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Thermophilic enrichment and preservation of microbial communities tolerant to the ionic liquids tetrabutylphosphonium chloride and tributylethylphosphonium diethylphosphate

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Running headline: IL-tolerant microbial communities

Abstract

Aims: The aims of this study were to identify thermophilic microbial communities that degrade green waste in the presence of the ionic liquids (IL) tetrabutylphosphonium chloride and tributylethylphosphonium diethylphosphate and examine preservation methods for IL tolerant communities.

Methods and Results: High-solids incubations with step-wise increases in IL concentration were conducted to enrich for thermophilic IL tolerant communities. 16S rRNA sequencing of enriched communities revealed microorganisms capable of tolerating high levels of IL. Cryogenic preservation of enriched communities reduced the IL tolerance of the community and decreased the relative abundance of IL tolerant organisms. The use of cryoprotectants did not have an effect on microbial activity on green waste of the stored community.

Conclusions: Step-wise increases in IL facilitated the enrichment of thermophilic, IL tolerant microbial communities that decomposed green waste. Alternative community storage and revival methods are necessary for maintenance and recovery of IL-tolerant communities.

Significance and Impact of Study: A successful approach was developed to enrich communities that decompose green waste in thermophilic high-solids environments in the presence of IL. The enriched communities provide a targeted source of enzymes for the bioconversion of IL-pretreated green waste for conversion to biofuels.

Keywords

Phosphonium ionic liquids, microbial communities, bioenergy, high-solids, pretreatment, storage

Introduction

Efficient and sustainable biofuel production from renewable lignocellulosic feedstocks is strategic for providing a viable alternative to traditional fossil fuel sources (Eichorst et al. 2014). Lignocellulose found in plant cell walls is one of the largest reserves of convertible energy (Simmons et al. 2008;

Somerville et al. 2010), however, efficient degradation of the cell wall has proven to be very difficult without added physical and chemical pretreatment. Ionic liquids (ILs) have been used as a pretreatment technique to improve enzymatic hydrolysis of cellulose by disrupting the inter- and intra- molecular hydrogen bonds within plant cell wall polymers (Quijano et al. 2010; Gladden et al. 2014). At elevated temperature and pressure, pretreatment of biomass with ILs has been shown to improve the digestibility of feedstocks (Li et al. 2011) yet at certain concentrations, they are inhibitory to enzymes and microorganisms used in the conversion steps performed later in the biofuel production process (Samori et al. 2011).

Phosphonium ILs have been shown to make cellulose more accessible without degrading it (Ohno and Fukumoto 2007; Keskar et al. 2011; Abe et al. 2012). Furthermore phosphonium ILs have high pretreatment beta numbers ($x > 1.0$), indicating efficient extraction of lignin (Doherty et al. 2010). Several studies have demonstrated compatibility between microorganisms and phosphonium ILs (Doherty et al. 2010; Abe et al. 2012). This feature could be beneficial during the biological conversion steps in biofuel production.

The aims for this study were to identify thermophilic microbial communities tolerant to ionic liquids (ILs) that can efficiently decompose lignocellulose in a high solids environment, and to evaluate cryogenic preservation methods for the enriched community. Green waste was selected as the feedstock for all experiments. In an effort to divert organic wastes from landfills, municipalities throughout California have invested in infrastructure for collection and processing of green waste to compost. Because green waste is widely and abundantly available on a year round basis, it has great potential for biomass based bioenergy production. High lignin woody residues in green waste, however, make green waste particularly recalcitrant to bioconversion and IL pretreatment is one approach to address recalcitrance (Watteau and Villemin 2011). Tetrabutylphosphonium chloride (tetra butyl) and tributylethylphosphonium diethylphosphate (diethyl) ILs were selected for experiments due to their efficiency in lignin extraction, de-crystallization of cellulose, and biocompatibility (Keskar et al. 2011). Tetra butyl was specifically selected based on the presence of chlorine, which is considered a good anion

for biomass processing (Doherty et al. 2010; Abe et al. 2012). Preservation methods included plating and cryopreservation with the addition of cryoprotectants. Methods were evaluated based on tolerance of the stored community to IL upon revival on green waste.

Materials and Methods

Green waste feedstock and inoculum preparation

Synthetic green waste (SGW) was prepared to simulate municipal green waste in this study. The SGW components and mass fractions were based on the California 2008 Statewide Waste Characterization Study and included leaves (41.0 % dry basis), grass (11.4 % dry basis), prunings and trimmings (40.4 % dry basis), and branches and stumps (7.2 % dry basis) (Board 2009). All components were collected from the University of California, Davis, USA and milled to less than 10 mm prior to mixing. The resulting cellulose, hemicellulose, and lignin contents of the SGW were 28.9 % wt wt^{-1}, 17.9 % wt wt^{-1}, and 15.0 % wt wt^{-1} of dry matter respectively. Finished green waste compost was used as inoculum for enrichments based on prior success with community tolerance to IL (Reddy et al. 2012); it was obtained from a commercial facility that composts agricultural residues including tree and vine prunings (Northern Recycling, Zamora, CA, USA). Compost was solar-dried and stored at room temperature until applied as inocula.

