Title: *Bacillus coagulans* tolerance to 1-ethyl-3-methylimidazolium-based ionic liquids in aqueous and solid-state thermophilic culture

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Abstract

The use of ionic liquids (ILs) to disrupt the recalcitrant structure of lignocellulose and make polysaccharides accessible to hydrolytic enzymes is an emerging technology for biomass pretreatment in lignocellulosic biofuel production. Despite efforts to reclaim and recycle IL from pretreated biomass, residual IL can be inhibitory to microorganisms used for downstream fermentation. As a result, pathways for IL tolerance are needed to improve the activity of fermentative organisms in the presence of IL. In this study, microbial communities from compost were cultured under high-solids and thermophilic conditions in the presence of 1-ethyl-3-methylimidazolium-based ILs to enrich for IL-tolerant microorganisms. A strain of *Bacillus coagulans* isolated from this IL-tolerant community was grown in liquid and solid-state culture in the presence of the ionic liquids 1-ethyl-3-methylimidazolium acetate ([C2mim][OAc]) or 1-ethyl-3-methylimidazolium chloride ([C2mim][Cl]) to gauge IL tolerance. Viability and respiration varied with the concentration of IL applied and the type of IL used. *B. coagulans* maintained growth and respiration in the presence of 4 wt% IL, a concentration similar to that present on IL-pretreated biomass. In the presence of both [C2mim][OAc] and [C2mim][Cl] in liquid culture, *B. coagulans* grew at a rate approximately half that observed in the absence of IL. However, in solid-state culture, the bacteria were significantly more tolerant to [C2mim][Cl] compared to [C2mim][OAc]. *B. coagulans* tolerance to IL under industrially-relevant conditions

makes it a promising bacterium for understanding mechanisms of IL tolerance and discovering IL tolerance pathways for use in other microorganisms, particularly those used in bioconversion of IL-pretreated plant biomass.

Introduction

Pretreatment of lignocellulosic biomass with ionic liquids (ILs) is a promising technique for improving saccharification and ultimately realizing commercial production of lignocellulosic biofuels [1, 2]. However, residual ionic liquid in pretreated biomass can inhibit fermentative microorganisms downstream [3-5]. As a result, there is a need to discover and characterize ionic liquid-tolerant microorganisms to better understand pathways and mechanisms associated with IL tolerance. Such pathways could be used to engineer fermentative microorganisms to tolerate residual IL in pretreated biomass. Existing research into IL tolerance has focused primarily on aqueous systems. However, IL tolerance in high-solids systems must also be considered, as industrial biofuel production processes will minimize water use to improve process economics [6, 7]. Differences between aqueous and high-solids systems, such as the lack of a well-mixed medium, may affect the pathways that microorganisms use to tolerate IL.

In this study, microbial communities from compost were enriched in a high-solids mixture of switchgrass, a potential biofuel feedstock, wetted with carbon-free media. Thermophilic conditions were applied during culture, as biomass pretreatment typically occurs at high temperature. The compound 1-ethyl-3-methylimidazolium acetate ([C2mim][OAc]), an IL that has been the focus of much pretreatment research [8, 9], was added to the biomass to simulate residual IL remaining after pretreatment. In a previous study that applied thermophilic conditions and IL simultaneously, it was difficult to select for a community that tolerated both extreme environments[10]. For this reason, enrichment under thermophilic conditions was completed first and IL was applied to the enriched community.

Tolerance to two ionic liquids, [C2mim][OAc] and 1-ethyl-3-methylimidazolium chloride ([C2mim][Cl]), was investigated for a strain of *Bacillus coagulans*, isolated from the enriched microbial community. Tolerance was tested both in the context of liquid and solid-state systems to examine potential applications in lignocellulosic bioconversion processes.

Materials and Methods

Switchgrass and green waste compost preparation

Switchgrass was grown, dried, milled, screened and stored as described previously [11]. Mature green waste compost was collected from a commercial composting facility (Grover Soil Solutions, Zamora, CA, USA). Compost was dried outdoors under ambient conditions and then stored at 4 °C until use. To prepare biomass for solid-state culture, switchgrass was wetted to 400 wt% moisture content (dry weight basis) with carbon-free M9TE minimal medium [12] and stored overnight at 4 °C to equilibrate. This moisture content was selected to maintain a moist environment during thermophilic incubation and because prior solid culture enrichments used a

similar moisture content[13, 14]. To inactivate spores on the plant biomass, switchgrass for use in isolate cultures was autoclaved once after equilibration (121 °C for 30 min), stored overnight at 4 °C and autoclaved again prior to inoculation with bacteria.

