Community Dynamics and Glycoside Hydrolase Activities of Thermophilic Bacterial Consortia Adapted to Switchgrass

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Abstract

Microbial consortia adapted to switchgrass at 60°C were cultivated using green waste compost as the inocula. Microbial community analysis using marker gene amplicon sequencing demonstrated that thermophilic adaptation to switchgrass resulted in low diversity bacterial consortia with a few dominant members. Reconstruction of full length SSU rRNA genes from short read metagenomic sequencing revealed these communities had abundant populations closely related to thermophilic Paenibacilli, *Rhodothermus marinus*, and *Thermus thermophilus.* At lower abundances, ubiquitous populations of *Thermobaculum* from the *Chloroflexi* phylum and an uncultivated lineage of the Gemmatimonadetes phylum were observed. Supernatants isolated from these consortia had high levels of xylanase and endoglucanase activity. Compared to commercial enzyme preparations the endoglucanase enzymes had a higher thermotolerance and were more stable in the presence of 1-ethyl-3-methylimazolium acetate ([C2mim][OAc]), an ionic liquid used for biomass pretreatment. The supernatants were used to saccharify [C2mim][OAc]-pretreated switchgrass at elevated temperatures (up to 80°C) demonstrating that these consortia are an excellent source of enzymes for the development of enzymatic cocktails tailored to more extreme reaction conditions. The work described here provides a template to adapt microbial consortia to specific biomass substrates for efficient lignocellulose deconstruction.

Introduction

Enzyme cocktails that hydrolyze plant cell wall polysaccharides are a critical component of bioprocessing configurations designed to transform lignocellulosic biomass to biofuels (22, 39). The large variety of potential biomass feedstocks and pretreatments available require tailored glycoside hydrolase cocktails that function optimally under diverse conditions, including high temperatures, extreme pH, high biomass loadings, and the presence of residual pretreatment chemicals and inhibitors (23, 40). Current commercial biomass-deconstructing enzyme cocktails consist of preparations of fungal-derived glycoside hydrolases, namely cellulases and hemicellulases (6, 27, 33, 50, 51). However, fungal enzymes are often deactivated by elevated temperatures or by residual chemicals from pretreatment and are optimized for a limited number of feedstock/pretreatment combinations (6). For example, ionic liquids (ILs) such as 1-ethyl-3-methyl imidazolium acetate ([C2mim][OAc]) can dissolve lignocellulosic biomass and dramatically improve cellulose hydrolysis kinetics (21, 37, 49), yet multiple studies have shown that fungal endoglucanases are deactivated at low levels of ILs that may persist in the biomass after pretreatment (10, 56). In contrast, thermophilic bacterial and archaeal endoglucanases have been shown to be active in >20% [C2mim][OAc], suggesting that thermophilic prokaryotes may be an important source of enzymes for the development of alternative enzyme cocktails that are compatible with biomass pretreatments that utilize ILs or high temperatures (12).

Aerobic, thermophilic bacteria are a potentially rich source of robust glycoside hydrolases for biomass deconstruction. However, these bacteria generally secrete low

levels of glycoside hydrolases, especially cellulolytic enzymes (3, 25, 39). Recent efforts to identify these enzymes have involved functional screening of expression libraries or bioinformatic homology-based searches of sequences derived from isolated organisms or environmental samples (2, 58). However, these approaches have significant limitations: expression libraries may miss relevant genes due to low representation within the library and poor expression in laboratory host strains, while bioinformatic approaches suffer mainly from the limited ability to predict the specific characteristics of an enzyme (activity, thermostability, etc.), which is usually based on homology to known enzymes (52). The complexity of microbial communities in environmental samples also hampers bioinformatic enzyme discovery efforts because it often prevents assembly of full-length genes from metagenomic databases generated by current sequencing technologies (2, 13). Enrichment cultures on lignocellulosic biomass provide a method to reduce the complexity of the microbial communities and provide more tractable samples for detailed investigations. In previous studies, simplified bacterial communities have been enriched at elevated temperature with cellulose or biomass as substrates (38, 47, 60). These studies have focused on the isolation of thermophilic bacteria from the enrichments and the characterization of glycoside hydrolases secreted by these isolates. Here, we describe an alternative approach to discovering robust and highly active lignocellulosic-biomass degrading glycoside hydrolases, in which enrichments are performed using a targeted biomass feedstock, switchgrass, and the enriched microbial consortia are studied directly using culture-independent approaches. Amplicon pyrosequencing of small-subunit (SSU) rRNA marker genes and reconstruction of full length SSU rRNA genes from metagenomic sequencing data demonstrated that the bacterial consortia were simplified

and enriched for bacterial populations closely related to known biomass deconstructing lineages, as well as entirely new, uncultivated lineages. Functional characterization of glycoside hydrolases secreted by the consortia revealed that they are more thermotolerant and tolerant to an ionic liquid used for biomass pretreatment than commercial fungal cocktails when assayed on purified biomass substrates and pretreated switchgrass.

Materials and Methods

Environmental Samples

The compost inocula for switchgrass-adapted cultures were collected from two municipal green waste composting facilities. The first facility was Grover Soil Solutions located in Zamora, CA. Samples collected from this site are refered to as Zamora (Z) and were collected as described in (2). The second facility was Jepson Prairie Organics located in Vacaville, CA. Samples collected from this site are referred to as Jepson Prairie (JP). This facility composts municipal green waste in watered and turned windrows. Compost was collected from windrows in the mesophilic (7 days) and thermophilic (30 and 60 day) composting stages. A spade was used to remove the top 12 inches of each windrow and the exposed biomass was placed into 50 mL Falcon tubes, transported at room temperature, and frozen at -80°C within 2 h.

Cultivation Conditions

Green waste compost microbial communities were adapted to ground switchgrass (*Panicum virgatum* L.) as their sole carbon source by serially passing the community through nine liquid cultures (Table 1). To remove soluble sugars and other nutrients from the switchgrass, it was exhaustively extracted with water and ethanol in a Soxhlet apparatus, then dried at 50°C prior to use. The first culture was set up by adding 0.5 g of Zamora (Z) or Jepson Prairie (JP) green waste compost to a 250 mL shaker flask containing 50 mL M9TE and 2.5 g of extracted switchgrass. M9TE medium was prepared as in (13). Cultures were shaken at 200 rpm for two weeks at 60°C. The JP compost added to the culture consisted of a 1:1:1 mixture of 7, 30, and 60 day compost

samples. The eight subsequent serial cultures were set up by adding 2 mL of the previous JP or Z culture to a 250 mL shaker flask containing 50 mL M9TE and 3 g of extracted switchgrass, and shaking at 200 rpm for two weeks at 60°C. An additional 60°C culture containing 0.5 g of extracted switchgrass was added to the series at culture eight. It was seeded with 2 mL of the 60°C culture containing 3 g of switchgrass from the seventh passage. One more additional 60°C culture containing 0.5 g of extracted switchgrass and 0.25 g carboxymethyl cellulose (CMC) was added to the series at culture nine. It was seeded with 2 mL of the 60°C culture containing 3 g of switchgrass from the eighth passage. No growth or enzymatic activity was observed in cultures containing only switchgrass without compost inoculation (data not shown).

Isolation of Culture Supernatants

The final cultures (passage #9) were used for glycoside hydrolase activity analysis and all passages were prepared for DNA isolation. The culture supernatant was clarified by decanting 30 ml of the culture supernatant into several 2 ml centrifuge tubes, and spinning at 21,000 x *g* for 10 min. The supernatant was removed and the pellets from four tubes consisting of the switchgrass-adapted microbial community and particles of switchgrass were combined, transferred into 2 ml Lysing Matrix E tubes (Qbiogene**,** Montreal, QN), and frozen at -80°C for DNA extraction. The clarified supernatants were pooled in 50 mL Falcon tubes and passed through a 0.2 μm filter; this supernatant was used directly for measuring glycoside hydrolase activity. For zymography, contaminating lignin-derived phenolic compounds were removed from the clarified supernatant by adding polyethyleneimine (Sigma, St. Louis, MO) to a final concentration

of 0.1% to 1 ml of supernatant, shaking for 2 h at 4°C, and centrifuging at 10,000 *x g* for 20 min at 4°C.

SSU rRNA Amplicon Pyrosequencing

DNA was isolated from the pellets generated during isolation of culture supernatants described above. DNA isolation and sequencing were performed as previously described (13). Sequencing tags were quality trimmed and analyzed using the pyroclust version of the software tool PyroTagger (http://pyrotagger.jgi-psf.org) with a 220 bp sequence length threshold and an accuracy of 10% for low quality bases (20, 35). To reduce noise in statistical analysis all singleton OTUs were removed from the data set. Calculation of Shannon's diversity indices and Bray-Curtis dissimilarities were performed using the vegan package in the R software environment (http://CRAN.R-project.org/package=vegan).

Illumina Sequencing and EMIRGE Reconstruction of SSU rRNA Genes

Illumina libraries were constructed and sequenced from DNA isolated from Z-9 1% SG and JP-9 1% SG using protocols developed at the Joint Genome Institute (Supplemental Files 2 and 3). Libraries of paired-end 76bp reads were created for JP-9 1% SG (4.8 GB) and Z-9 1% SG (1.3 GB). Full length ribosomal small subunit genes were reconstructed from Illumina sequencing reads using EMIRGE (41). For both communities, all reads were trimmed from the 3' end until a base with quality score ≥ 3 was encountered. Paired-end reads where both reads were at least 60 nucleotides in length after trimming were used as inputs to EMIRGE, with the reference database described in Miller et al.

(41). Data from each community was processed separately for 80 iterations, and SSU sequences with relative abundances > 1% were kept for further analysis.

Phylogenetic Tree Construction

Maximum Likelihood trees were built with RAxML (54), using the GTRGAMMA model of nucleotide substitution and 100 bootstrapped replicates. Sequences were first aligned with Muscle (19) using default parameters, and columns in the full alignment with gaps were removed from the alignment for tree construction and pairwise percent identity calculations. For Figure S2, the alignment was manually edited to span the region covered by pyrotag sequencing, and columns in the alignment with a majority of gaps were removed. *Methanococcus jannaschii* (Genbank: M59126.1) was used as the outgroup to root the trees.