High-Solids Incubations

Protocols for biomass preparation and high-solids incubations followed previous work, with slight modifications (Reddy et al. 2011). For the first incubation, prior to inoculation, SGW was wetted with M9 minimal media with no additional carbon source to a target moisture content of 400 wt % dry basis (g water per g dry solid) and stored at 4 ^{o}C overnight to equilibrate. The wetted SGW was inoculated with 10 wt% compost (g dry compost per g dry solid) just before incubation.

Bioreactors with a 0.2 L working volume were loaded with 6-10 g dry weight of SGW and compost mixture. To maintain aerobic conditions, humidified air was supplied to each bioreactor at 15-

20 mL min^{-1}. For the first incubation, the incubator temperature was maintained at 35 °C for 24 hours and then ramped up to 55 °C over 24 hours and maintained at 55 °C for the extent of the experiment. Every 3-4 days, water lost during incubation was replaced on a weight basis in order to maintain moisture content for microbial activity.

The respiration rate of the microbial community, represented as CO_2 evolution rate (CER), was measured for all incubated samples (Reddy et al. 2009). An infrared CO_2 sensor (Vaisala, Woburn, MA, USA) measured CO_2 concentration on the influent and effluent air of the bioreactors and a thermal mass flow meter (Aalborg, Orangeburg, NY, USA) measured the air flow. The data acquisition system recorded carbon dioxide and air flow data every 20 minutes. Carbon dioxide evolution rate (CER) and cumulative respiration (cCER) were calculated as previously described (Reddy et al. 2009).

High-solids enrichments followed prior IL enrichments and were completed with stepwise increases in the concentrations of tetra butyl and diethyl (Reddy et al. 2012). The enrichment pathways are presented in Figure 1. Each enrichment time point consisted of seven days of continuous incubation and monitoring. Fresh SGW wetted with ionic liquid (IoLiTec, Tuscaloosa, AL, USA) incorporated into the M9 media was inoculated with 10 wt. % (g dry enriched sample per g total dry weight) of the previously enriched community and transferred to a new bioreactor every 7 days. Both ILs were solubilized in water prior to incorporation into the M9 media. Samples from the highest IL concentration tested at each time point were used as inoculum for the subsequent time point. Every 3-4 days, respiration data from incubated treatments were examined and used to determine IL concentrations for the subsequent time point. Iterative methods were used to narrow the range for highest possible ionic liquid concentration tolerance based on respiration data. An initial high to low concentration range of 1 mmol L^{-1} to 10 mmol L^{-1} was selected based on preliminary data. Respiration was detected at 10 mmol L^{-1} IL for both ILs. The concentration range increased from 10 mmol L^{-1} to 50 mmol L^{-1} for T3 and from 50 mmol L^{-1} to 75 mmol L^{-1} for T4. The enrichment experiment ran for four weeks yielding a total of four time points (T1, T2, T3, and T4) for microbial community analyses.

At the end of each incubation, samples were taken from each reactor to measure microbial community composition and moisture content. Moisture content was measured gravimetrically after drying samples at 105 °C for 24 hours.

Microbial Community Preservation

At the end of time point T4, incubated SGW samples enriched with 75 mmol L^{-1} IL were stored under four different conditions. Three conditions involved cryogenic storage at -80 °C, where two methods used either the cryopreservant dimethyl sulfoxide (DMSO) or glycerol, and no cryopreservant was used for the third condition. The samples with cryopreservant were stored in a 50 % solution of cryopreservant (3 g cryopreservant per 3 g wet sample). All three cryo-storage sample sets were snap-frozen in liquid nitrogen prior to storage at -80 °C.

For the plated storage technique, colonies were obtained from the 75 mmol L^{-1} incubated samples. To remove the microorganisms from the SGW, 1 mL of M9 was added to 0.5 g of fermented SGW from the T4 sample and vortexed for 20 minutes. The media was plated on $M9^{+}$ plates without the presence of ILs. Plates consisted of M9 media with addition of 3 % agar and 0.5 % each of dextrose, yeast extract, cellobiose, Birchwood xylan, and starch from potatoes. Plates were incubated at 55 °C for two days and then stored at 4 °C.

High-solids incubations were conducted to test the viability of each storage treatment after four weeks of storage. Sources of inoculum for the enrichment were selected based on IL concentration levels and respiration data from T4 for both tetra butyl and diethyl. Equal amounts of DMSO and glycerol were added to each reactor containing SGW wetted with IL incorporated into M9 media prior to inoculation to control for the possibility of the cryopreservants serving as additional substrate. Wetted SGW was inoculated with 10 wt. % (g dry enriched sample per g total dry weight) of the stored enriched community. The bioreactors were incubated for seven days at 55 °C.