Solid-state culture

Solid state cultures were performed using an aerated bioreactor system described previously [15]. Respiration in bioreactors was monitored via measurement of effluent CO_2 concentration, as described elsewhere [15, 16]. For enrichment of ionic liquid-tolerant microbial communities, two reactors were loaded with 30 g of wetted switchgrass inoculated with green waste compost (10% inoculation, dry mass basis) and aerated at 15 ml/min. Reactors were initially incubated for 24 h at 35 °C and then underwent a temperature ramp from 35 to 55 °C over 24 hours by increasing the temperature by 5 °C every 6 hours. Reactors were then held at 55 °C for the remainder of the experiment. Minimal media was added to reactors every 3-4 days to replenish lost moisture. Cultures were passaged every 7 days by inoculating 27 g of freshly wetted switchgrass with biomass from active cultures to achieve a concentration of 10% (fresh weight basis). During each passage, the ionic liquid [C2mim][OAc] was mixed into the biomass of one reactor according to Table I while the second reactor remained free of ionic liquid as a control. IL concentration was ramped up to 4 wt%, as this is the amount of residual IL typically present after IL pretreatment of biomass and washing [17]. Cultures ended 35 days after the initial inoculation. Samples of biomass from each passage were stored at -80 °C for DNA extraction.

Solid-state cultures to measure activity of *Bacillus coagulans* in isolation were performed as previously described for enrichment of ionic liquid-tolerant microbial communities with several exceptions. Sterile switchgrass was inoculated with 3 ml of *B. coagulans* culture broth (prepared as described in the liquid culture methods) instead of compost. Reactors were incubated at 55 °C immediately after inoculation in lieu of a temperature ramp and were fed sterile-filtered air at 15 ml/min. Reactors were treated with either [C2mim][OAc] or [C2mim][Cl] according to the schedule in Table I for 3 passages. Cultures without ionic liquid and cultures inoculated with sterile water served as positive and negative controls, respectively.

Isolation of bacteria

Bacteria were isolated from solid-state cultures containing 4% [C2mim][OAc] 7 days after the fourth passage. Isolates were obtained by mixing 0.5 g of cultured biomass with 3 ml of phosphate buffered saline (PBS), vigorously mixing the suspension for 30 s, diluting the liquid phase 1/100 in PBS, and plating the dilution onto plates of MI media [18] (with glucose substituted for starch) containing 30 g/l agar. Plates were incubated at 55 °C and individual colonies were streaked onto fresh media after 3 days. Isolate cultures were acclimated to ionic liquid by subculturing onto media with 0 to 2 to 4% (mass %) [C2mim][OAc] over three passages. Isolates were maintained on MI medium with 4% [C2mim][OAc] at 55 °C with weekly subculturing.

Liquid culture

Bacteria were grown in liquid MI medium at 55 °C with 115 rpm agitation. To prepare inoculum for isolate solid-state cultures, bacteria were grown to OD_{590}= 0.8 in MI medium supplemented with 4 wt % [C2mim][OAc]. Tolerance to ionic liquids in liquid culture was determined by culturing bacteria in 15 ml of MI media supplemented with 4 wt % (236 mM) [C2mim][OAc] or an equimolar quantity of [C2mim][Cl]. Media supplemented with equimolar amounts of NaCl or NaOAc were used to elucidate the effects of ionic liquid anions on bacterial tolerance. Culture lacking ionic liquid and mock-inoculated media served as positive and negative controls, respectively. Bacterial growth was monitored by measuring OD_{590} in the cultures over time.

Isolation of DNA

Genomic DNA was isolated from solid-state cultures by freezing samples of biomass in liquid nitrogen, disrupting the biomass with an oscillating ball mill (MM400; Retsch Inc., Newtown, PA, USA), and purifying DNA using CTAB extraction [11]. DNA was purified from isolates in liquid culture using a FastDNA Spin Kit (MP Biomedicals, Santa Ana, CA) according to the manufacturer's protocol. DNA concentration was measured using a Qubit fluorometer and reagent system (Invitrogen, Carlsbad, CA).

