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Screening for novel bacteria from the bioenergy feedstock switchgrass (*Panicum virgatum* L.)

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Switchgrass is considered as a good candidate for biofuel, especially ethanol production due to its huge biomass output and high cellulose content. In a search for novel microorganisms capable of using and degrading switchgrass to produce sugars and ethanol, enrichment experiments were established to screen for microorganisms from soil samples obtained at the University of Tennessee Agricultural Research Station, Jackson, Tennessee. Three enrichments were prepared and incubated at different pH and temperatures: (1) 30°C, pH 5, (2) 30°C, pH 8 and (3) 60°C, pH5. Bulk community DNA was directly extracted from the enrichments. Microbial community structures were determined by phylogenetic analysis of 16S rRNA gene sequences retrieved from the enrichment cultures containing switchgrass as the carbon source. The mesophilic enrichments were dominated by *Sarcina*, *Anaerobacter*, and *Clostrium*, which were not found in the thermophilic enrichment. The thermophilic enrichment selected for two types of bacteria belonging to the class Bacilli (*Geobacillus* and *Saccharococcus*). The thermophilic enrichments were dominated by the *Geobacillus* spp. (*Firmicutes*, class Bacilli), and *Saccharococcus* (*Firmicutes*, class Bacilli); both containing thermophilic microorganisms with some cellulolytic members. Enzymatic assays detected the presence of enzymes involved in cellulose (β-glucosidase and cellobiohydrolase) and hemicellulose degradations (β-xylosidase); and the activity tends to be higher in the enrichments incubated at 30°C.

Keywords: switchgrass; cellulose; *Geobacillus*, *Saccharococcus*; soil enrichment

1. Introduction

Switchgrass is (*Panicum virgatum* L.) a perennial grass with large geographical distribution and high biomass and has been proposed as a promising bioenergy feedstock.[1,2] It is considered as a good candidate for biofuel, especially ethanol production due to its huge biomass output and high cellulose content.[3] Cellulose is the most abundant renewable fuel resource on Earth, accounting for about half of the organic material in the biosphere, and is the major polysaccharide found in plant biomass.[4] It is totally insoluble in water. It is a linear, unbranched homopolysaccharide consisting of glucose subunit joined together via β 1–4 glycosidic linkages. The hydrolysis of cellulose, aided by various enzymes, produces glucose, an easily fermentable monosaccharide.[4,5] Cellulolytic enzymes are synthesized by a number of microorganisms. Fungi and bacteria are the main natural agents of cellulose degradation.[4] The cellulose utilizing population includes aerobic and anaerobic mesophilic bacteria, filamentous fungi, thermophilic and alkaliphilic bacteria, actinomycetes and certain protozoa.[6] Cellulose-degrading bacteria have been isolated from various environments such as compost,[7] soils,[8] wastewaters,[9] thermal springs [10] and deep terrestrial subsurface environments.[11] Cellulose does not typically exist in nature by itself and so other enzymes are needed for effective biomass utilization. Xylanases hydrolyse the β-1,4-xylan linkage in hemicellulose to produce xylose, a five-carbon sugar.[12]

Intense research is currently aimed at the conversion of cellulose to sugars and ethanol by microbes because this process has great economic potential and is environmentally friendly. Unfortunately, the main impediment for ethanol production via enzymatic saccharification of cellulose is the low level of activity in native cellulose. One of the best strategies of fermentable sugars from cellulosic wastematerials is to develop novel enzymes that will enable much improved hydrolysis of cellulosic substrates in a shorter retention time. Thus, screening and characterization of new cellulosic bacteria isolates and their enzymes may provide key targets in the bioenergy conversion process and development of alternative fuels including sugars and bioethanol. The biorefining process also remains economically unfeasible due to lack of biocatalysts that can overcome costly hurdles such as cooling from high temperature, pumping oxygen, and neutralization from acidic or basic pH. Currently, industrial bioconversions of lignocellulose requires the application of high temperature and acidic conditions to breakdown lignin, decrease crystallinity, increase pore volume and solubilize cellulose and hemicellulose to allow enzymatic hydrolysis of target polysaccharides.[13]

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Isolation of novel microorganisms able to use switchgrass and efficiently degrade this material to produce sugars and ethanol is an important line of investigation in bioenergy research. In this study, enrichments were established to screen for microorganisms capable of degrading cellulose from soil samples obtained at the University of Tennessee (UT) Agricultural Research Station, Jackson, TN. Enrichments were set up under aerobic conditions at different pH and temperature combinations (30°C and 60°C, pH 5 and 8) using a mineral medium base with milled and autoclaved switchgrass. Bacterial 16S ribosomal RNA fragments were amplified from the bulk enrichment DNA were cloned and sequenced to characterize the mesophilic and thermophilic cellulose-degrading enrichment cultures. Enzymatic assays involved in cellulose (β-glucosidase and cellobiohydrolase) and hemicellulose degradations (β-xylosidase) were also performed for each enrichment.