DNA extraction and 16S rRNA gene sequencing

Samples from all time points, including post-storage incubations, were taken from each bioreactor and snap-frozen in liquid nitrogen, then homogenized with an oscillating ball mill (MM400; Retsch Inc., Newtown, PA, USA). DNA was extracted using the MoBio PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA). The quantity of the purified products was measured with a Qubit® fluorometer (Life Technologies, Grand Island, New York) using the manufacturer's protocol. Sequencing of a hypervariable region of the broadly conserved 16S rRNA gene was performed on purified DNA by the United States Department of Energy Joint Genome Institute using the Illumina Miseq platform, as previously described (Eichorst et al. 2014).

Data Analysis

The VEGAN package in R software (http://CRAN.R-project.org/package=vegan) was used to perform ecological analyses to characterize enriched microbial communities as described elsewhere (Reddy et al. 2012). Richness, Shannon index, and Pielou index of each community, as well as Bray Curtis dissimilarity values between each community in a pairwise approach, were calculated based on sequencing data. Similarity percentage (SIMPER) analyses were performed as previously described (Clark 1993) for paired communities with BC values larger than 0.5 to identify the species most responsible for dissimilarity and two dimensional nonmetric multidimensional scaling (NMDS) plots were used for ordination of enriched communities over time and treatment.

Respiration rates and diversity indices of preserved samples were compared using a Tukey's means comparison test in JMP v11 (SAS Institute, Cary, NC).

Results

The microbial community enriched for T1 used compost as the initial inocula and was grown without the presence of ionic liquid. After the community adapted to thermophilic conditions, the average cumulative respiration after 7 days of incubation was 121 mg CO_2 g^{-1} dw (Table 1). This community was used as inoculum for each treatment in the subsequent time point, T2. No IL controls

were enriched for three additional time points. Cumulative respiration significantly increased by 48 % between T1 and T4 suggesting enrichment of a green waste decomposing community.

Microbial response to tetra butyl

The treatments and lineage for each time point are shown in Figure 1. In the T2 enrichments respiration levels for 1 mmol L^{-1} tetra butyl and 10 mmol L^{-1} tetra butyl ranged from 20 mg CO_2 g^{-1} dw to 173 mg CO_2 g^{-1} dw (Figure 2). However, low respiration levels for T2 replicate 1 at 1 mmol L^{-1} were due to a drop in airflow provided to the reactor. Optimal airflow rate was restored for T3. For samples containing 1 mmol L^{-1} tetra butyl, cumulative respiration was significantly similar to no IL controls with the exception of replicate 1 (Table 1). For samples containing 10 mmol L^{-1} tetra butyl in T2 enrichments, respiration was also similar to the controls with the exception of replicate 2, which was nearly two times lower than its corresponding no-IL control.

The communities from the 10 mmol L^{-1} tetra butyl incubations were used as inocula for T3 enrichments (Figure 1). The respiration levels were similar within replicates between T2 enrichments and T3 enrichments containing 10 mmol L^{-1} tetra butyl (Figure 2). Cumulative respiration decreased with increasing tetra butyl concentration for all replicates in T3, except for replicate 2 (Figure 2). For replicate 1 activity decreased from 166 CO_2 g^{-1} dw to 1 mg CO_2 g^{-1} dw while for replicate 3 activity decreased from 145 mg CO_2 g^{-1} dw to 13 mg CO_2 g^{-1} dw. However, for replicate 2, the cumulative respiration increased with increased IL concentration to a cCER value of 101 mg CO_2 g^{-1} dw from the inoculum's cCER value of 81 mg CO_2 g^{-1} dw.

T4 enrichments used inocula from 50 mmol L^{-1} tetra butyl T3 communities (Figure 1). The respiration levels were similar between T3 enrichments and T4 enrichments containing 50 mmol L^{-1} tetra butyl with the exception of replicate 2 in which respiration increased by 50 %. As observed with T3 enrichments, cCER decreased with increasing tetra butyl concentration for replicates 1 and 3. Inocula from replicate 2 of 50 mmol L^{-1} tetra butyl from the T3 enrichment appeared to tolerate increasing concentration with only a slight decrease in respiration observed between T3 50 mmol L^{-1} and T4 75

mmol L^{-1} tetra butyl incubations (Figure 2). Respiration levels indicate there was microbial activity present in the community for tetra butyl replicate 2 at 50 mmol L^{-1} and 75 mmol L^{-1} in T3 and T4 and at 10 mmol L^{-1} for replicates 1 and 3.

Microbial response to diethyl

The respiration levels for 1 mmol L^{-1} diethyl in T2 incubations (Figure 3) were slightly lower than the respiration levels of the control samples (Table 1). The respiration levels for 10 mmol L^{-1} diethyl were lower than 1 mmol L^{-1} diethyl in T2, and varied from 36-112 mg CO_2 g^{-1} dw and 117-137 mg CO_2 g^{-1} dw, respectively (Figure 3) and were lower than the T2 controls which had an average cCER of 146 mg CO_2 g^{-1} dw.

The communities from the 10 mmol L^{-1} diethyl T2 enrichment were used as inocula for T3 enrichments (Figure 1). Cumulative respiration decreased with increasing diethyl concentrations for all lineages in T3, except for replicate 3 (Figure 3). Activity for replicate 1 and replicate 2 decreased from 112 to 21 mg CO_2 g^{-1} dw and 49 to 18 mg CO_2 g^{-1} dw, respectively, while activity increased for replicate 3 from 36 to 49 mg CO_2 g^{-1} dw.