Small subunit rRNA gene amplicon sequencing

High-throughput sequencing of a hypervariable region of the broadly conserved small subunit (SSU) rRNA gene was performed on DNA obtained from solid-state culture microbial communities. Sequencing was performed by the United States Department of Energy Joint Genome Institute using the Illumina Miseq platform, as previously described [19]. Amplification of the 16s rRNA gene from bacterial isolate DNA was performed by using the primer pair 27F and 1492R [20]. PCR was performed using a reaction mix comprised of 1X ExTaq buffer (Takara, Otsu, Shiga, Japan), 0.8 mM dNTPs (Takara), 1 mg/ml BSA, 300 nM of each primer, 0.025 activity units/µl ExTaq polymerase (Takara), and 0.7 ng/µl template. Amplicons were sequenced via dye-terminator sequencing at the DNA Sequencing Facility at the University of California, Davis. Primers 27F, 1492R, 336R (5'- ACT GCT GCS YCC CGT AGG AGT CT-3'), 337F (5'- GAC TCC TAC GGG AGG CWG CAG-3'), 518R (5'- GTA TTA CCG CGG CTG CTG G-3'), 533F (5'- GTG CCA GCM GCC GCG GTA A-3'), 785F (5'- GGA TTA GAT ACC CTG GTA-3'), 805R (5'- GAC TAC CAG GGT ATC TAA TC-3'), 907R (5'- CCG TCA ATT CCT TTR AGT TT-3'), 928F (5'- TAA AAC TYA AAK GAA TTG ACG GG-3'), 1100F (5'- YAA CGA GCG CAA CCC-3'), and 1100R (5'- GGG TTG CGC TCG TTG-3') were used for sequencing to provide complete coverage of the 16s SSU rRNA gene. Dye-terminator reactions were performed using a BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA) with BetterBuffer BigDye dilution buffer (Gel Company, San Francisco, CA). A PureSeq Magnetic Bead Purification kit (Aline Biosciences, Woburn, MA) was used for post-

cycle sequencing purification. Capillary electrophoresis and dye detection of extension products was conducted on an ABI Prism 3730 DNA Analyzer (Applied Biosystems).

Data analysis

Sequences obtained through high-throughput sequencing of DNA isolated from compost-derived microbial communities were quality trimmed, filtered, assembled and phylogenetically binned using methods described previously [19]. 16s rRNA gene sequences from isolated bacteria were quality-trimmed to remove base calls from either fragment end with more than a 5% chance of erroneous prediction based on Phred quality scores using Geneious Pro software (version 5.6.2. Biomatters, Auckland, New Zealand). Trimmed sequences were assembled using the de novo assembly function in Geneious Pro. The assembled sequence was aligned against the National Center for Biotechnology Information nucleotide collection using the nucleotide basic local alignment search tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi#) with default parameters.

Respiration data from solid-state cultures was used to calculate CO_2 evolution rates (CER) and cumulative respiration (cCER) based on measurements of CO_2 concentration in reactor influents and effluents and gas mass flow measurements, as described previously [16]. Respiration data from solid-state cultures inoculated with bacterial isolates were analyzed by fitting cCER versus time data from the exponential phase of cumulative CO_2 evolution to the following equation:

$$cCER = cCER_0 e^{kt} \quad (1)$$

where $cCER_0$ is the initial amount of cumulative respiration at the beginning of the exponential evolution phase (mg CO_2/g dry weight), k is the specific respiration rate (day^{-1}) – a measure of how quickly CO_2 is evolved by the culture and, by extension, of microbial activity, and t is the time post-inoculation (hr). Values of k were estimated from linear regression of semi-log plots of cCER versus time data after every subculture. Specific respiration rates for cultures with IL were normalized as percentage of the k values measured in IL-free control reactors that were run in parallel.

Absorbance data from bacterial growth in liquid cultures was used as a measure of cell density. The logarithmic and stationary phases of growth were represented by a logistic model of the following form:

$$\frac{dA}{dt} = K\left(1 - \frac{A}{A_{max}}\right)A \quad (2)$$

with analytical solution

$$A = \frac{A_{max}A_0}{A_0 + (A_{max} - A_0)e^{-K(t-t_{lag})}} \quad (3)$$

where A is the absorbance of the culture at 590 nm, A_{max} is the maximum absorbance possible in the culture, A_0 is the initial absorbance of the culture, t is the time post-inoculation (hr), t_{lag} is the duration of the lag phase of the culture (hr), and K is a proportionality constant describing growth rate (hr^{-1}). The value of t_{lag} was defined as the time beyond which the absorbance increased by more than 5% of the initial absorbance value, as estimated by linear interpolation of

the data. Only time points for which $(t\text{-}t_{lag})\geq 0$ were considered for parameter fitting. The nlinfit function of Matlab (version 7.4.0.739, Mathworks, Natick, MA) was used to estimate values of K and A_{max} for each culture. The coefficients determined upon fitting the data from experimental replicates facilitated comparison of the liquid culture treatments.