Glycoside Hydrolase Activity Assays

The enzymatic activities present in each switchgrass-adapted supernatant were characterized using two types of assays: the DNS reducing sugar assay and the *p*-nitrophenol (pNP) assay. Activity units for all assays were calculated as μmole sugar liberated min^{-1} ml^{-1} and reported as U/ml. Endoglucanase and xylanase activities were assayed by the DNS assay (12). The other glycoside hydrolase activities were measured using the pNP assay (cellobiohydrolase, β-D-glucosidase, β-D-xylosidase, and α-L-arabinofuranosidase)(53). To eliminate background, heat-killed samples generated by heating the supernatant to 95°C for 16 h were used as blanks. pNP assays were conducted by mixing 80 μl of each pNP substrate at 1 mg/ml in 50 mM NaOAc pH 5.0

with 20 µl of sample and heating for 1 h at 70°C, then adding 50 µl of cold 2% $NaCO_3$. Absorbance was measured at 410 nm and the concentration of sugars liberated was calculated using the extinction coefficient 18.5 ml/µmol/cm.

For the DNS assay, 32 µl of supernatant (diluted 1:10 in samples with high activity) were added to 8 µL water and 40 µL of either 2% CMC (Sigma, St. Louis, MO) or 2% oats spelt xylan (soluble fraction, Sigma, St. Louis, MO**)** in 100mM NaOAc pH 5.0, and incubated for 30 min at 70°C. DNS reagent (80 µl) was then added to each sample and heated to 95°C for 5 min. The absorbance at 540 nm was measured for each sample, values from the blanks were subtracted, and the concentration of sugars was determined by comparing to a standard curve of glucose or xylose. Temperature profiles were determined by repeating the steps above but incubating the samples at 60, 70, 80, 90, and 99°C. Activity in ionic liquid was determined by mixing the supernatant, [C2mim][OAc], and 40 µl of 2% CMC or xylan in a total reaction volume of 80 µl containing a final concentration of 0, 10, 15, 20, 25, and 30% [C2mim][OAc], then incubating for 30 min at 50°C. Sugar liberated was calculated per µl of supernatant in each reaction and the percent activity was calculated relative to the activity of the sample with 0% [C2mim][OAc]. Comparisons were made to Novozymes enzyme cocktails: NS50030 for xylanase activity and NS50013-10 (a mixture of 10 µL NS50013 and 1 µlL of NS50010) for cellulase activity, each diluted 1:10000.

Saccharification of IL-Pretreated Switchgrass

The supernatant from the JP-9 1%SG switchgrass-adapted community was tested for its ability to saccharify ionic liquid pretreated switchgrass. The switchgrass was pretreated with [C2mim][OAc] at 140°C for three hours and recovered using a proprietary method (17). Duplicate 10 mL saccharification reactions were set up with 250 mg of IL-pretreated switchgrass. The JP-9 1% SG reaction consisted of 9.5 ml of pre-warmed supernatant mixed with 0.5 ml of 1 M NaOAc pH 5.0. For comparison, a Novozymes cocktail containing both cellulase and xylanase activities was mixed using recommended enzyme/glucan content of biomass (w/w) loadings; 8.32 µl of NS50013 (1% w/w) and 0.832 µl each of NS50010 and NS50030 (0.1% w/w) were added to 10 ml pre-warmed 100 mM NaOAc pH 5.0 buffer. Lower amounts of enzymes were used to test thermo- and IL-tolerance due to limited amounts of the JP-9 1% SG supernatant. Lyophilized supernatant (1 mL) was resuspended in 1 mL of 100 mM NaOAc pH 5.0 and added to 9 ml of the same pre-warmed buffer. The Novozymes enzyme preparation was adjusted to match more closely the endoglucanase and xylanase activity of the JP-9 1%SG supernatant: 0.25 µl of the cellulase mix (8.32 µl of NS50013 and 0.832 µl of NS 50010) and 2.5 µl of the xylanase NS50030 were added to 10 ml pre-warmed buffer. For IL-tolerance, the reaction buffer used was 100 mM NaOAc/15% [C2mim][OAc] at pH 5.0. All samples were incubated in a shaker for 72 h at 70°C or 80°C, and 150 µl was withdrawn for each time point and frozen at -20°C. Time point samples were then spun at 21,000 *x g* for 5 min at 4°C and 5 µl was added to 55 µl water and 60 µl DNS reagent. Samples were incubated at 95°C for 5 min, and ABS 540 nm was taken. A background subtraction blank was made by adding 5 µl of each sample to 115 µl of water. The total sugars were calculated by comparing to a standard curve of glucose. The percent total

sugar was calculated using the estimated glucan and xylan content of switchgrass (47% and 33%, respectively).

Zymography

Zymography was conducted using a modified protocol from (48). One quarter volume of 4x sample application buffer was added to the PEI-treated secretome samples, heated to 95°C for 3 min, and 5 to 40 µl of each sample was loaded on a 10% polyacrylamide gel containing either 0.1% soluble oat spelts xylan or 0.2% CMC for SDS-PAGE. Gels were then washed 4x for 15 min each in 25% (v/v) isopropanol followed by 4x washes 15 min each in 50 mM NaOAc pH 5.0 at room temperature. Gels were then incubated at 50°C for 0.5 to 2 h in 50 mM NaOAc pH 5.0, washed 2x for 5 min each with water, stained with 0.1% Congo Red in 50 mM NaOAc pH 7.0 for 15 min, and washed with 1 M NaCl to remove the residual Congo Red.

Results

Adaptation of Bacterial Consortia to Switchgrass

Liquid enrichment cultures with inocula sourced from two green-waste compost facilities in Northern California, Jepson Prairie Organics in Vacaville, CA (JP) and Grover Soil Solutions in Zamora, CA (Z), were adapted to switchgrass (6% w/v) as a sole carbon source through multiple passages at 60ºC (Table 1). The switchgrass was extracted with water/ethanol to remove soluble material and promote microbial deconstruction of complex plant cell wall polymers. Changes in community composition were monitored by SSU rRNA amplicon pyrosequencing after each of the nine serial passages for both JP and Z switchgrass-adapted (SG-adapted) cultures (Figure 1). The compost inocula were complex communities composed of 259 taxa for JP and 593 taxa for Z, most of which were clustered with bacterial taxa**.** The microbial community profiles of the JP and Z inocula were divergent (Bray Curtis dissimilarity 0.81). A significant reduction in taxonomic diversity was observed in the initial adaptation to switchgrass at 60°C. This reduction in complexity was demonstrated by a reduction in the Shannon diversity index from 3.87 in the initial JP inoculum to 2.04 in the first JP enrichment and from 4.42 to 2.28 for the Z enrichment. By the third passage, the microbial community structures of the third passage of the JP and Z enrichments converged (Bray-Curtis dissimilarity 0.23). These low diversity communities were maintained through nine passages on switchgrass, with the Shannon diversity index for the JP-9 enrichment (2.06) and the Z-9 enrichment (2.26) remaining close to the initial enrichment, however the community structures diverged somewhat compared to early enrichments (Bray-Curtis dissimilarity 0.58). Preliminary measurements of glycoside

hydrolase activity in culture supernatants from the 6% switchgrass enrichments indicated relatively low activities, so at an advanced state of enrichment, the JP and Z enrichments were perturbed by lowering switchgrass loadings to 1% (JP-9 1%SG/Z-9 1%SG) and by amending 1% switchgrass cultures with carboxymethylcellulose (JP-9 SGCMC/Z-9 SGCMC) (Table 1). The community composition of the cultures containing lower amounts of biomass was almost identical at the phylum level compared to the cultures with 6% switchgrass (Figure 1), however the glycoside hydrolase activities recovered in the supernatants from the lower biomass cultures were higher (see below).

Community Composition of Enrichment Cultures

Comparison of SSU rRNA amplicons at the level of individual OTUs (97% identity) demonstrated that the enrichment cultures after nine passages were dominated by only a few phylotypes (Fig 2A and B)(20). Despite the different inocula from which they were derived, the JP and Z enrichment cultures had remarkably similar community profiles. The most abundant sequences recovered from both the high and low biomass JP and Z enrichments were closely related to thermophilic gram-positive Firmicutes of the Paenibacillaceae family (*Thermobacillus, Paenibacillus*) and *Rhodothermus marinus*, a known biomass deconstructing member of the Bacteriodetes typically isolated from hot springs. Sequences related to thermophilic Chloroflexi (*Thermobaculum, Sphaerobacter, Thermomicrobium*), were recovered at lower abundances, as were sequences of a population related to an uncultivated lineage, Gemm-5, in the Gemmatimonadetes phylum. A significant difference between the JP and Z enrichments was the prominence of a population closely related to *Thermus thermophilus* in the JP enrichments (27% in

the high biomass; 39% in the low biomass) that was almost absent in the Z enrichments. However, in the Z enrichments, sequences related to the family *Truperaceace* in the *Deinoccocus/Thermi* phylum were recovered (10% in high biomass; 1% in low biomass). Though, there was not significant alterations in community membership when CMC was added as a co-substrate, the proportion of amplicons related to *Rhodothermus marinus* increased in both the JP and Z cultures (Figure 2)

Recovery of Full Length Sequences from Illumina Metagenomic Sequencing Data

To provide a more detailed understanding of the population structure of the bacterial consortia enriched on switchgrass, full-length SSU rRNA sequences were reconstructed from short-read metagenomic data obtained for JP-9 and Z-9 1% SG enrichment cultures. These sequences were reconstructed by the EMIRGE method, which uses an expectation maximization algorithim to reconstruct SSU rRNA gene sequences and estimate abundances from a low diversity acid mine drainage community using Illumina metagenomic sequencing data (41). Comparison of the full-length SSU sequences with a maximum likelihood tree confirmed that the two enrichments contained closely related populations (Figure 3). For most members of the enrichment cultures, the reconstructed full length SSU sequences contained identical ≈200bp segments to a corresponding representative pyrotag cluster sequence, providing an independent confirmation of the community structure and validating the ability of the EMIRGE method to reconstruct SSU genes (Figure S2). However, a maximum likelihood tree indicated that the thermophilic *Paenibacilli* were more diverse than predicted by the shorter pyrotag sequences, and were more divergent from the nearest *Paenibacilli* representatives (Figure

3). Only about half (49 %) of all possible reconstructed full-length *Paenibacillus* and *Thermobacillus* sequence pairs shared an average pairwise identity ≥ 95 %. However, when only considering the aligned V8 hypervariable region covered by the pyrotag sequencing, nearly all of the *Paenibacillus* and *Thermobacillus* sequence pairs share ≥ 95 % identity, obscuring diversity in the community.