T4 enrichments used inocula from 50 mmol L^{-1} diethyl T3 communities (Figure 1). As observed with T3 enrichments, cCER decreased with increasing diethyl concentrations for replicate 1 and replicate 3, however, respiration increased for replicate 2. In the T4 75 mmol L^{-1} replicate 2 enrichment, respiration was 2.8 times greater than respiration in the T3 50 mmol L^{-1} replicate 2 enrichment. For replicate 2, this level of respiration indicates there was continued activity in the community and adaptation to IL.

Microbial response to ionic liquid, post-storage

After four weeks of storage, the preserved samples from T4 replicate 2 of both 75 mmol L^{-1} tetra butyl and diethyl were used as inocula for the high-solids incubations. SGW wetted with 75 mmol L^{-1} IL in M9 was inoculated with stored samples and incubated at 55 °C for 7 days. Tetra butyl samples

cryogenically stored yielded approximately the same respiration values across storage techniques (Table 2), where respiration values ranged from 2.9 to 8.6 mg $CO_2\,g^{-1}$ dw. The communities stored on enriched plates yielded slightly lower cCER values at 1.6 mg $CO_2\,g^{-1}$ dw. Cumulative respiration values overall for the stored samples were significantly lower than the respiration value observed prior to storage at 96 mg $CO_2\,g^{-1}$ dw (Figure 2).

Respiration results from revived diethyl storage treatments showed higher respiration levels than tetra butyl and ranged from 7-16.2 mg $CO_2\,g^{-1}$ dw for stored treatments revived on 75 mmol L^{-1} diethyl (Table 2); however, the communities were not able to reach the same activity level achieved prior to storage, where cCER level was 57 mg $CO_2\,g^{-1}$ dw (Figure 3). These results suggests that the microbial communities active in the presence of either 75 mmol L^{-1} diethyl or tetra butyl were not preserved during storage or were not active upon reinoculation. It is possible that lower concentrations of IL could have been tolerated, however lower IL concentrations were not tested in this experiment.

In order to test the viability of the stored community in the absence of IL, SGW was wetted with only M9 media. Biological replicates 1 and 3 from T4 for 75 mmol L^{-1} tetra butyl and 75 mmol L^{-1} diethyl stored at -80 $^{\circ}$C without cryopreservants were used as inocula. Both inoculum sources contained IL so the viability test included trace amounts of IL. Respiration values for this test exceeded cCER values achieved for each sample prior to storage. This was most likely due to the low concentration of ILs in the post storage tests. It is possible that the presence of ILs in enrichments suppressed the activity of some microorganisms in the community, but did not completely inactivate them. Prior studies have shown that the activity of lignocellulose degrading communities can be retained upon cryopreservation (Yu et al. 2015). In this study communities were able to maintain functional respiration traits despite being enriched in stressed environments and stored at -80 ^{o}C for four weeks. The results indicate that viable microorganisms active on SGW under thermophilic conditions can be preserved during storage at -80 ^{o}C.

Changes in microbial community structure with increasing ionic liquid concentration

All communities from T1, T2, T3, and T4 were selected for 16S rRNA gene sequencing. Ordination analysis of the control and 1 mmol L^{-1}, 10 mmol L^{-1}, 50 mmol L^{-1}, and 75 mmol L^{-1} communities for both tetra butyl and diethyl revealed a distinct shift when thermophilic conditions were applied. NMDS of communities showed that the community structure at T1, which is common to all lineages sequenced and was not exposed to IL, is distanced from all other communities at subsequent time points and concentrations of tetra butyl (Figure 4) and diethyl (Figure 5). For tetra butyl, communities became more distant with enrichment and as IL concentration increased (Figure 4). Furthermore, for T4 replicate 2 at 75 mmol L^{-1} tetra butyl (T4.R2.75mmol L^{-1}, the community was distanced from all other communities. A similar trend of divergence from inoculum was also seen for diethyl, with T4 replicate 1 75 mmol L^{-1} diethyl (T4.R1.75mmol L^{-1}) showing distance from all other samples (Figure 5). These data indicate that the introduction of IL resulted in a shift in microbial community structure away from that initially present in the early thermophilic enrichment.

Ecological measures were calculated to quantify the changes in microbial structure under stress from ILs and thermophilic conditions (Table 3 and Table 4). Richness of the controls decreased over each enrichment (Table 3). In T1 the presence of ILs showed decreased richness, diversity, and evenness in microbial communities compared to treatments with no ILs present. For tetra butyl communities, richness increased more for replicates 1 and 3 than replicate 2 during T3 at 50 mmol L^{-1} and continued this trend for 50 mmol L^{-1} and 75 mmol L^{-1} during T4. This same trend for replicates 1 and 3 was seen with the Pielou index and Shannon index. Greater symmetry was detected amongst replicates 1 and 3 than replicate 2 at the same concentrations and time points starting at T3. Diversity of replicates 1 and 3 increased more than replicate 2 and had higher Shannon index values for 50 mmol L^{-1} and 75 mmol L^{-1} concentrations of tetra butyl. Loss of diversity for replicate 2 and other community members was due to decreases in both community richness and evenness. Replicate 2 had the lowest diversity and evenness at the highest concentration of tetra butyl.