Comparison of means within experiments was performed using Tukey's Honestly Significant Difference (HSD) test with JMP software (version 10.0.0, SAS Institute, Cary, NC). Tukey's HSD test is a post-hoc analysis that expands upon the information provided in an ANOVA analysis by conducting pair-wise comparisons of means to determine specific treatments that differ with statistical significance. The type I error probability threshold (α) for determining significance of each pairwise comparison is adjusted such that the value of α for all comparisons combined is 0.05. Two-way ANOVA of solid-state culture respiration data was conducted with the data analysis toolpak of Excel (version 15.0.4420.1017, Microsoft, Redmond, WA).

Results

Enrichment of ionic liquid-tolerant microorganisms in solid-state culture

Respiration data from bioreactors containing compost-derived microbial communities showed that microorganisms were active in the high-solids, thermophilic environment in the absence of ionic liquid (Figure 1). Respiration was detected in the community containing 2 wt % (147 mM in aqueous phase) [C2mim][OAc]. However, respiration was not detected once [C2mim][OAc] concentration was increased to 4 wt % (294 mM in aqueous phase) during the third and fourth passages. The control reactor lacking ionic liquid retained activity during these passages, suggesting that the decrease in activity resulted from addition of [C2mim][OAc].

High-throughput 16s rRNA gene sequencing revealed the structure of enriched microbial communities in both the culture containing ionic liquid and the control culture. For both cultures, sequencing data indicated that *Bacillus coagulans* was the dominant species after 21 days of enrichment (Table II).

Isolation and 16s rRNA gene sequencing of bacteria from solid-state culture

Colonies that grew readily on solid media containing 4 wt % [C2mim][OAc] underwent 16s rRNA gene sequencing to determine their identity. Assembly of sequenced regions of the 16s rRNA gene yielded a 1,491 bp sequence, representing near complete coverage of the ~1.5 kb gene, with a mean sequencing depth of 5.8±0.9 (± standard deviation) across all assembled bases. Alignment of the assembly against the NCBI nucleotide collection database showed that the sequence shared >99% identity with the 16s rRNA gene sequences for several *B. coagulans* strains in the database.

Growth of Bacillus coagulans *in liquid culture in the presence of ionic liquids*

B. coagulans was grown in liquid media containing various salts to determine tolerance to ionic liquids. Absorbance data from liquid cultures captured sigmoidal growth curves for all

treatments (Figure 2). These data were used to calculate metrics of cell growth (Table III). The data showed that addition of any of the tested salts (at 236 mM, equivalent to 4 wt % [C2mim][OAc]) significantly lowered the maximum cell density achievable in the culture. Moreover, salt addition in general had a significant negative effect on the bacterial growth rate. Furthermore, cultures with [C2mim][OAc] and [C2mim][Cl] had significantly lower growth rates compared than those observed in cultures containing NaOAc or NaCl. Lag times for cultures with acetate salts did not significantly differ from the control culture. Alternately, cultures containing chloride salts exhibited significantly longer lag phases than the control culture, with [C2mim][Cl] cultures having a significantly longer lag time compared to NaCl cultures.

Activity of Bacillus coagulans *in solid-state culture in the presence of ionic liquids*

Cumulative respiration data from solid-state cultures of isolated *B. coagulans* on switchgrass revealed changes in bacterial activity in response to ionic liquid addition (Figure 3A). Control reactors lacking ionic liquid and the reactor containing 147 mM [C2mim][Cl] maintained activity through the second passage. However, addition of [C2mim][OAc] to 118 mM resulted in a marked decline in activity. Attenuated respiration in cultures containing [C2mim][OAc] persisted at 236 mM of ionic liquid, while cultures with an equivalent concentration of [C2mim][Cl] maintained a higher level of activity. Estimates of specific respiration rates, K, were used to quantify changes in respiration in response to increased ionic liquid levels (Figure 3B). ANOVA of specific respiration rate data showed that bacterial activity is significantly affected by both the type of ionic liquid applied ($p<0.001$) and the concentration of ionic liquid ($p<1e-4$). Furthermore, a significant interaction effect between IL type and concentration was observed ($p<0.001$). The interaction effect is evidenced by the bacteria retaining higher activity in the presence of 147 mM [C2mim][Cl] compared to equivalent levels of [C2mim][OAc].

