level of resistance to ionic liquids (Table 3). When these three strains were incubated in plates containing 5% [C4mim][Cl], no growth was observed under the same conditions, indicating that these strains are incapable of growing on [C4mim][Cl]. However, when 1% NaOAc was added to plates containing 5% [C4mim][Cl], radial growth comparable to the radial growth observed for 5% [C2mim][OAc] was observed.

Table 3 Radial growth (diameter in mm) of *Aspergillus* strains in presence of ionic liquid

Strain	1% NaOAc	2·5% [C2mim] [OAc]	5·0% [C2mim] [OAc]	7·5% [C2mim] [OAc]	10% [C2mim] [OAc]	5% [C4mim] [Cl]/1% NaOAc
JF1	18·0 ± 0·5	13·0 ± 1·0	14·0 ± 1·0	10·0 ± 0·5	8·0 ± 1·0	11·0 ± 0·5
JF4	16·0 ± 0·5	16·0 ± 1·5	10·0 ± 0·5	7·5 ± 0·5	5·0 ± 0·5	10·5 ± 1·5
UCDF1	19·0 ± 2·0	16·0 ± 1·0	13·0 ± 1·0	13·0 ± 0·5	11·0 ± 0·5	13·0 ± 0·5

Table 4 Endoglucanase and xylanase activity of *Aspergillus fumigatus* JF1 supernatants grown on biomass substrates

Substrate	Avicel	Xylan	Corn Stover	Switchgrass	Switchgrass/5% [C2mim][OAc]	Switchgrass/5% [C4mim][Cl]
CMC	ND*	0·27 ± 0·12†	0·71 ± 0·08	1·4 ± 0·15	0·04 ± 0·01	1·7 ± 0·08
Xylan	0·04 ± 0·01	0·38 ± 0·04	0·78 ± 0·14	3·3 ± 0·10	0·16 ± 0·01	4·2 ± 0·15

*Not detected.
†Enzymatic activity expressed in μmol of sugar (glucose or xylose) released min^{-1} ml^{-1} of supernatant.

IL tolerance of *Aspergillus fumigatus* enyzmes in the context of lignocellulose deconstruction

Of relevance to this work is the impact of ionic liquids on the expression and activity of lignocellulose-deconstructing enzymes. As *Aspergillus* strains have been extensively employed in deconstructing biomass and enzyme production, the isolation of ionic liquid-tolerant *Aspergilli* provides an opportunity to directly test the effect of ionic liquids on biomass deconstruction (Grigorevski-Lima *et al.* 2009). Incubations of the three representative *Aspergillus* strains (JF1, JF4 and UCDF1) in the presence of biomass substrates indicated that the *A. fumigatus* JF1 strain growing at 37°C possessed significantly higher levels of secreted endoglucanase and xylanase activities in comparison to the *A. ustus* and *A. oryzae*, so detailed studies were performed on the *A. fumigatus* strain (data not shown). Initial growth studies demonstrated that the highest endoglucancase and xylanase activities were obtained for supernatants isolated from *A. fumigatus* JF1 cultured in the presence of switchgrass; minimal activity in the supernatant was recovered from cultures grown with purified biomass substrates (Avicel, birchwood xylan) (Table 4). When *A. fumigatus* JF1 was cultivated on switchgrass in the presence of 1% [C2mim][OAc], fungal growth was observed, but no enzymatic activity was detected in the supernatant, consistent with the use of [C2mim][OAc] as the primary carbon source. In contrast, in the presence of 1% [C4mim][Cl]/switchgrass, comparable levels of enzymatic activity were recovered in the supernatant compared to the switchgrass-only culture. The levels of endoglucanase and xylanase activities are similar to previously reported results for other strains of *A. fumigatus* grown on biomass substrates (Stewart *et al.* 1983; Grigorevski-Lima *et al.* 2009).

To test the tolerance of the secreted enzymes to ionic liquids, endoglucanase and xylanase assays were performed on the switchgrass-derived supernatant in the presence of increasing levels of [C2mim][OAc]. Endoglucanase assays in the presence of increasing [C2mim][OAc] concentration demonstrated that the ionic liquid did inhibit enzymatic activity; however, 43% of the residual activity was retained at 20% IL (Fig. 4). Comparable levels of inhibition were also observed for in the xylanase activity assay, although the loss of activity was more pronounced at higher concentrations of [C2mim][OAc]. Incubating the