Communities enriched in the presence of diethyl showed decreased evenness and diversity as IL concentration increased (Table 4). Richness tended to increase in samples between T1 and T4 with the

greatest increase observed in replicate 1. The large increase for replicate 1 at 75 mmol L^{-1} diethyl was also reflected in the Pielou index and Shannon index results, where the community increased in symmetry and diversity as it adapted to a thermophilic environment with the presence of increasing concentrations of diethyl.

Changes in community diversity corresponded to enrichment of specific phyla. Small changes in relative abundance were observed with enrichment in the absence of ILs (Figure 6a). Lineages with and without the presence of ILs exhibited enrichment primarily of *Firmicutes* (Figure 6), however there were a few treatments in which the dominant phylum was *Proteobacteria*. For example, for replicate 2 at 75 mmol L^{-1} tetra butyl (Figure 6b), *Proteobacteria* accounted for 93% of the community. Microorganisms from this phylum may have been responsible for the high activity levels detected in this sample while undergoing high stress conditions at elevated tetra butyl concentrations. The relative abundance of *Firmicutes* increased in diethyl treated communities when compared to the communities enriched without the presence of ILs. Organisms from this phylum may have contributed to the sustained respiration and survival in the presence of diethyl.

Bray-Curtis values indicated that with more IL the communities began to shift and became more dissimilar (Table 4); once 50 mmol L^{-1} diethyl was introduced as a stress the new communities had a dissimilarity greater than 90% to the inoculum. This divergence was also observed in the tetra butyl communities but to a slightly lesser extent.

Bray-Curtis values indicated that the pooled T1 (grown without IL) and replicate 2 from T4 at 75 mmol L^{-1} tetra butyl were very dissimilar, greater than 90% (Table 4). SIMPER analysis performed between these two communities (Table S1) revealed *Chelatococcus (p-Proteobacteria)* to be the largest contributor to the dissimilarity with an increased relative abundance in T4 followed by *Paenibacillus (p-Firmicutes)* which had a decreased relative abundance in T4. Bray-Curtis dissimilarity values also indicated that the 75 mmol L^{-1} diethyl replicate 2 community was most dissimilar to T1 at greater than 90%. SIMPER analysis performed between the two communities showed *Bacillaceae (p-Firmicutes)* as

the largest contributor to dissimilarity at 46.2% with increased relative abundance in T4, followed by *Paenibacillus (p-Firmicutes)* whose relative abundance decreased in T4 (Table S2).

Changes in microbial community structure after storage and revival

Communities from revived stored treatments of the T4 community from 75 mmol L^{-1} tetra butyl replicate 2 and 75 mmol L^{-1} diethyl replicate 2 were selected for sequencing analysis. Ecological measures were calculated to quantify microbial community changes resulting from preservation followed by incubation with IL (Table 5). For both tetra butyl and diethyl revived communities, there was not a significant difference between storage techniques, except for richness in the glycerol treatment in tetra butyl. However, compared to the profile of the community before storage, richness, evenness, and diversity all increased for both tetra butyl and diethyl stored communities revived in the presence of ionic liquid. The microbial community profile was consistent among storage techniques for both tetra butyl and diethyl based on evenness, diversity, and richness measures.

As shown in Figure 7, *Proteobacteria* relative abundance decreased for all storage techniques of the tetra butyl preserved communities. SIMPER analysis between replicate 2 at 75 mmol L^{-1} tetra butyl from T4 and the communities revived after storage determined *Chelatococcus (p_Proteobacteria)* to be the largest contributor to dissimilarity (Tables S3-S6) where its relative abundance decreased from 82.3 % to 5.0-10 %. This was consistent for all storage treatments where *Chelatococcus (p_Proteobacteria)* was no longer the dominant member in the tetra butyl community (Table 6). The dominant OTU in the revived tetra butyl communities shifted to *Acinetobacter (p-Proteobacteria)*; it was the second largest contributor to Bray-Curtis dissimilarity values between the revived and pre-stored communities where percent relative abundance increased from 4.2 % to 21.6 % (Tables S3-S6).

Diethyl treatments also showed a large difference between the original pre-stored community and the revived communities (Table 7). The dominant OTU in the community prior to storage and after revival was *Bacillaceae (p-Firmicutes)*. Despite this dominance its percent relative abundance decreased from 89.2% in the T4 sample to 38-43% in the stored and revived communities (Table 7) and it had the

largest contribution to Bray-Curtis dissimilarity between samples (Tables S7-S10). Like the tetrabutyl treatments, the second largest contributor to Bray-Curtis dissimilarity was *Acinetobacter (p-Proteobacteria)*. Its relative abundance increased from 3% to 14-15% between T4 and revived samples.