Discussion

Previous characterization of microbial communities present on the switchgrass and compost used in this study showed that *Bacillus* sp. comprised a minor fraction of the community present on uncultured biomass [14]. This suggests that the observed dominance of *B. coagulans* on cultured biomass, both with and without ionic liquid addition, represents an enrichment of these bacteria specifically in response to the culture conditions. Somewhat surprisingly, *B. coagulans* dominated microbial communities enriched both with and without ionic liquid added. This may stem from the fact that all bioreactors underwent an initial thermophilic enrichment period without the addition of ionic liquid – a strategy that essentially screens for thermotolerance first and then selects for halotolerance among the enriched thermophiles. As a thermophile, *B. coagulans* may have outcompeted other bacteria within the compost during this period, leaving it as the dominant community member by the time ionic liquid was added. The extreme dominance of *B. coagulans* in both the control and IL-treated

communities may be have been assisted by secretion of coagulin, a bactericidal protein produced by *B. coagulans* [21]. That there were no perfect matches in the NCBI gene collection (i.e., both complete sequence coverage and 100% identity) for the16s rRNA gene sequence of *B. coagulans* isolated from the IL-tolerant community may suggest that the isolated bacterium represents a novel strain. However, many entries in the database are partial sequences and deeper sequencing is needed for both the isolated and database strains to conclude the novelty of the isolated strain.

While halotolerance has been reported in a number of *Bacillus* species [22-24], halotolerance in *B. coagulans* is not well-studied and tolerance to ionic liquids under conditions relevant to biofuel production has not been previously reported. A previous study examined the toxicity of several imidazolium-based ionic liquids (1-butyl-, 1-hexyl-, and 1-octyl-3-methylimidazolium hexafluorophosphate) on a *B. coagulans* strain under mesophilic conditions and found that cells survived and continued to produce lactic acid in the presence of 5% (volume basis) IL, albeit with significantly reduced viability and production rates [25]. Moreover, Matsumoto et al. found that tolerance to IL, as indicated by bacterial lactic acid production, correlated to the water solubility of the IL used, with less soluble ILs being more toxic. The 1-ethyl-3-methylimidazolium cation used in this paper is more water soluble than those used by Matsumoto et al. [26]. Thus, *B. coagulans* may exhibit greater tolerance to [C2mim]-based ILs, such as those currently under consideration for biomass pretreatment processes, compared to other imidazolium-based ILs with longer alkyl chains. Additionally, *B. coagulans* growth kinetic data indicated that the IL cation was more inhibitory compared to the anion for the ILs tested. However, there was also evidence that the anion could affect lag phase duration. Together, these observations highlight the importance of choosing an appropriate combination of IL cation and anion for biomass pretreatment that balance plant deconstruction efficacy with inhibition of downstream fermentative microorganisms.

The data also suggest that the availability of free liquid may affect IL tolerance. While logarithmic and stationary phase growth data in liquid culture did not show a significant difference between [C2mim][OAc] and [C2mim][Cl], significant differences in activity were observed in solid-state culture depending on the IL applied. This result raises the possibility that IL tolerance pathways may differ based on culture mode. Previous research of IL tolerance in bacteria has indicated several potential mechanisms for tolerance [3]. These include changes in the fatty acid profile of the cell membrane, uptake of compatible solutes, changes in the regulation of certain transporters, efflux pumps, and porins. *Bacillus coagulans* may utilize some or all of these mechanisms. Additional research is needed to understand if IL tolerance mechanisms differ between liquid and solid-state culture. In solid-state culture, it is possible that some mechanisms may be favored over others. For instance, uptake of compatible solutes and membrane restructuring may be more effective than efflux pumps, due to the lack of a well-mixed medium to remove pumped ions from the cell exterior.

Conclusions

Tolerance to ionic liquids was demonstrated in *Bacillus coagulans* by showing that *B. coagulans* can be enriched from a diverse community in the presence of ionic liquid and grows in isolation in media containing ionic liquid. Moreover, ionic liquid tolerance was shown under high temperature, high-solids conditions relevant to biomass pretreatment for biofuel production. *Bacillus coagulans* warrants further study to elucidate pathways responsible for IL tolerance, particularly in high-solids and thermophilic environments. Such pathways from *B. coagulans* could prove very useful in fermentative microorganisms used for bioconversion of IL-pretreated biomass into biofuels.

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Tables

Table 1. Schedule of ionic liquid addition to solid-state culture to enrich for ionic liquid-tolerant microorganisms.

Passage number	Time (days)	[C2mim][OAc] in culture (mass %)	[C2mim][OAc] in aqueous phase (mM)
0	0	0	0
1	7	0	0
2	14	2	147
3	21	4	294
4	28	4	294

Table 2. Relative abundance of species comprising >95% of microbial communities in high-solids, thermophilic enrichments with and without addition of [C2mim][OAc] after 21 days of culture.