The EMIRGE method also predicts the relative abundances of microbes in mixed consortia by probabilistically measuring the relative proportions of reads recruited to each reconstructed SSU rRNA gene sequence. The predicted abundances have been shown to be accurate for an artificial community with a defined mixture of eukaryote and prokaryote members (41). For the JP and Z enrichments, EMIRGE abundance estimates showed good general concordance with abundance estimates made from pyrotag sequencing (Table 2). However, the estimated abundance of the thermophilic *Paenibacilli* was lower for the full length sequences compared to the pyrotags and for the JP enrichment, the abundance of the *Thermus thermophilus* population was higher compaed to the pyrtoags. For both enrichments, the estimated abundance of the Gemmatimonadetes populations was higher than predicted by pyrotag abundance, and Trueperaceae were predicted at higher abundance in the Z enrichment by EMIRGE.

Glycoside Hydrolase Activities in Switchgrass-Adapted Cultures

Comparison of endoglucanase and xylanase activity at 70°C and pH 5 in supernatants obtained from the high and low biomass cultures indicated that more activity was recovered from the low biomass cultures, so the activity profiles of these supernatants were studied in detail. The supernatants (JP-9 1%SG, Z-9 1% SG, JP-9 SGCMC, and Z-

9 SGCMC) displayed significant enzymatic activity in standard assays on purified substrates, including endo/exogluconase, β-glucosidase, endoxylanase, β-xylosidase, and α-L-arabinofuranosidase activities (Figure 4A-B and Supplemental Figure 1). To benchmark these activities against known glycoside hydrolases, endoglucanase and xylanase activities were compared to commercial enzymes preparations produced by Novozymes diluted to comparable activity levels.

Temperature profiles of endoglucanase activity demonstrate that these supernatants possess more thermostable enzymes than the commercial cocktails from Novozymes (Fig 5A). Higher optimum temperatures were observed for the enzyme mixtures recovered from the low biomass enrichments compared to Novozymes enzymes preparations (T_{opt}- 80 °C vs, 60°C). The supernatants from the low biomass cultures even retained endoglucanase activity (15-50%) at 99ºC. The supernatant endoglucanase enzymes also exhibited high levels of tolerance to the ionic liquid [C2mim][OAc], retaining ~50% activity in the presence of 30% [C2mim][OAc], while the Novozymes cocktails were essentially inactive at 10% [C2mim][OAc] (Fig 6A). In contrast, xylanase activities of the culture supernatants exhibited only a slightly higher thermotolerance and tolerance to [C2mim][OAc] than the Novozymes xylanase preparation (NS50030) (Fig 5B and 6B).

Glycoside Hydrolase Activities on Ionic Liquid-pretreated Switchgrass

Though the supernatants obtained from the switchgrass-adapted communities performed well on model biomass substrates, a more stringent test of their utility is their ability to efficiently deconstruct pre-treated biomass. The supernatant obtained from the JP-9 1% SG enrichment performed well on model substrates and was selected to saccharify

switchgrass pretreated with [C2mim][OAc]. For comparison, Novozymes enzyme preparations were mixed at the recommended enzyme/biomass loadings (w/w), which amounts to approximately 15x the endoglucanase and 0.25x the xylanase activity compared to the JP-9 1% SG supernatant at 70°C. A reaction temperature of 70°C was chosen because this temperature represented an intermediate temperature between the T_{opt} of the endoglucanase activity of the culture supernatant and the Novozymes preparation. Despite the comparatively low endoglucanase activity in the JP-9 1% SG supernatant, both cocktails liberated virtually all the sugars from the [C2mim][OAc]-pretreated switchgrass at 70°C after 72 h, demonstrating that the enzymes secreted by the bacterial consortia were active on complex, insoluble biomass substrates (Figure 7A).

The enzyme cocktails were then compared at a higher temperature (80°C) or in 15% [C2mim][OAc] at 70°C (Fig 7B-C). Due to limited amounts of sample, the JP-9 1% SG supernatant was diluted ten-fold in these saccharifications. For a more balanced comparison, the Novozymes cocktail enzyme loads were adjusted to approximate the activity levels of the JP-9 1% SG supernatant enzymes using endoglucanase and xylanase assays. At these enzyme loadings only about 40 to 50% of the total sugars were liberated from [C2mim][OAc]-pretreated switchgrass at 70°C. The Novozymes cocktail had a faster initial rate of saccharification than the JP-9 1%SG supernatant, though to a lesser extent than seen at higher enzyme loadings, and again the total sugars liberated after 72 h were comparable to the JP-9 1%SG enzymes. At 80°C, the Novozymes cocktail liberated 73% less sugars in 72 h than at 70°C, while the JP-9 1% SG secretome liberated about 31% less sugars. In 15% IL, the Novozymes cocktail also liberated fewer sugars after 72 h (81% less than in 0% IL), while the JP-9 1% SG secretome liberated only 29% less

sugars. At 80°C and at 70°C in the presence of 15% [C2mim][OAc], the sugar release profile suggests that the Novozymes cocktail was inactivated within the first 2 h, while the JP-9 1% SG secretome was active throughout the 72 h incubation.

Zymography to Identify Endoglucanase and Xylanase Enzymes

To compare the biomass-deconstructing enzyme profiles of the switchgrass-adapted microbial consortia, zymograms with CMC and xylan as substrates were performed on the JP-9 1%SG, Z-9 1%SG, JP-9 SGCMC, and Z-9 SGCMC secretomes (Figure 8A-B). Greater than 10 bands were observed for each activity, indicating that the enriched consortia each produced multiple endoglucanase and xylanase enzymes. Comparison of the JP and Z samples indicates that the active enzyme complement in both samples is remarkably similar, suggesting that similar populations secrete the endoglucanase and xylanase enzymes responsible for biomass deconstruction in these cultures. Increased intensity of the some of the endoglucanase bands was observed in the cultures amended with CMC, consistent with the observed increase in endoglucanase activity (Figure 4A).

Discussion

We found that growing compost microbial communities on extracted switchgrass to be an effective means to generate simplified bacterial consortia that possess higher activity than commercial fungal cocktails when assayed on pretreated switchgrass at 80°C and in the presence of ionic liquids. Adaptive cultivation on other feedstocks such as lignin, cellulose and CMC has also successfully generated simplified communities and, along with this study, indicate that this method is a useful tool for making a range of simplified biomass-degrading consortia tailored to deconstruct a designated feedstock under defined conditions such as temperature or pH (13, 38, 47). These adaptations also generate communities that are amenable to detailed characterization using genomic, transcriptomic and proteomic techniques, which will provide a comprehensive picture of which community members and glycoside hydrolases are responsible for biomass deconstruction (8, 57).

Following the composition of the communities using SSU rRNA gene amplicon pyrosequencing revealed that the members of the native compost microbial community rapidly adapted to switchgrass at elevated temperature. A similar approach using amplicon pyrosequencing has been used to document the changes in microbial community composition of mesophilic and thermophilic fermentations of alkaline-pretreated sorghum to organic acids (29). Despite the differences in the community composition of the initial compost inocula, both the JP and Z enrichments converged to closely related consortia with similar microbial community compositions and levels of secreted glycoside hydrolase activities. Perturbation of the enriched communities by inoculation into cultures with low biomass (1% switchgrass) and amendment with CMC

did not significantly alter the community composition of the enrichments, however the glycoside hydrolase activities were higher. T his observation indicates that biomass loading is a critical component of enrichment cultivation studies and can be adjusted to maximize titers of enzymes of interest without dramatically affecting the community composition.

The success of this enrichment strategy was demonstrated by the selection of biomass-deconstructing populations. Analysis of amplicon pyrosequencing data and reconstructed SSU rRNA genes from metagenomic sequencing demonstrated that thermophilic *Paenibacilli* and *Rhodothermus* populations were abundant in both enrichments. Both of these populations have cultured relatives known to degrade biomass, suggesting that they are the source of many of the glycoside hydrolases present in the culture supernatants. *Rhodothermus marinus*, closely related to the populations in the JP and Z enrichments, was isolated from a marine hot spring, and several biomass-degrading enzymes have been identified in this species, including endoglucanases, xylanases, and α-L-arabinofuranosidases, activities of which have been recovered in the JP and Z enrichment culture supernatants (1, 3, 9, 11, 25, 26, 32, 45). The thermophilic Paenibacilli present in the cultures are related to *Thermobacillus xylanilyticus* and *T. composti*, both of which have been reported to have xylanase activity (55, 59); a thermostable xylanase and an α-L-arabinofuranosidase have been cloned and characterized from *T. xylanilyticus* (14, 15, 43, 44). Additionally, compost and swine waste-derived microbial communities enriched on microcrystalline cellulose at elevated temperatures under aerobic conditions also contained thermophilic *Paenibacilli* related to

the populations enriched from switchgrass, suggesting that these microbes are critical components of biomass deconstruction under thermophilic aerobic conditions (38, 47).

Members of the Chloroflexi phylum are ubiquitous in the enrichments, and amplicon sequences related to *Thermobaculum terrenum* often were very abundant (10-30% percent) in the early enrichment passages at 6% biomass loading (5)(Figure 1). As the Chloroflexi have relatively few cultivated and sequenced representatives, their metabolic capabilities are not well understood (16, 30, 31, 61). Inspection of genomes available for three thermophilic type strains belonging to this phylum (*T. terrenum*, *Sphaerobacter thermophilus*, *Thermomicrobium roseum*), indicates that they possess a number of glycoside hydrolase enzymes, including cellulases and hemicellulases (36). Another prominent population present in both the JP and Z enrichments is a member of an uncultivated lineage, (Gemm-5), in the Gemmatimonadetes (62) distantly related to the sole cultivated representative of this phylum, *Gemmatimonas aurantica* (SSU rRNA genes have 88% identity). Since both the Chloroflexi and Gemmatimonadetes are commonly found in soils and sediments, sequence information from these populations will illuminate how they function in the biomass-deconstructing enrichment cultures as well as terrestrial ecosystems (7, 42, 46).