Discussion

Green waste is a promising feedstock for biofuels and products due to infrastructure for its collection and availability. This is the first time that microbial communities that are enriched during thermophilic green waste decomposition have been examined using next-generation 16S rDNA gene sequencing technology. The top five phylum that were enriched included *Firmicutes*, *Proteobacteria*, *Actinobacteria*, *Cyanobacteria*, and *Bacteroidetes*. Several of these phyla including *Firmicutes*, *Proteobacteria*, and *Actinobacter* were also observed when thermophilic communities were enriched on switchgrass and corn stover (Reddy et al. 2011) indicating they may be a universal source of microorganisms for deconstruction of lignocellulosic feedstocks. *Cyanobacter* and *Bacteroidetes* were unique to green waste. Further study into these newly enriched phyla could yield organisms and enzymes that are directed to green waste decomposition.

Thermophilic conditions vastly shifted the microbial community, even without the presence of ionic liquid. In this study, decrease in diversity in thermophilic green waste enrichments was observed, which indicates selection for a few dominant community members adapted to decompose this feedstock in a high-solids, thermophilic environment. The dominant members in the final enrichment were *Bacillaceae (p_Firmicutes)* and *Acinetobacter (p-Proteobacteria)*. Members of the family *Bacillaceae* have well documented cellulolytic activity (Eichorst et al. 2013) and have been found in cellulose-rich environments including landfill refuse (Pourcher et al. 2001), green waste (Bru-Adan et al. 2009) and cattle waste fed biodigesters (Singh and Jain 1986). *Acinetobacter* belongs to the family *Moraxellaceae,* a proteobacteria with reported ability to degrade cellulose and hemicellulose of sugarcane (Rinke et al. 2011).

Results demonstrate that a community tolerant to a thermophilic, high-solids environment containing 75 mmol L^{-1} tetra butyl or diethyl can be enriched from compost. Gradual increases in tetra butyl and diethyl concentrations during the enrichment yielded communities capable of thriving on green waste in a high-solids, thermophilic environment in the presence of phosphonium ionic liquids. The gradual increases in IL concentration aided in microbial adaptation to the stressor, as advised by previous high-solids incubation studies with ionic liquids (Reddy et al. 2012). Overall, increased tetra butyl and diethyl concentration caused a decline in microbial activity, as indicated by decreased respiration rates, which is consistent with prior thermophilic enrichments on switchgrass (Reddy et al. 2012; Simmons et al. 2014a). Identification of the exact role of each organism in green waste decomposition requires further studies.

The dominant OTU in the 75 mmol L^{-1} tetra butyl enrichment with the highest respiration was *Chelatococcus*. *Chelatococcus*, classified as an aerobic methanogenic bacterium, belongs to an alphaproteobacterial family that can grow on a wide range of substrates (Liebner et al. 2009). This organism may be a promising source of IL tolerant cellulases due to high microbial activity detected while in the presence of tetra butyl IL.

The dominant OTU in the 75 mmol L^{-1} diethyl enrichment was *Bacillaceae (p_Firmicutes)* which is the same family that was previously found to be tolerant of 1-ethyl-3-methylimidazolium acetate on switchgrass (Reddy et al. 2012; Simmons et al. 2014a; Simmons et al. 2014b). Here we report the dominant organism was from the family *Bacillaceae* but in previous studies organisms were identified down to the genus level as *Bacillus coagulans* (Simmons et al. 2014b) and *Geobacillus* (Reddy et al. 2012). In several different studies with different ILs and different feedstocks the family *Bacillaceae* has been shown to thrive and dominate in the community. This family warrants further study for IL tolerance. .

The high-solid incubations, with post-storage inoculum, showed that communities enriched for their ability to grow in thermophilic conditions with elevated IL concentrations maintain that ability through storage, though at much lower activities. *Chelatococcus (p_Proteobacteria)*, which was dominant

in enriched samples containing 75 mmol L^{-1} tetra butyl, decreased in relative abundance upon storage. This drop in relative abundance could have been due to the inability of *Chelatococcus* to form spores; storage could have inactivated the majority of the viable population (Ibrahim and Steinbüchel 2010; Panday and Das 2010). Alternatively, slow growth upon re-inoculation may have contributed to a reduction in relative abundance. It is possible that given more cultivation time *Chelatococcus* would have become the dominant organism in the community. The reduction of this organism in the community may explain why there was a significant decrease in microbial activity for all of the 75 mmol L^{-1} tetra butyl treatments post-storage. *Chelatococcus* is a microorganism of interest that should be studied in more detail to determine survival rates on substrates with high IL concentrations as well as better storage techniques.

Acinetobacter (p-Proteobacteria) increased for both 75 mmol L^{-1} tetra butyl and 75 mmol L^{-1} diethyl treatments post-storage. This ability to survive cryopreservation and increase in abundance upon re-inoculation could be have been due to the ability of this microorganism to produce biofilms (Rodríguez-Baño et al. 2008).

Current storage techniques appear to be too harsh for total community recovery; there was a substantial decrease in respiration, which can be attributed to a decrease in IL tolerant microorganisms. In addition the community shift after storage indicates that the entire community cannot be adequately stored with standard techniques. Further studies are needed to explore effective storage, stabilization and revitalization techniques.