Species	Relative abundance in IL-treated cultured (%)	Relative abundance in IL-free culture (%)
Bacillus coagulans	91.4	90.6
Pseudoxanthomonas taiwanensis	1.7	1.8
Bacillus sp.	1.1	1.1
Geobacillus pallidus	1.3	1.0
Sporolactobacillus kofuensis	0.7	0.9

Table 3. Parameters describing growth of *Bacillus coagulans* in liquid culture in the presence of various salts.

Treatment[a]	A_{max}[b]	K (hr)[b]	t_{lag} (hr)[b]
no salt (control)	0.78 (0.01) A	0.51 (0.01) A	0.10 (0.01) C
[C2mim][OAc]	0.62 (0.01) B	0.29 (0.01) B	0.47 (0.02) C
[C2mim][Cl]	0.61 (0.01) B	0.25 (0.01) B	4.06 (1.08) B
NaOAc	0.66 (0.03) B	0.42 (0.04) C	0.44 (0.16) C
NaCl	0.62 (0.01) B	0.42 (0.03) C	8.00 (0.27) A

[a]All treatments are the molar equivalent of 4 wt % [C2mim][OAc].

[b]Values are given as means with standard deviations in parentheses. Within each column, values that do not share a letter are significantly different (α=0.05). n=3.

Figures

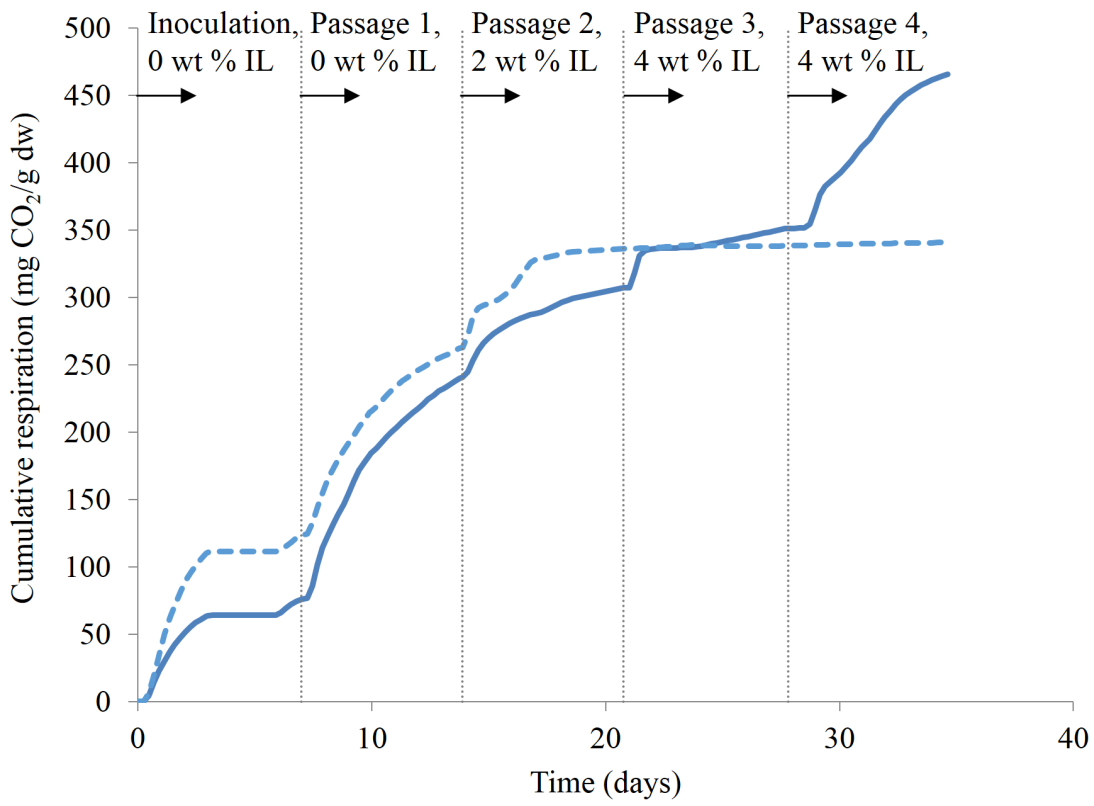


Figure 1. Cumulative respiration for microbial communities enriched under thermophilic, high-solids conditions. Communities were cultured without ionic liquid (solid line) or with increasing levels of [C2mim][OAc] over time (dashed line). Vertical dotted lines indicate when cultures were subcultured onto fresh biomass with or without ionic liquid (IL). IL concentrations refer to the amount of [C2mim][OAc] present in the fresh switchgrass used for subculture of IL-treated bioreactor.