The bacterial population closely related to *Thermus thermophilus* in the JP enrichment is puzzling, because it is a dominant member of the population in this enrichment but is absent in the Z enrichment. This observation suggests that the *T. thermophilus* population may not be involved in biomass deconstruction as the glycoside hydrolase activities and the CMC/xylan zymography are very similar in both enrichments. The genomes of sequenced strains of *T. thermophilus* do not encode

glycoside hydrolases necessary for biomass deconstruction, however, *T. thermophilus* strains have been isolated from hot composts (60-80°C) and shown to express highly active xylanases (28). Detailed proteomic analysis of the secreted proteins in the supernatant and isolation of *T. thermophilus* strains from the enrichment cultures will resolve its role.

Comparison of microbial community profiles between the SSU amplicon pyrosequencing and the SSU rRNA gene sequences recovered from the metagenomic sequencing revealed similar community structures that diverged in two notable ways. The full length sequences of the thermophilic *Paenibacilli* were more diverse than predicted by amplicon pyrosequencing, reflecting that this family has variable regions of the SSU gene that are found outside of the V8 region used for cluster assignment for the pyrotag sequences. The two methods also differed slightly in their estimates of population abundance, though the amplicon pyrosequencing and the Illumina metagenomic sequencing were performed on the same DNA samples for both the JP and Z enrichments. These differences likely stem from inherent biases specific to the sequencing technology used. The estimates of abundance of the thermophilic *Paenibacilli* and Gemmatimonadetes are the most divergent between the two methods, and the overrepresentation of the thermophilic *Paenibacilli* may be related to the large number of rRNA gene copies present in these bacteria (4, 18). Further comparisons between these two methods of determining species abundance will be required to identify and control for potential sources of bias.

A significant amount of glycoside hydrolase activity, especially xylanase activity, was recovered from supernatants isolated from the switchgrass-adapted consortia. The

xylanase activity is comparable to highly productive *Bacillus* and *Paenibacillus* strains cultivated under optimized conditions, suggesting that mixed consortial cultivation on switchgrass is an effective method to generate highly xylanolytic cultures (34). Celluloytic activity was relatively low and cultivations on pretreated switchgrass are being explored to enhance the cellulolytic activity of the recovered supernatants. However, comparison of the endoglucanase activity between the culture supernatants and the Novozymes preparations indicates that the thermophilic bacteria secrete enzymes that are both more thermotolerant and more active in the presence of ionic liquid [C2mim][OAc]. These assays confirmed the relationship between thermotolerance and tolerance to [C2mim][OAc] observed in purified endoglucanases from fungal, bacterial and archaeal sources (12). Interestingly, the thermotolerance and the [C2mim][OAc]-tolerance of the xylanase activities of the culture supernatants and the Novozymes preparations were comparable, suggesting the properties of the xylanases in the culture supernatants and the Novozymes xylanase preparation (NS50030) may be similar.

Both the Novozymes enzymes preparations and the culture supernatants were able to saccharify [C2mim][OAc]-pretreated switchgrass at 70°C. These observations indicate that both systems possess the necessary complement of glycoside hydrolase enzymes to release sugars from complex biomass. Surprisingly, the Novozymes preparations efficiently released sugars from pretreated switchgrass at 70°C despite that temperature being 10°C above the cellulase optimum temperature and 20°C higher than the recommended reaction temperature (24). Perhaps this is due to the greatly enhanced hydrolysis kinetics of [C2mim][OAc]-pretreated switchgrass compared to acid-pretetreated switchgrass, allowing for rapid polysaccharide hydrolysis before enzyme

denaturation (37). However, at 80°C or in the presence of ionic liquids, the enhanced stability of the glycoside hydrolase enzymes from the thermophilic bacterial cultures is evident (Figure 7), demonstrating their value as sources of enzymes for enzymatic cocktails adapted to more extreme reaction conditions than fungal enzymes can tolerate.

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Table 1: Switchgrass-adapted Communities

Inoculum	Feedstock (% w/v)	Temp (°C)	Culture ID	2 wk Passage
JP compost	SG (6)	60	JP-9	9x
JP passage #7	SG (1)	60	JP-9 1%SG	2x
JP passage #8	SG (1) CMC (0.5)	60	JP-9 SGCMC	1x
Z compost	SG (6)	60	Z-9	9x
Z passage #7	SG (1)	60	Z-9 1%SG	2x
Z passage #8	SG (1) CMC (0.5)	60	Z-9 SGCMC	1x

The Culture ID represents the final culture used for analysis of the culture supernatant.

Table 2. Comparison of Estimated Abundances for Pyrotag and Metagenomic Sequencing fro JP-9 1%SG and Z-9 1%SG[1]

Population	JP-Estimated Abundance from Pyrotags	JP-Estimated Abundance from EMIRGE	Z-Estimated Abundance from Pyrotags	Z-Estimated Abundance from EMIRGE
Thermophilic Paenibacilli[2]	40.43	20.38	69.66	52.55
Thermus thermophilus	39.33	49.00	<1.00	<1.00
Rhodothermus marinus	9.62	10.37	17.17	18.38
Gemm-5	2.05	7.70	3.15	13.50
Thermomicrobia[3]	1.18	<1.00	2.41	2.85
Thermobaculum terrenum	1.43	1.40	<1.00	<1.00
Sphaerobacter thermophilus	<1.00	1.16	<1.00	<1.00
Trueperacae	<1.00	<1.00	1.01	7.88

[1]Populations with >1% estimated abundance in pyrotag and EMIRGE-derived sequences are depicted in the table. Total estimated abundance of these depicted populations is 90-95% of the total population.

[2]Pyrotags abundances are represented by sum of the abundances for pyrotag clusters related to Paenbacillus sp. str. SAFN-007, Paenibacillus kobensis DSM 10249, Paenibacillus D273a and Thermobacillus sp. str. KWC4. EMIRGE-derived sequence abundance is the sum of individual sequences presented in Figure 3 that cluster with Paenibacillacae family (JP 211, JP 261, JP 2339, JP 2453; JP 2459, Z 19, Z51, Z 137, Z 146, Z 261, Z 484, Z 1266, Z 1300). The individual estimated abundances of each of these sequences is are listed on the phylogenetic tree in Figure S2.

Figure Legend

Figure 1. Microbial community profiles of the native compost inoculum and serial passages of the switchgrass-adapted comunities. Profiles are based on the number of SSU rRNA amplicon clusters and are reported as bacterial phylum, all archaea, fungi, and all other eukaryotes.

Figure 2. Plot of the relative abundance of the top 1% of SSU rRNA amplicon clusters (OTUs) from the compost inoculum, and the switchgrass-adapted communities: passages 1 and 9, low biomass (1%SG), and CMC amended (SGCMC) cultures. (A) Jepson Prairie compost (B) Zamora compost. A cutoff of 1% abundance was chosen to highlight the most abundant organisms present in the community. OTUs that were assigned the same name by Pyrotagger are distinguished by adding the pyrotagger cluster number after the name. GenBank accessions numbers for each cluster assigned by Pyrotagger are included in Supplemental Table 1A (JP) and 1B (Z).

Figure 3. Maximum likelihood phylogenetic tree of 1% SG enrichment communities. Full-length SSU sequences reconstructed with EMIRGE are shown for both Z (red circles) and JP (blue diamond) communities, along with selected reference sequences identified from pyrotag sequencing of the initial inocula, the first switchgrass enrichment and the ninth enrichment. Bootstrap support values >50% are shown at the nodes on the tree. Units are base substitutions per site. GenBank accession numbers for sequences used in this tree are listed in Figreu S1.

Figure 4. (A) endoglucanase and (B) endoxylanase activities measured in the supernatant of the final switchgrass-adapted cultures at 70°C and pH 5.0. Novozymes cellulase and xylanase cocktails were used as positive controls. The Novozymes NS5003-10 and NS50030 enzymes were diluted 1:1000 and 1:10000, respectively, concentrations that are similar to those used to saccharify biomass at 2.5% w/v loadings in Figure 7A. Endoxylanase values reported were extrapolated from 1:10 dilutions of supernatant sample.

Figure 5. Temperature profiles of the (A) endoglucanase and (B) endoxylanase activities of the low biomass and CMC amended switchgrass-adapted community culture supernatants. NS50013-10 is the Novozymes cellulase positive control, and NS50030 is the Novozymes xylanase positive control.

Figure 6. Ionic liquid tolerance profiles of the (A) endoglucanase and (B) endoxylanase activities of the supernatants from the low biomass and CMC amended switchgrass-adapted cultures. The reactions were incubated at 50°C and pH 5.0. NS50013-10 is the Novozymes cellulase positive control, and NS50030 is the Novozymes xylanase positive control.

Figure 7. Saccharification plot of ionic liquid-pretreated switchgrass incubated with either the JP-9 1%SG secretome or a Novozymes cellulase (NS50013-10)/xylanase (NS50030) cocktail. Saccarification using (A) undiluted supernatant and the Novozymes cocktail mixed at recommended enzyme loadings, incubated at 70°C pH 5.0 or (B-C) 1:10 diluted supernatant and the Novozymes cocktail mixed with endoglucanase and xylanse enzyme activity levels matching the supernatant, incubated at 70°C and either (B) 80°C or (C) with 15% of the IL-[C2mim][OAc] at 70°C, each reaction at pH 5.0.

Figure 8. Zymography of the the supernatants from the low biomass and CMC amended cultures. (A) CMC zymogram (B) oat spelts xylan zymogram. Lanes are 1) JP-9 1%SG, 2) JP-9 SGCMC, 3) Z-9 1%SG, 4) Z-9 SGCMC. The enzymatic reaction was incubated at 50°C and pH 5.0. Similar results were obtained when zymography was performed at 70 °C and pH 5.0 (data not shown).