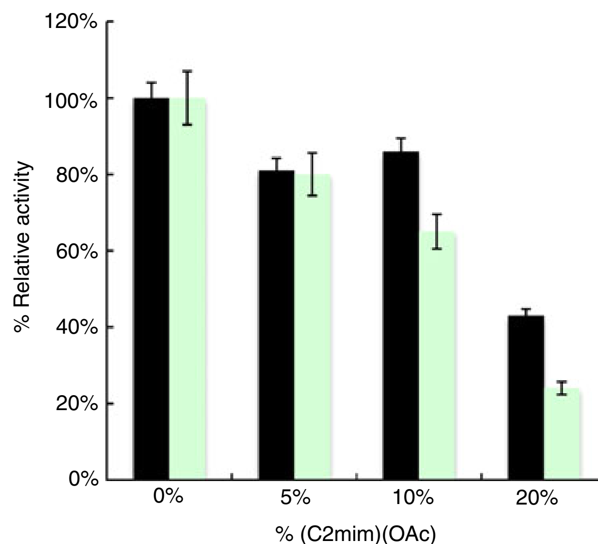

Figure 4 Endoglucanase and xylanase assays in the presence of [C2mim][OAc]. Incubations of *Aspergillus fumigatus* JF1 supernatant with carboxymethylcellulose and xylan were performed in the presence of varying concentrations of [C2mim][OAc] and release of sugars measured by DNS assay. (■) Endoglucanase and (□) Xylanase.

supernatant from strain JF1 in the presence of varying levels of [C2mim][OAc] demonstrated that the endoglucanase activity was quite stable in the presence of [C2mim][OAc], retaining approximately the same amount of activity after 12 h in the presence of 10% [C2mim][OAc] relative to an unamended control. In contrast, xylanase activity was dramatically reduced by incubation in the presence of 10% [C2mim][OAc] (Fig. 5a,b).

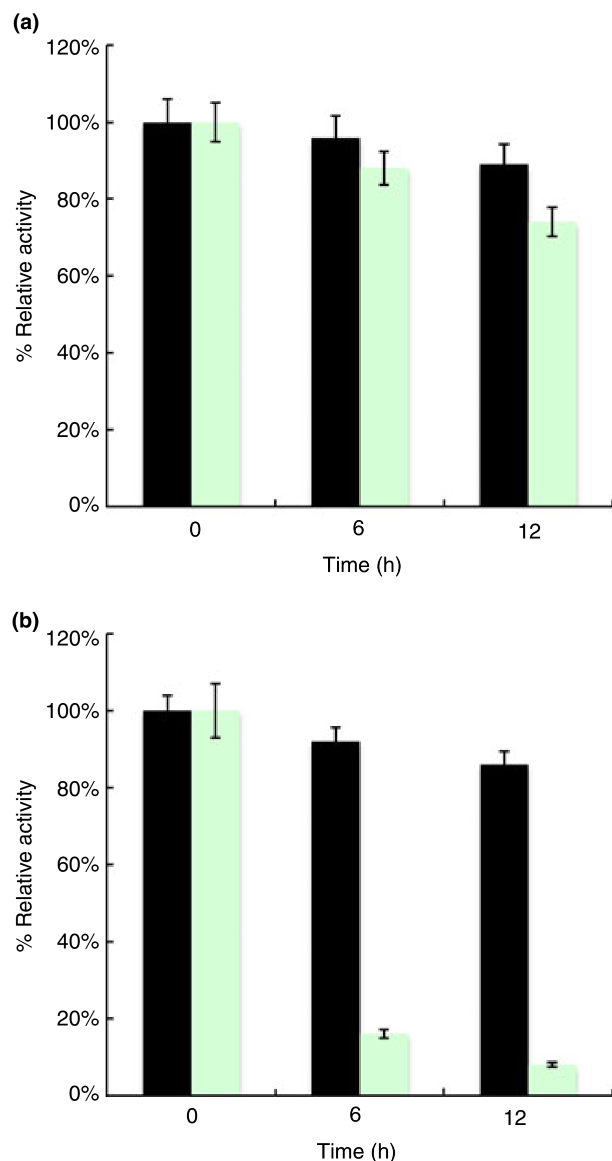

Figure 5 Stability of endoglucanases and xylanases in the presence of [C2mim][OAc]. *Aspergillus fumigatus* JF1 supernatant was preincubated for 12 h with varying concentrations of [C2mim][OAc]. Aliquots were removed at 0, 6 and 12 h and assayed for release of sugars from carboxymethylcellulose and xylan: (a) 0% [C2mim][OAc]; (b) 10% [C2mim][OAc]. (■) Endoglucanase and (□) Xylanase.