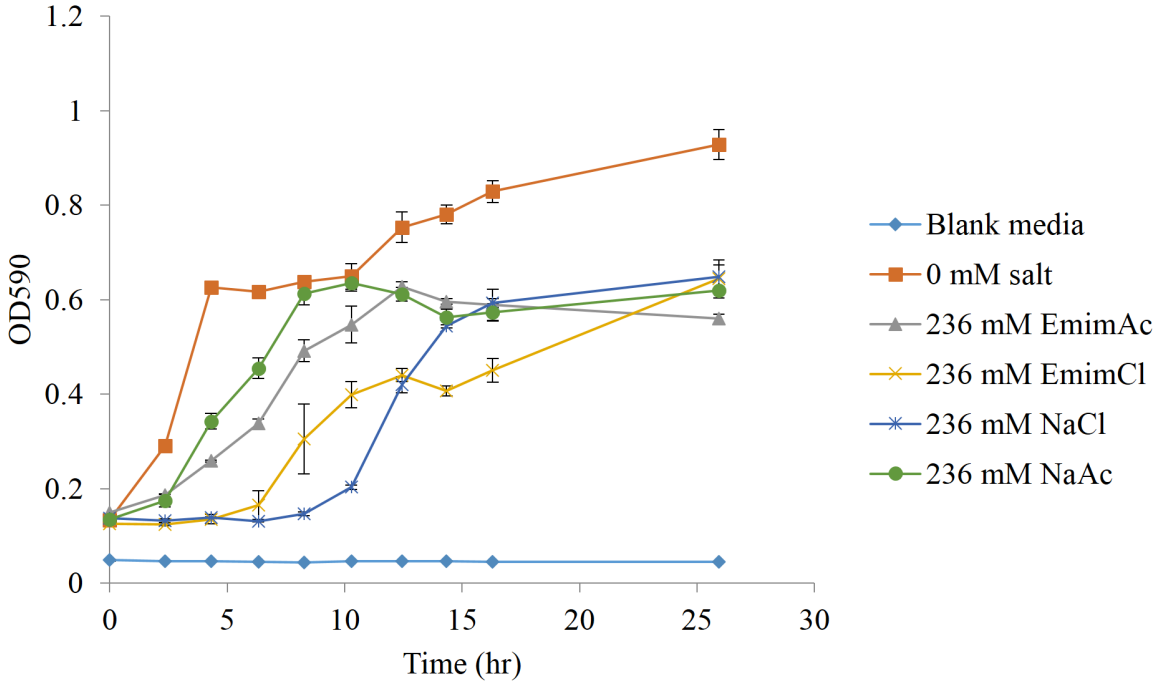
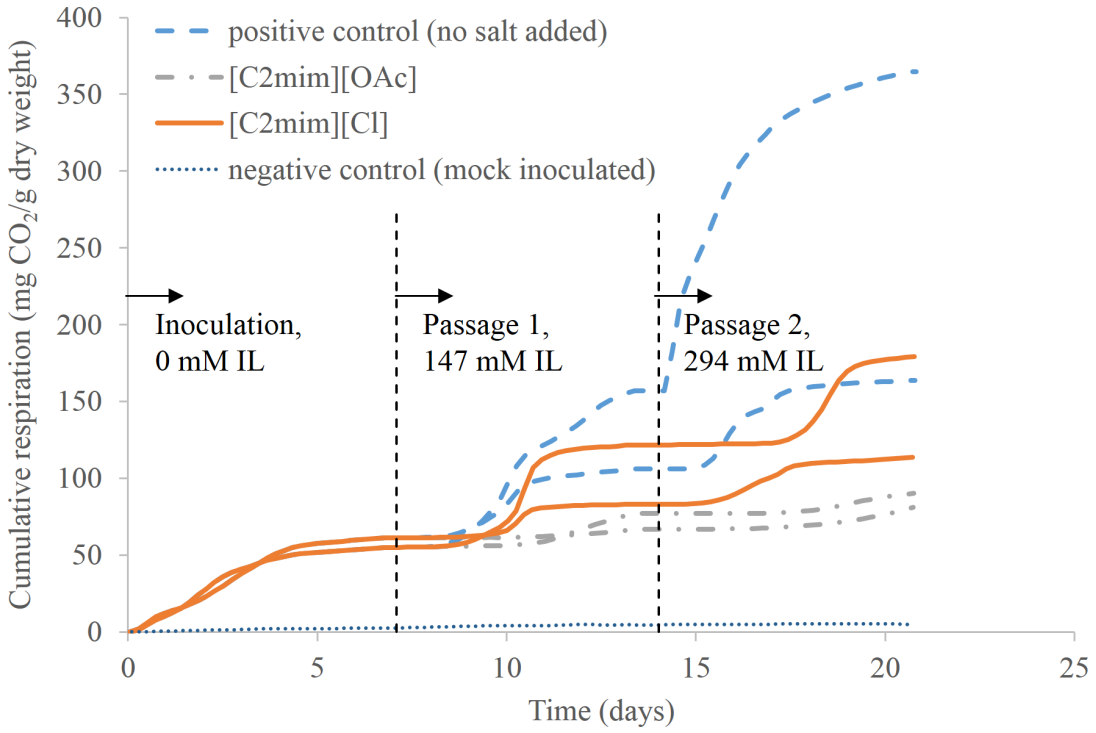