Figure 1

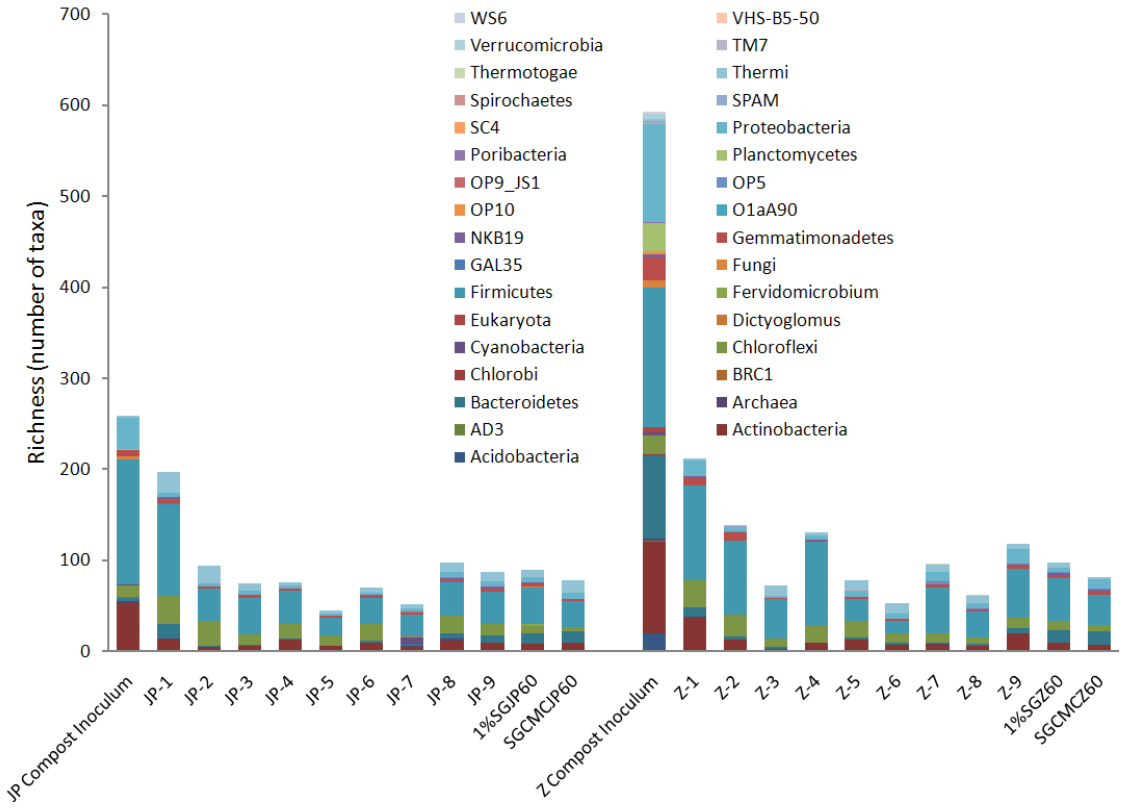
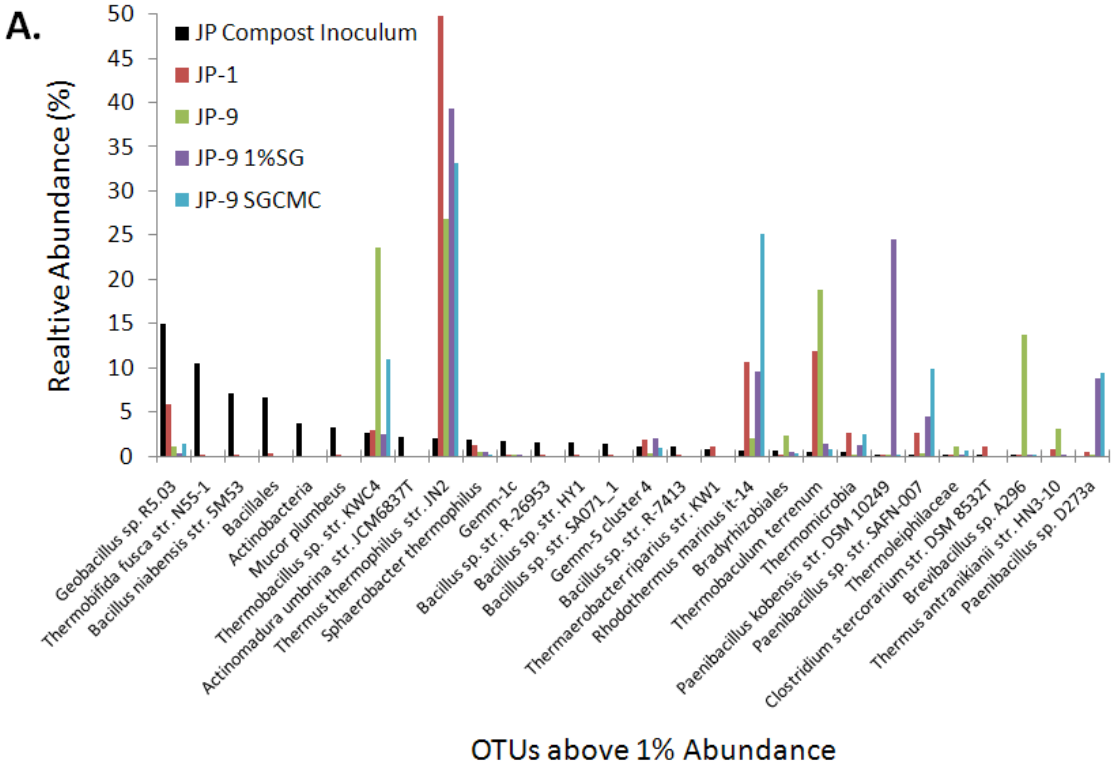

Richness (number of taxa)
700
600
500
400
300
200
100
0
WS6
Verrucomicrobia
Thermotogae
Spirochaetes
SC4
Poribacteria
OP9_JS1
OP10
NKB19
GAL35
Firmicutes
Eukaryota
Cyanobacteria
Chlorobi
Bacteroidetes
AD3
Acidobacteria
VHS-B5-50
TM7
Thermi
SPAM
Proteobacteria
Planctomycetes
OP5
O1aA90
Gemmatimonadetes
Fungi
Fervidomicrobium
Dictyoglomus
Chloroflexi
BRC1
Archaea
Actinobacteria
JP Compost Inoculum
JP-1
JP-2
JP-3
JP-4
JP-5
JP-6
JP-7
JP-8
JP-9
1%SGJP60
SGCMCJP60
Z Compost Inoculum
Z-1
Z-2
Z-3
Z-4
Z-5
Z-6
Z-7
Z-8
Z-9
1%SGZ60
SGCMCZ60

Figure 2


A.
JP Compost Inoculum
JP-1
JP-9
JP-9 1%SG
JP-9 SGCMC
Realtive Abundance (%)
50
45
40
35
30
25
20
15
10
5
0
Geobacillus sp. R5.03
Thermobifida fusca str. N55-1
Bacillus niabensis str. 5M53
Bacillales
Actinobacteria
Mucor plumbeus
Thermobacillus sp. str. KWC4
Actinomadura umbrina str. JCM6837T
Thermus thermophilus str. JN2
Sphaerobacter thermophilus
Gemm-1c
Bacillus sp. str. R-26953
Bacillus sp. str. HY1
Bacillus sp. str. SA071_1
Gemm-5 cluster 4
Bacillus sp. str. R-7413
Thermaerobacter riparius str. KW1
Rhodothermus marinus it-14
Bradyrhizobiales
Thermobaculum terrenum
Thermomicrobia
Paenibacillus kobensis str. DSM 10249
Paenibacillus sp. str. SAFN-007
Clostridium stercorarium str. DSM 8532T
Thermoleiphilaceae
Brevibacillus sp. A296
Thermus antranikianii str. HN3-10
Paenibacillus sp. D273a
OTUs above 1% Abundance

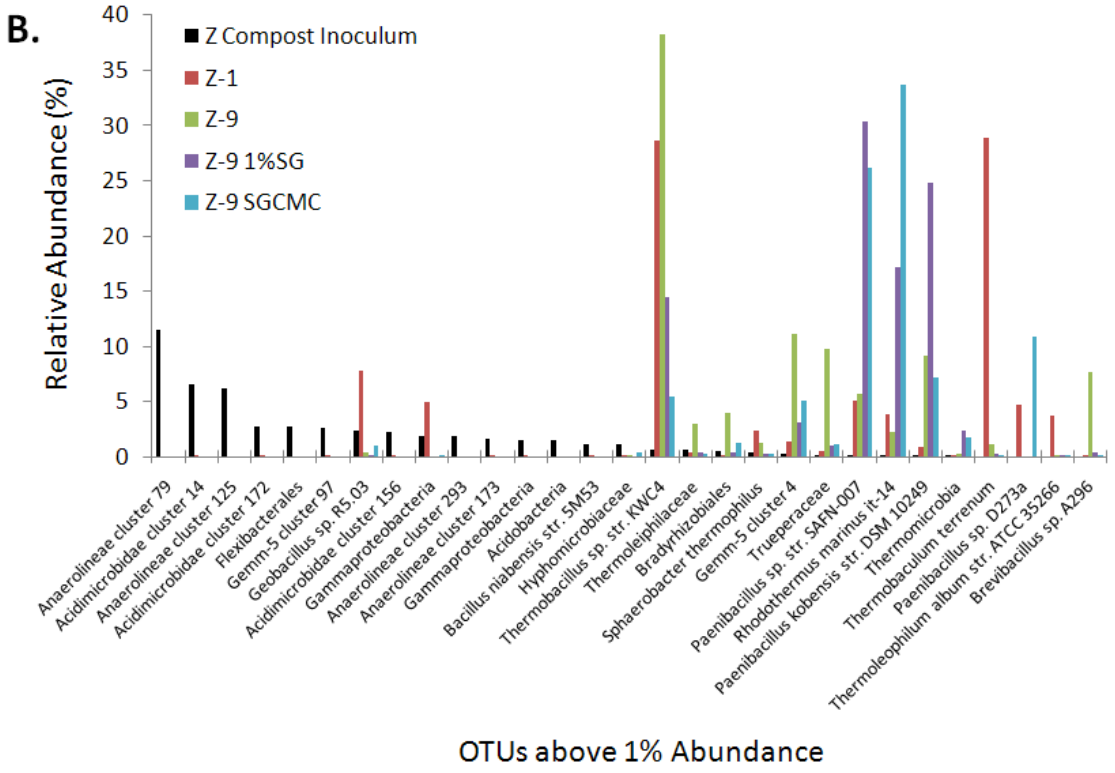

B.
Z Compost Inoculum
Z-1
Z-9
Z-9 1%SG
Z-9 SGCMC
Relative Abundance (%)
40
35
30
25
20
15
10
5
0
Anaerolineae cluster 79
Acidimicrobidae cluster 14
Anaerolineae cluster 125
Acidimicrobidae cluster 172
Flexibacterales
Gemm-5 cluster-97
Geobacillus sp. R5.03
Acidimicrobidae cluster 156
Gammaproteobacteria
Anaerolineae cluster 293
Anaerolineae cluster 173
Gammaproteobacteria
Acidobacteria
Bacillus niabensis str. 5M53
Hyphomicrobiaceae
Thermobacillus sp. str. KWC4
Thermoleiphilaceae
Bradyrhizobiales
Sphaerobacter thermophilus
Gemm-5 cluster 4
Trueperaceae
Paenibacillus sp. str. SAFN-007
Rhodothermus marinus it-14
Paenibacillus kobensis str. DSM 10249
Thermomicrobia
Thermobaculum terrenum
Paenibacillus sp. D273a
Thermoleophilum album str. ATCC 35266
Brevibacillus sp. A296
OTUs above 1% Abundance