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Conflict of Interest

No conflict of interest declared.

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Table 1: Cumulative respiration (cCER) for controls (no ILs present) for time points T1 through T4 after 7 days of incubation. Values in parentheses represent standard errors.

Enrichment	Mean cCER, mg CO_2 g^{-1} dry wt*
T1	121 (21)
T2	146 (6)
T3	115 (18)
T4	180 (4)

*n=3 for all enrichments except T1 where n=5

Table 2: Cumulative respiration at 7 days for samples inoculated with preserved communities.

Community origin			Preservation conditions and treatment post storage		
IL	Concentration (mmol L^{-1})	Replicate	Storage Treatment	IL Concentration (mmol L^{-1})	Mean cCER (mg CO_2 g^{-1} dry material)*
Tetra butyl	75	2	Liquid Nitrogen	75	2.9 A
Tetra butyl	75	2	DMSO	75	8.6 A
Tetra butyl	75	2	Glycerol	75	7.6 A
Tetra butyl	75	2	Plated	75	1.6 A
Diethyl	75	2	Liquid Nitrogen	75	16 A
Diethyl	75	2	DMSO	75	7.0 A
Diethyl	75	2	Glycerol	75	11 A
Diethyl	75	2	Plated	75	15 A
Tetra butyl	75	1	Liquid Nitrogen	0	98 A
Tetra butyl	75	3	Liquid Nitrogen	0	74 A
Diethyl	75	1	Liquid Nitrogen	0	102 A
Diethyl	75	3	Liquid Nitrogen	0	86 A

*for a given IL treatment, means followed by the same letter within columns are not significantly different at α = 0.05

Table 3: Ecological measures for microbial communities from thermophilic enrichments on SGW without the presence of ionic liquids. Bray-Curtis dissimilarity values represent comparison to the T1 community.

Community			Richness [R]	Pielou Index [J]	Shannon Index [H]	Bray-Curtis Dissimilarity [BC]
Timepoint	Concentration (mmol L^{-1})	Replicate				
T1	0	na	154	0.55	2.75	
T2	0	1	129	0.43	2.10	0.54
T2	0	2	119	0.47	2.27	0.44
T2	0	3	122	0.42	2.03	0.50
T3	0	1	101	0.42	1.95	0.50
T3	0	2	104	0.38	1.76	0.54
T3	0	3	95	0.39	1.77	0.54
T4	0	1	94	0.50	2.29	0.40
T4	0	2	87	0.48	2.12	0.43
T4	0	3	97	0.42	1.91	0.52

Table 4: Ecological measures for microbial communities from thermophilic enrichments on SGW in the presence of ionic liquids, tetra butyl and diethyl. Bray-Curtis dissimilarity values represent comparison to the T1 community.

Community			Richness [R]		Pielou Index [J]		Shannon Index [H]		Bray-Curtis Dissimilarity [BC]	
Timepoint	Concentration (mmol L^{-1})	Replicate	Diethyl	Tetra butyl	Diethyl	Tetra butyl	Diethyl	Tetra butyl	Diethyl	Tetra butyl
T1	0	na	153	154	0.55	0.55	2.75	2.75		
T2	1	1	128	127	0.41	0.34	1.98	1.63	0.56	0.73
T2	1	2	127	128	0.38	0.44	1.84	2.13	0.59	0.57
T2	1	3	124	112	0.39	0.44	1.88	2.09	0.57	0.55
T2	10	1	120	127	0.31	0.30	1.48	1.44	0.68	0.81
T2	10	2	124	126	0.22	0.31	1.06	1.48	0.82	0.81
T2	10	3	136	132	0.18	0.30	0.90	1.48	0.84	0.81
T3	10	1	96	94	0.28	0.31	1.29	1.39	0.74	0.83
T3	10	2	108	100	0.23	0.29	1.07	1.32	0.85	0.81
T3	10	3	107	99	0.26	0.30	1.20	1.39	0.85	0.80
T3	50	1	142	154	0.21	0.41	1.03	2.05	0.92	0.87
T3	50	2	115	110	0.15	0.24	0.69	1.12	0.94	0.91
T3	50	3	156	168	0.28	0.39	1.39	1.99	0.91	0.86
T4	50	1	132	138	0.20	0.36	0.99	1.78	0.94	0.91
T4	50	2	90	100	0.06	0.27	0.25	1.26	0.97	0.88
T4	50	3	134	148	0.30	0.41	1.47	2.06	0.93	0.90
T4	75	1	156	137	0.52	0.25	2.64	1.23	0.87	0.94
T4	75	2	111	104	0.14	0.21	0.64	0.99	0.95	0.91
T4	75	3	103	154	0.09	0.44	0.40	2.20	0.96	0.90

Table 5: Ecological measures for microbial communities from thermophilic enrichments on SGW in the presence of ionic liquids, tetra butyl and diethyl after inoculation and incubation with preserved samples. Bray-Curtis (BC) dissimilarity values represent comparison to the T4 75 mmol L^{-1} IL community.