Figure 2. Growth of *Bacillus coagulans* in liquid media containing [C2mim][OAc], [C2mim][Cl], NaCl, or NaOAc. Error bars represent one standard deviation. n=3.

A


positive control (no salt added)
[C2mim][OAc]
[C2mim][Cl]
negative control (mock inoculated)
Inoculation, 0 mM IL
Passage 1, 147 mM IL
Passage 2, 294 mM IL
Cumulative respiration (mg CO2/g dry weight)
Time (days)
0
50
100
150
200
250
300
350
400
0
5
10
15
20
25

B

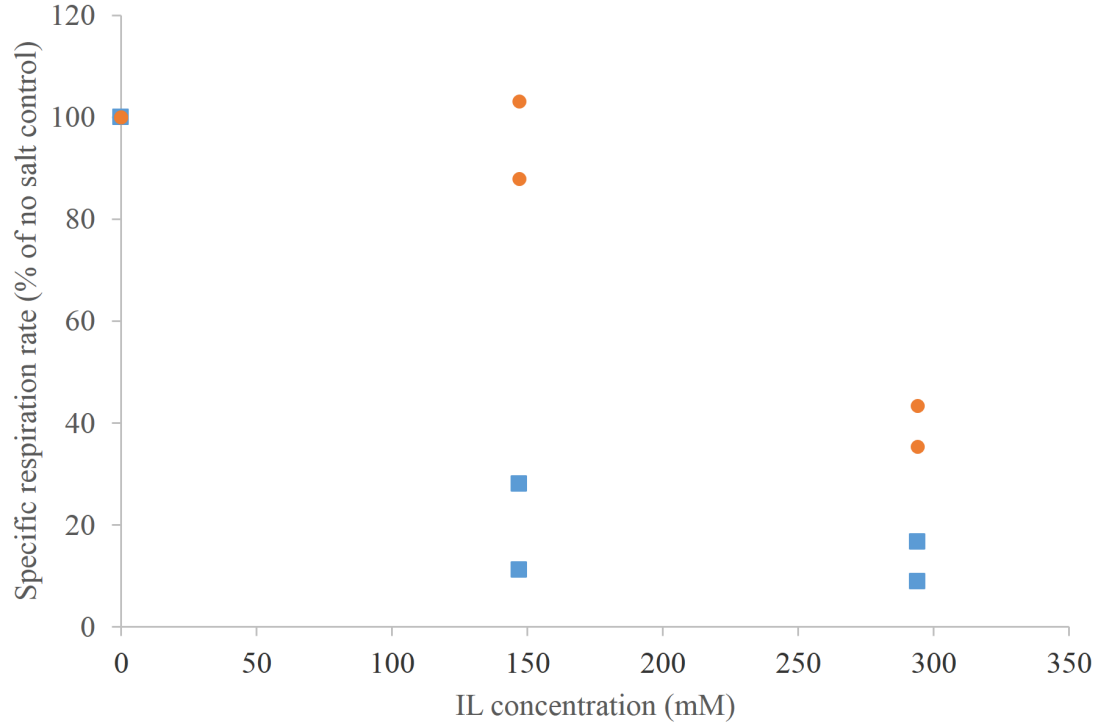

Specific respiration rate (% of no salt control)
IL concentration (mM)
0
20
40
60
80
100
120
0
50
100
150
200
250
300
350

Figure 3. Cumulative respiration for solid-state cultures of *Bacillus coagulans* on switchgrass in the presence of ionic liquids. (A) Cumulative respiration of cultures with increasing concentrations of ionic liquid added during subculture. Vertical dashed lines indicate subculture times. Ionic liquid (IL) concentrations refer to the amount of IL present in the fresh switchgrass used for subculture of the [C2mim][OAc], [C2mim][Cl], and negative control treatments and are given as the concentration of IL in the aqueous phase of the culture. (B) Specific respiration rates of solid-state cultures containing varying levels of ionic liquid presented in (A). Circles and squares represent cultures with [C2mim][Cl] and [C2mim][OAc], respectively.