Figure 3

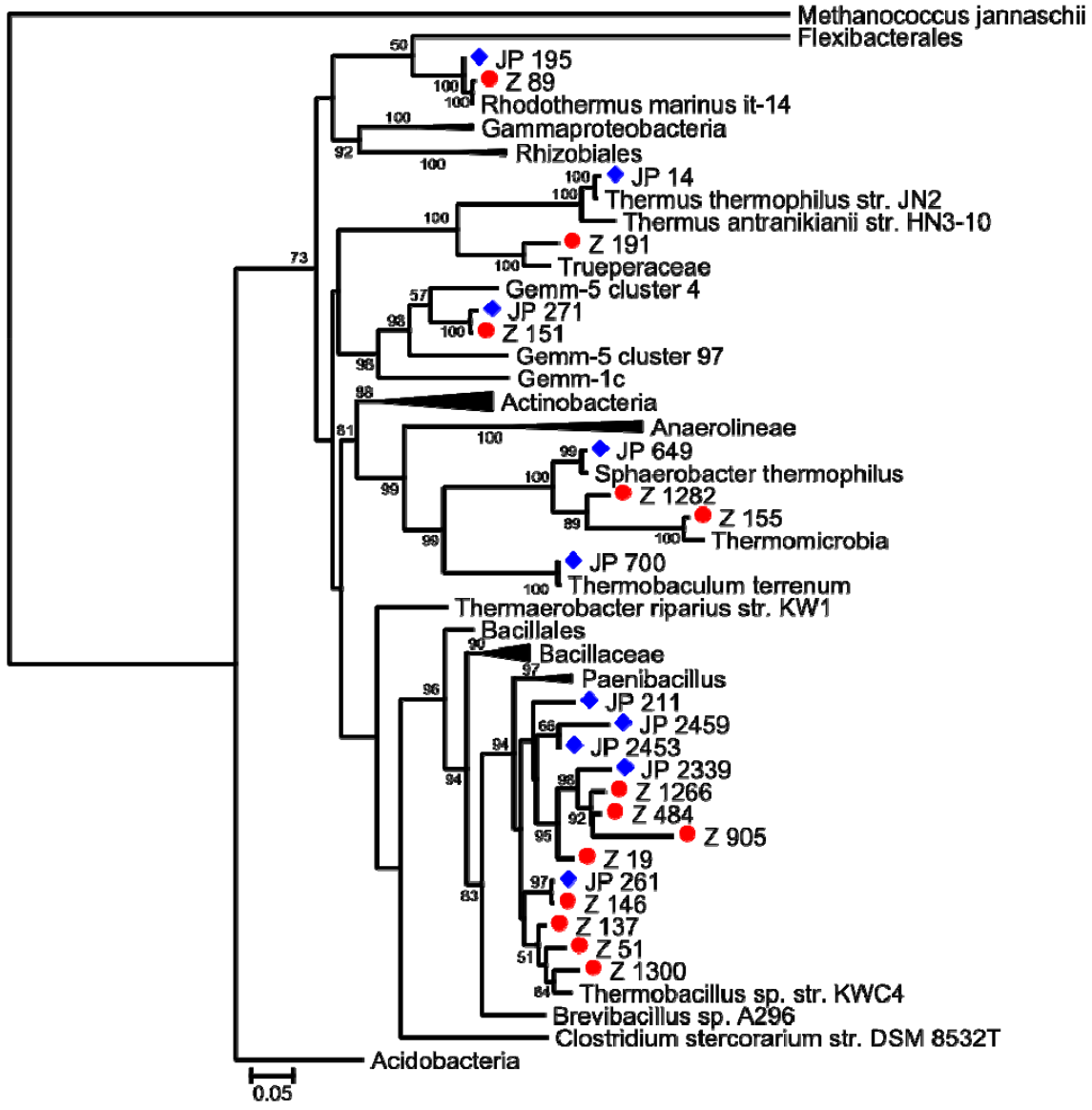

Methanococcus jannaschii
Flexibacterales
JP 195
Z 89
Rhodothermus marinus it-14
Gammaproteobacteria
Rhizobiales
JP 14
Thermus thermophilus str. JN2
Thermus antranikianii str. HN3-10
Z 191
Trueperaceae
Gemm-5 cluster 4
JP 271
Z 151
Gemm-5 cluster 97
Gemm-1c
Actinobacteria
Anaerolineae
JP 649
Sphaerobacter thermophilus
Z 1282
Z 155
Thermomicrobia
JP 700
Thermobaculum terrenum
Thermaerobacter riparius str. KW1
Bacillales
Bacillaceae
Paenibacillus
JP 211
JP 2459
JP 2453
JP 2339
Z 1266
Z 484
Z 905
Z 19
JP 261
Z 146
Z 137
Z 51
Z 1300
Thermobacillus sp. str. KWC4
Brevibacillus sp. A296
Clostridium stercorarium str. DSM 8532T
Acidobacteria
0.05

Figure 4

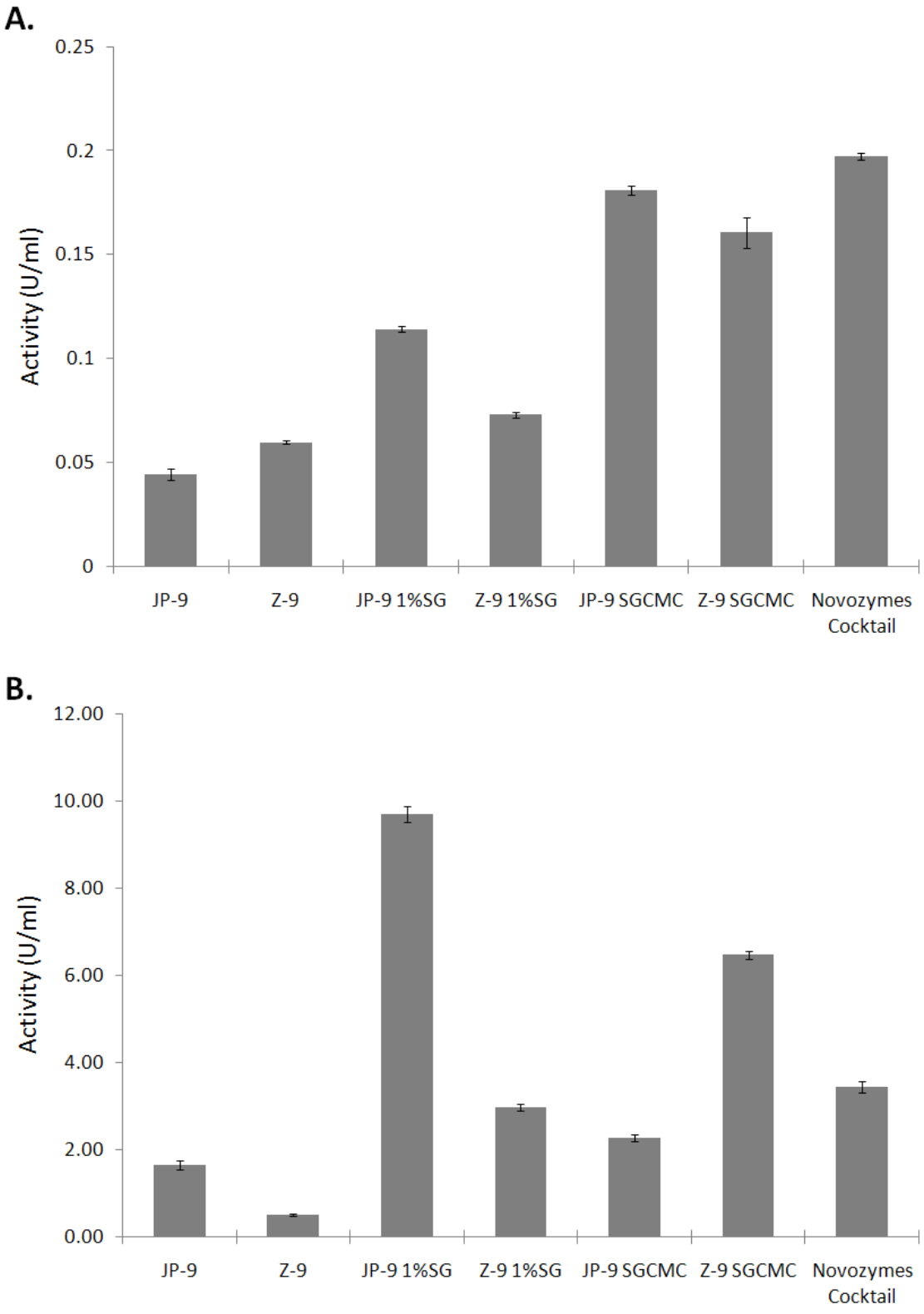

A.
Activity (U/ml)
0.25
0.2
0.15
0.1
0.05
0
JP-9
Z-9
JP-9 1%SG
Z-9 1%SG
JP-9 SGCMC
Z-9 SGCMC
Novozymes Cocktail
B.
Activity (U/ml)
12.00
10.00
8.00
6.00
4.00
2.00
0.00
JP-9
Z-9
JP-9 1%SG
Z-9 1%SG
JP-9 SGCMC
Z-9 SGCMC
Novozymes Cocktail