Discussion

We have demonstrated through three different cultivation methods that common *Aspergilli* isolated from soil and compost possess high tolerances to [C2mim][OAc] and can use this ionic liquid as their sole carbon source for growth. The absence of growth of these strains in the presence of [C4mim][Cl] suggests that the fungi utilize the acetate as the source of carbon; the ability of *Aspergillus* isolates to grow with acetate as the sole carbon source has been extensively documented (Collins and Kornberg 1960; Armitt *et al.* 1970; Meijer *et al.* 2009). These results are also consistent with previous observations that the imidazolium ring is resistant to biodegradation and may persist in the environment (Docherty and Kulpa 2005). The *Aspergilllus* strains described here grow at concentrations of ionic liquid ($\leq$0·6 mol l^{-1}) that are orders of magnitude higher than what has been previously reported for most eukaryotic organisms (Latala *et al.* 2010). Recently, however, substantial ionic liquid tolerance (up to 0·375 mol l^{-1} IL) was reported for *Penicillium* isolates (Petkovic *et al.* 2009). Therefore, some filamentous fungi may have inherent tolerance to ionic liquids. Although studies of the toxicity of ionic liquids are not extensive, reports have suggested that toxicity is related to oxidative stress response or disruption of cellular membranes (Docherty and Kulpa 2005).

High solids incubations with compost-amended switchgrass yielded a diverse prokaryotic-enriched microbial community in previous studies (Allgaier *et al.* 2010). A fungal mat was not observed in either the prior study or the treatment in the present study with no ionic liquid. The presence of a fungal mat on switchgrass with [C2mim][OAc] amendment suggests the ionic liquid inhibited the community that would normally establish during switchgrass decomposition and allowed the establishment of fungi, in particular *Aspergilli*.

Very little respiration was detected in incubations containing ionic liquid under thermophilic conditions. While *Aspergillus* ssp. have been identified in thermophilic composts (Le Goff *et al.* 2010), the enriched strains were not able to remain active under thermophilic conditions in the presence of ionic liquids. This may have been because of a number of factors including the thermotolerance of the selected community and their hydrolytic enzymes and the greater potential of the ionic liquids to solubilize polysaccharides in plant and fungal cell walls with increasing temperature.

Previous work on the tolerance of endoglucanases to ionic liquid demonstrated that endoglucanases from hyperthermophiles possessed substantial ionic liquid tolerance, retaining significant enzymatic activity at 30% [C2mim][OAc], while a commercial endoglucanase from

Trichoderma viridie was inactive at [C2mim][OAc] concentrations >5% (Datta *et al.* 2010). Additionally, commercial enzymatic endoglucanase cocktails obtained from Novozymes showed very low activity in reducing sugar assays with >10% [C2mim][OAc] (Gladden *et al.* 2011). Therefore, the endoglucanases secreted by the *A. fumigatus* strain isolated here, which retain significant residual activity in the presence of 20% [C2mim][OAc] and are stable in the presence of 10% [C2mim][OAc] at 50°C, tolerate [C2mim][OAc] at concentrations intermediate between commercial fungal enzymes and hyperthermophilic bacterial enzymes and may have application in deconstruction of biomass pretreated with [C2mim][OAc]. The xylanase enzymes secreted by the *A. fumigatus* strain show much lower stability on the presence of [C2mim][OAc]. The structural differences between these two types of enzymes from *A. fumigatus* may be instructive in understanding the stabilization of proteins in the presence of ionic liquids. Interaction of the acetate anion with hydrogen bonds in the protein structure has been proposed as a mechanism for protein denaturation in the presence of [C2mim][OAc] (Kaar *et al.* 2003). This work also demonstrated that the endoglucanases and xylanases were secreted and were active in the presence of ionic liquid [C4mim][Cl] while they were not secreted in the presence of [C2mim][OAc], consistent with the observation that *A. fumigatus* requires an additional carbon source in the presence of [C4mim][Cl], but preferentially grows on [C2mim][OAc] in the presence of switchgrass, which is a recalcitrant source of carbon.

Conclusions

In conclusion, we have shown that liquid and solid-state enrichment cultures in the presence of [C2mim][OAc] selected for fungal populations, especially *Aspergillus* species. *Aspergillus* isolates obtained from these enrichments were capable of growing in the presence of up to 10% [C2mim][OAc] and secreted glycoside hydrolase enzymes that were moderately stable to [C2mim][OAc]. The demonstration that *Aspergillus* species are tolerant to [C2mim][OAc] may lead to biotechnological applications that utilize this industrially important fungal genera in the presence of ionic liquids.

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