Community origin			Preservation conditions and treatment post storage					
IL	Timepoint	IL Concentration (mmol L^{-1})	Storage Treatment	IL Concentration (mmol L^{-1})	Mean Richness [R] *	Mean Pielou Index [J]*	Mean Shannon Index [H]*	Mean Dissimilarity [BC]*
Tetra butyl	T4	75	DMSO	75	149 A	0.58 A	2.90 A	0.78 A
Tetra butyl	T4	75	Glycerol	75	120 B	0.61 A	2.89 A	0.77 A
Tetra butyl	T4	75	Liquid Nitrogen	75	160 A	0.58 A	2.95 A	0.77 A
Tetra butyl	T4	75	Plated	75	151 A	0.58 A	2.89 A	0.73 A
Diethyl	T4	75	DMSO	75	155 A	0.48 A	2.42 A	0.50 A
Diethyl	T4	75	Glycerol	75	148 A	0.46 A	2.30 A	0.46 A
Diethyl	T4	75	Liquid Nitrogen	75	153 A	0.49 A	2.45 A	0.51 A
Diethyl	T4	75	Plated	75	160 A	0.48 A	2.44 A	0.51 A

* for a given IL, means followed by the same letter within columns are not significantly different at $\alpha = 0.05$

Table 6: Relative abundance of storage treatments for the five most abundant OTUs in the T4 mmol L^{-1} tetra butyl sample.

Treatment	Relative abundance (%)				
	Chelatococcus (p_Proteobacteria)	*Acinetobacter (p_Proteobacteria)*	*Exiguobacterium (p_Firmicutes)*	*Bacillaceae (p_Firmicutes)*	*Weissella (p_Firmicutes)*
T4	82.3	4.2	1.8	1.2	1.0
DMSO	5.0	21.6	11.3	0.1	5.7
Glycerol	6.0	22.2	11.3	0.2	5.2
Liquid N2	5.9	20.1	10.1	0.1	5.2
Plated	10.8	20.1	10.4	0.1	5.6

Table 7: Relative abundance of storage treatments for the five most abundant OTUs in the T4 75 mmol L^{-1} diethyl sample

Treatment	Relative abundance (%)				
	Bacillaceae (p_Firmicutes)	*Acinetobacter (p_Proteobacteria)*	*Exiguobacterium (p_Firmicutes)*	*Leclercia (p_Proteobacteria)*	*Stenotrophomonas (p_Proteobacteria)*
T4	89.2	3.0	0.9	0.9	0.7
DMSO	38.9	15.1	7.0	5.5	3.7
Glycerol	43.2	14.0	6.7	5.2	3.5
Liquid N2	38.0	14.9	6.7	5.4	3.9
Plated	38.2	14.5	5.0	5.4	4.6

Table headings for supplemental material

Table S1: SIMPER Analysis of OTUs responsible for >90% of the Bray-Curtis dissimilarity between microbial communities in T1 and T4 of replicate 2 at 0 mmol L^{-1} and 75 mmol L^{-1} tetra butyl concentration, respectively.

Table S2: SIMPER Analysis of OTUs responsible for >90% of the Bray-Curtis dissimilarity between microbial communities in T1 and T4 of replicate 2 at 0 mmol L^{-1} and 75 mmol L^{-1} diethyl concentration, respectively.

Table S3: SIMPER analysis of OTUs responsible for >90% of the Bray-Curtis dissimilarity between microbial communities in T4 at 75 mmol L^{-1} tetra butyl concentration and sample enriched using inoculum cryopreserved in DMSO.

Table S4: SIMPER analysis of OTUs responsible for >90% of the Bray-Curtis dissimilarity between microbial communities in T4 at 75 mmol L^{-1} tetra butyl concentration and sample enriched using inoculum preserved in liquid nitrogen.

Table S5: SIMPER analysis of OTUs responsible for >90% of the Bray-Curtis dissimilarity between microbial communities in T4 at 75 mmol L^{-1} tetra butyl concentration and sample enriched using inoculum cryopreserved in glycerol.

Table S6: SIMPER analysis of OTUs responsible for >90% of the Bray-Curtis dissimilarity between microbial communities in T4 at 75 mmol L^{-1} tetra butyl concentration and sample enriched using inoculum preserved by plating.

Table S7: SIMPER analysis of OTUs responsible for >90% of the Bray-Curtis dissimilarity between microbial communities in T4 at 75 mmol L^{-1} diethyl concentration and sample enriched using inoculum cryopreserved in DMSO.

Table S8: SIMPER analysis of OTUs responsible for >90% of the Bray-Curtis dissimilarity between microbial communities in T4 at 75 mmol L^{-1} diethyl concentration and sample enriched using inoculum preserved in liquid nitrogen.

Table S9: SIMPER analysis of OTUs responsible for >90% of the Bray-Curtis dissimilarity between microbial communities in T4 at 75 mmol L^{-1} diethyl concentration and sample enriched using inoculum cryopreserved in glycerol.

Table S10: SIMPER analysis of OTUs responsible for >90% of the Bray-Curtis dissimilarity between microbial communities in T4 at 75 mmol L^{-1} diethyl concentration and sample enriched using inoculum preserved by plating.