Figure 5

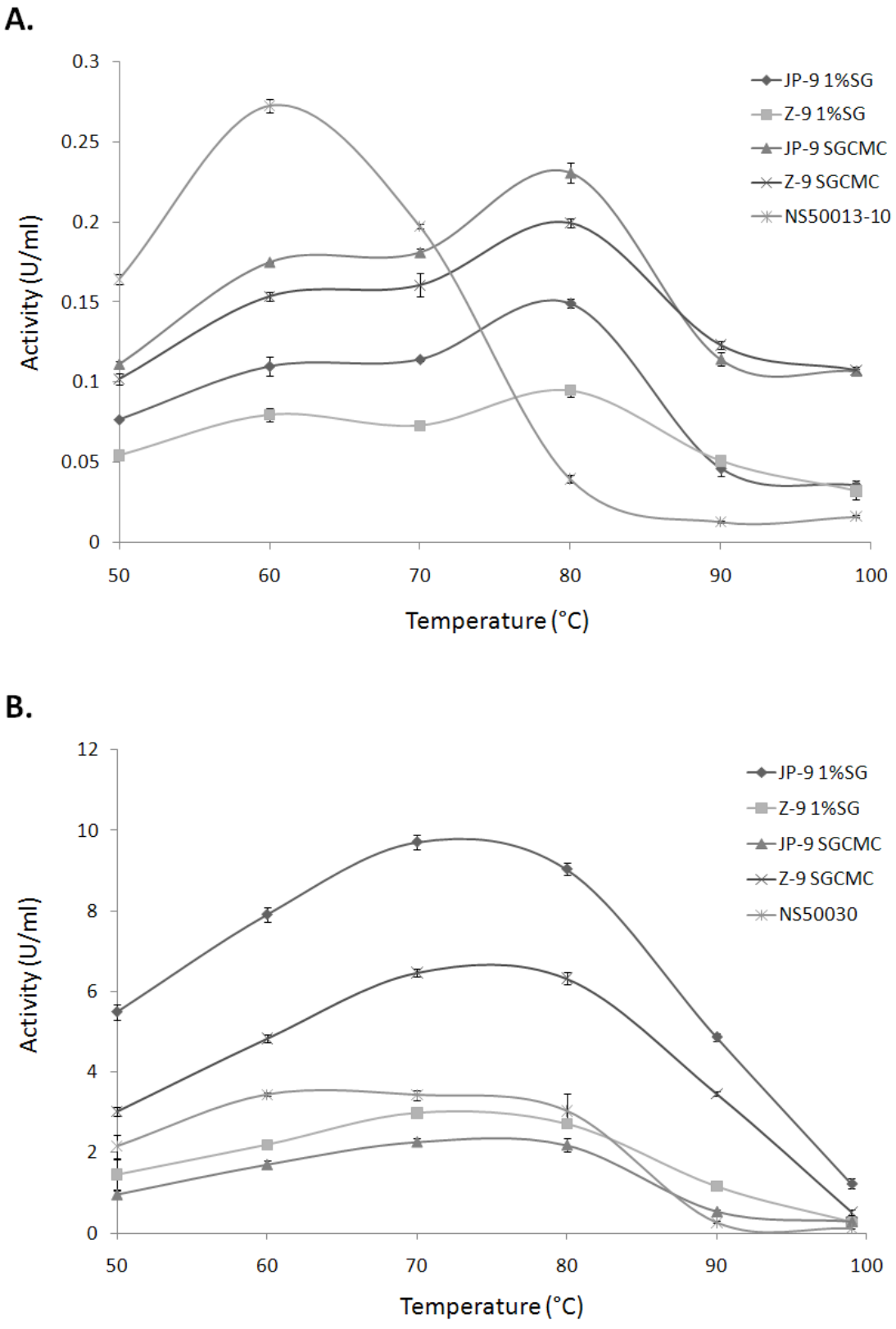
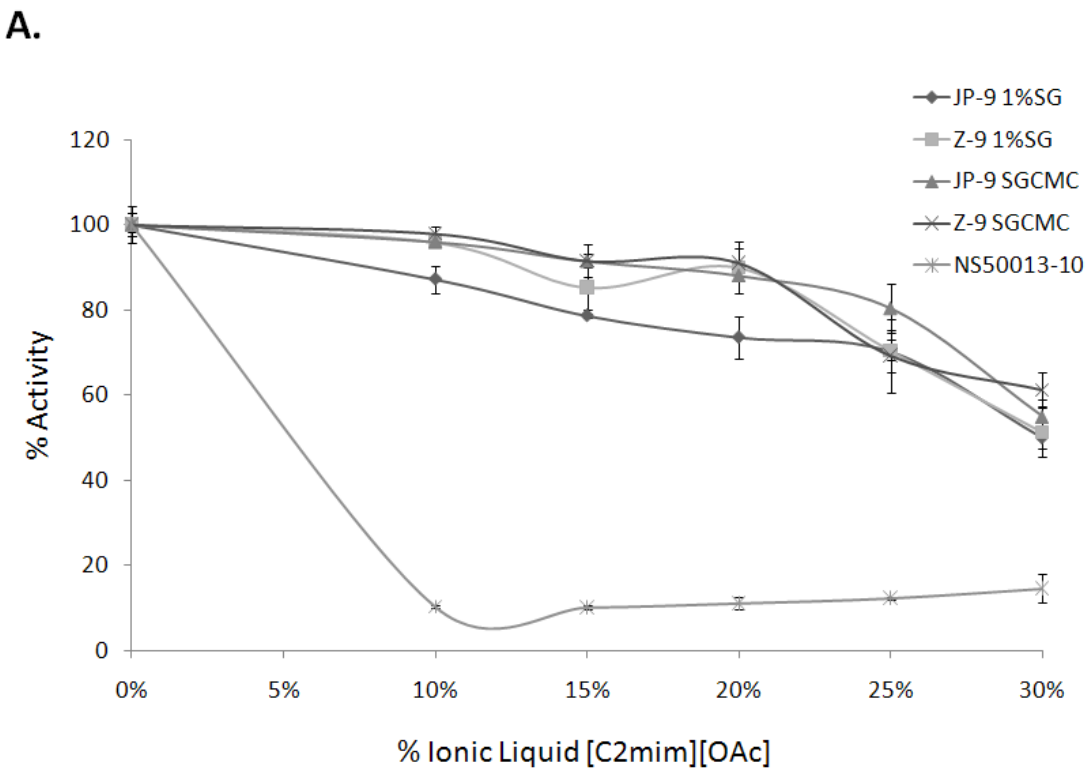

A.
0.3
0.25
0.2
0.15
0.1
0.05
0
Activity (U/ml)
50
60
70
80
90
100
Temperature (°C)
JP-9 1%SG
Z-9 1%SG
JP-9 SGCMC
Z-9 SGCMC
NS50013-10
B.
12
10
8
6
4
2
0
Activity (U/ml)
50
60
70
80
90
100
Temperature (°C)
JP-9 1%SG
Z-9 1%SG
JP-9 SGCMC
Z-9 SGCMC
NS50030

Figure 6


A.
JP-9 1%SG
Z-9 1%SG
JP-9 SGCMC
Z-9 SGCMC
NS50013-10
% Activity
120
100
80
60
40
20
0
0%
5%
10%
15%
20%
25%
30%
% Ionic Liquid [C2mim][OAc]

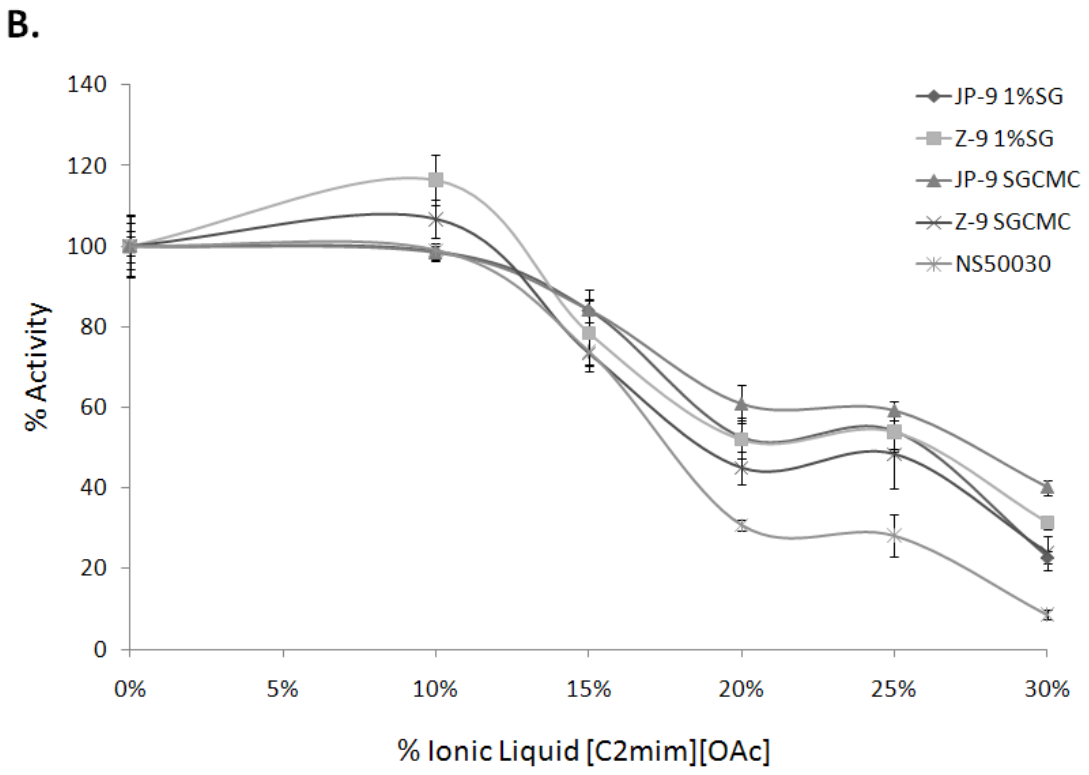
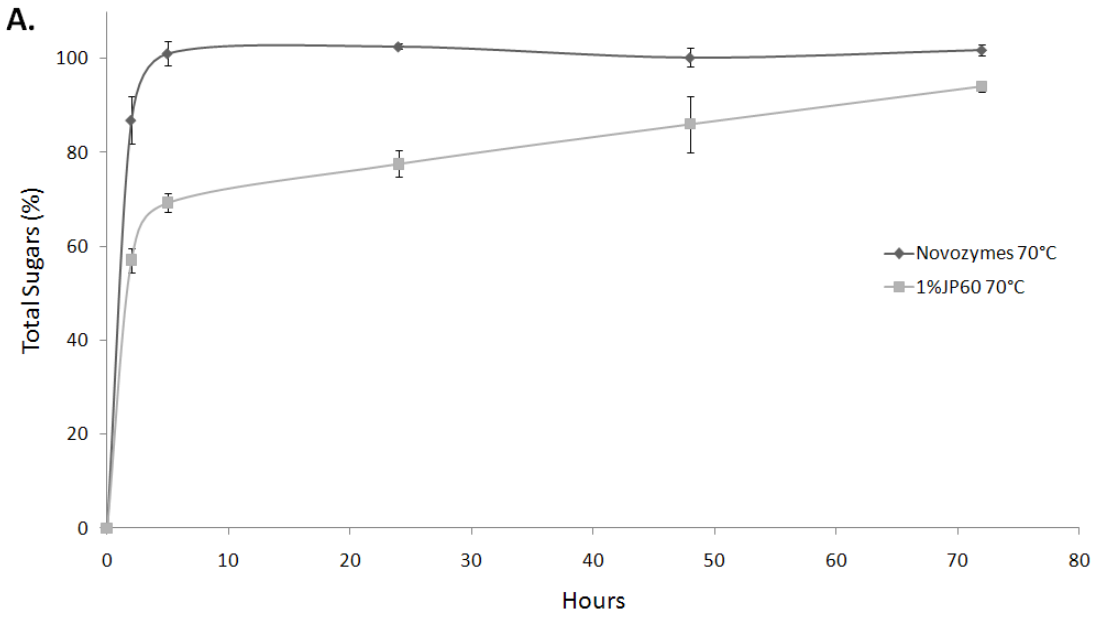
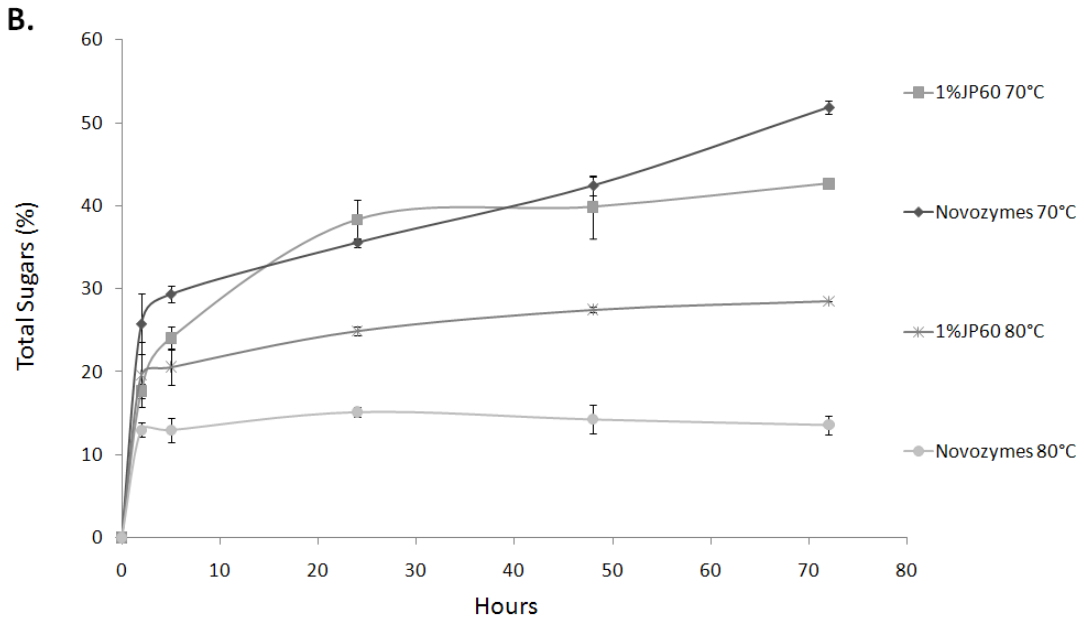

B.
JP-9 1%SG
Z-9 1%SG
JP-9 SGCMC
Z-9 SGCMC
NS50030
% Activity
140
120
100
80
60
40
20
0
0%
5%
10%
15%
20%
25%
30%
% Ionic Liquid [C2mim][OAc]

Figure 7


A.
Total Sugars (%)
Hours
Novozymes 70°C
1%JP60 70°C


B.
Total Sugars (%)
Hours
1%JP60 70°C
Novozymes 70°C
1%JP60 80°C
Novozymes 80°C

C.

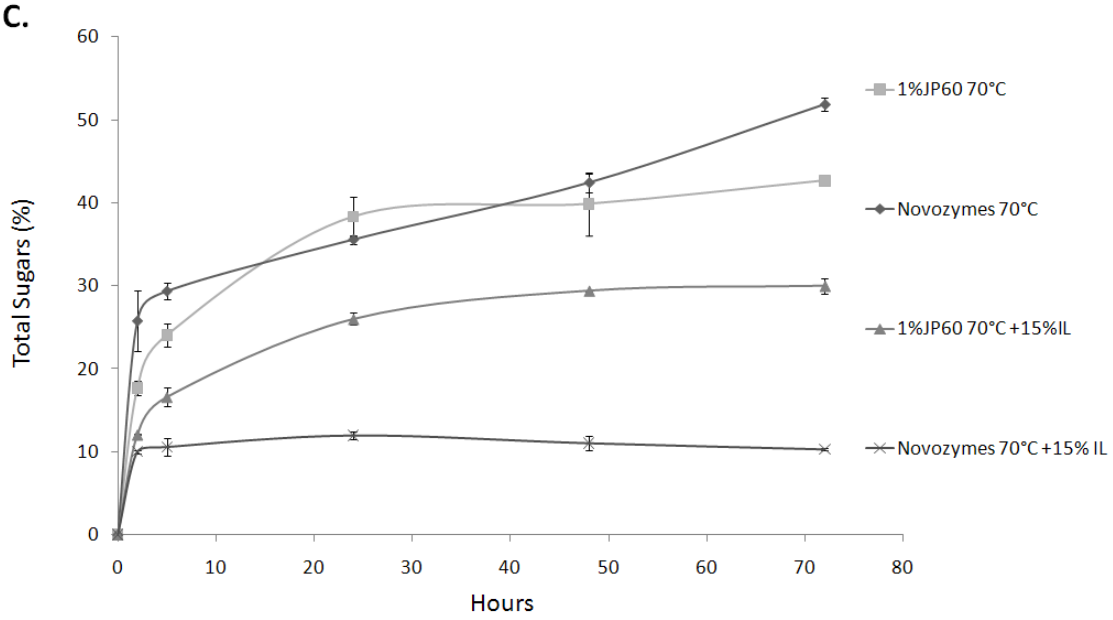
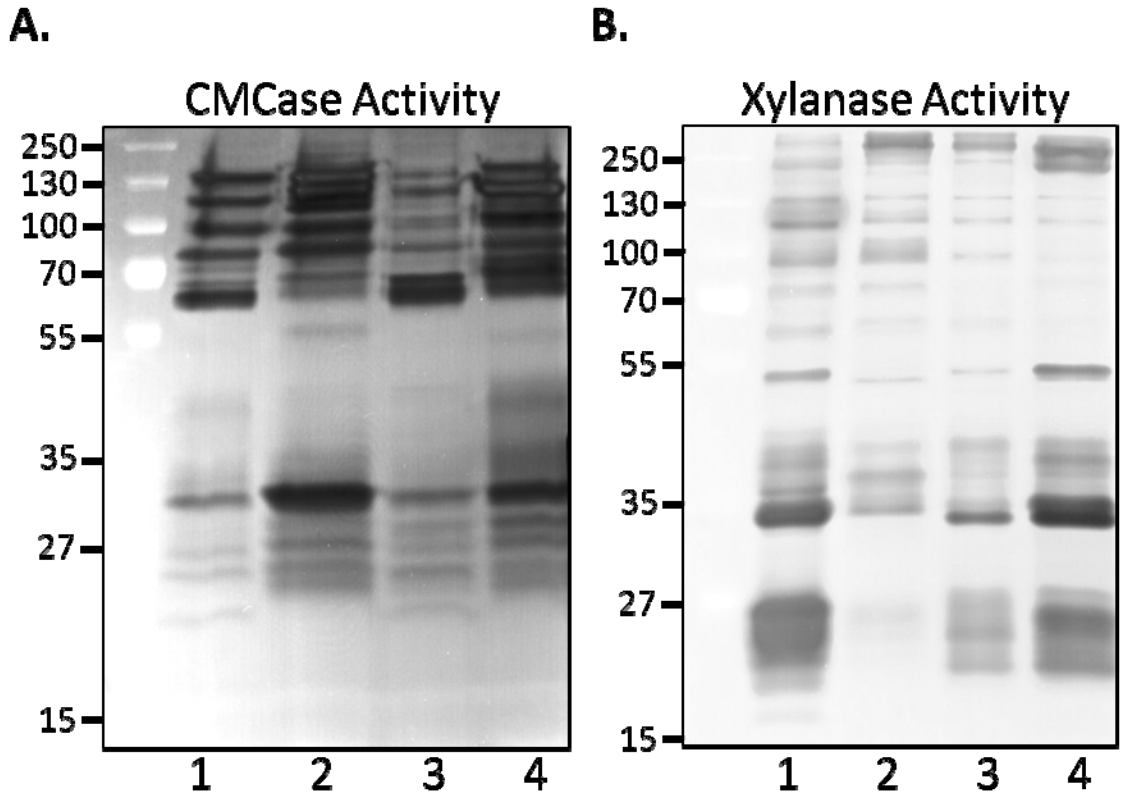

Total Sugars (%)
Hours
0
10
20
30
40
50
60
70
80
1%JP60 70°C
Novozymes 70°C
1%JP60 70°C +15%IL
Novozymes 70°C +15% IL

Figure 8


A.
CMCase Activity
250
130
100
70
55
35
27
15
1
2
3
4
B.
Xylanase Activity
250
130
100
70
55
35
27
15
1
2
3
4