2. Materials and methods

2.1. Enrichment and characterization of mixed enrichment cultures

Soil samples and switchgrass were obtained from the UT Agricultural Research Station in Western Tennessee and were used for the enrichment of switchgrass-degrading microbes. Three enrichments were prepared and incubated at different pH and temperatures: (1) 30°C, pH 5; (2) 30°C, pH 8; and (3) 60°C, pH 5. The enrichment medium contained (per litre): 0.1 g nitrilotriacetic acid, 1-ml $FeCl_3$ solution (0.03%), 0.05 g $CaCl_2$ $2H_2O$, 0.1 g $MgSO_4$ $7H_2O$, 0.01 g NaCl, 0.01 g KCl, 0.3 g NH_4Cl, 1.8 g of 85% H_3PO_4, 0.005 g methionine, 0.05 g yeast extract, 0.01 g casamino acids, and 1 ml of Nitsch's trace element solution.[14] The medium was supplemented with milled switchgrass as a sole source of carbon. The pH of the medium was adjusted to using either 1 M NaOH or 1 M KCl.

Dried switchgrass material (60 g) was extracted twice with 1 L hot water (near boiling) and filtered using Whatman #1 filter paper and a funnel. The filtered samples were weighed and divided into 12 equal parts and added in 250-ml media bottles containing 50 ml of basal medium. The bottles were autoclaved and allowed to cool at room temperature. Soil samples (10 g) were extracted in a waring blender containing 90 ml of 1X PBS (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4), by pulsing the blender for 5 s. In the laminar flow hood, 1 ml of soil slurry was added to each of the bottles containing switchgrass and basal medium. The enrichments were performed by incubating the serum bottles at 30°C and 60°C in an incubator shaker (200 rpm) for 7 days. Triplicate bottles were used for each enrichment experiment, and controls. Periodically, 1 ml of the samples were aseptically removed and analysed for total protein to measure the bacterial growth using quantitative colorimetric Coomassie assay.[15]

2.2. Molecular characterization of mixed enrichment cultures and isolates

Molecular characterization of mixed cellulose-degrading enrichment cultures and isolates grown at 30°C and 60°C were performed using 16S rRNA cloning and sequencing analysis. DNA extracts were prepared using the Power Soil™ DNA isolation kit (Mobio Laboratories, Inc. Carlsbad, CA). 16S rRNA genes were amplified in mixtures containing 50 ng μl^{-1} DNA; 1× High Fidelity PCR buffer (Invitrogen, Carlsbad, CA, USA); 0.2 mM of each of the four deoxynucleoside triphosphates; 2 mM $MgCl_2$; 0.2 μM each of the forward (FD1; 5′ AGA GTT TGA TCC TGG CTG AG 3′) and reverse (1540R; 5′ AAG GAG GTG ATC CAG CC 3′) primers [16–18]; and one unit of High Fidelity Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA) per 20 μl. The following PCR conditions were applied: initial denaturation at 94°C for 5 min; followed by 25 cycles of 94°C for 30 s, annealing for 58°C for 1 min, extension for 1 min; and completed with a final extension period of 72°C for 7 min. Negative PCR controls without DNA template were run concurrently for each sample. PCR products were visualized in a 1.5% (wt/vol) agarose Tris-acetate-EDTA gel to confirm the size of the product. Five replicate PCR runs were performed for each DNA extract. The replicate PCR products were combined (100 μl) and loaded into an 0.8% agarose gel, excised, extracted with Qiaquick gel extraction kit (Qiagen, Valencia, CA, USA), and eluted in 30 μl EB buffer (10 mM Tris-Cl, pH 5.0).

To construct clone libraries, purified PCR products from the enrichment cultures were cloned using TOPO TA PCR 2.1 cloning kit (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instruction. Transformants for each clone library were checked for inserts by PCR amplification using M13 primers. The applied PCR conditions were similar to the conditions described above except that 30 cycles were used with an annealing temperature of 60°C and a final extension time of 10 min.[19,20] The amplification products were analysed by gel electrophoresis. PCR products from each clone were purified with Montage PCR plate (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Purified PCR products of the 16S rRNA inserts were sequenced using Prism Big Dye Terminator sequencing kit (Applied Biosystems, Foster City, CA, USA) with 100 ng of template DNA. DNA sequences were determined on an ABI 3730 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

2.3. Phylogenetic and statistical analysis of clone libraries

The 16S rRNA sequences edited analysed using Chromas Pro version 1.5 (Digital River Inc., Eden Prairie, MN) and vector sequences were removed. Chimeric sequences were checked by Check_Chimera program available at the

Ribosomal Database Project (RDP II).[21] Sequences that appeared chimeric were excluded from further analysis. The closest known relative of the ground water microorganisms represented by the recovered sequences were identified with Sequence Match Program of RDP II. Unique 16S rRNA sequence were parsed into operational units (OTUs) based on 97% sequence identity using DOTUR.[22] Sequences were aligned with Clustal W [23] and the resulting alignments were used to construct phylogenetic trees. The maximum likelihood was used to generate tree topologies. Phylogenetic trees and evolutionary distance calculations were generated using distance Jukes–Cantor model (MEGA version 5.0).[24] Bootstrap resampling analysis for 10,000 replicates was performed to obtain confidence estimates for the phylogenetic trees within Mega version 5.0.[19] RDP classifier [25] was used to provide rapid taxonomic placement of 16S rRNA sequence data. The data provide taxonomic assignments from domain to genes, with confidence estimates for each assignment. Statistical parameters such as rarefaction curves and Shannon-Weaver (H') Index, with 95% confidence intervals (CI) were calculated to estimate the diversity of the phylotypes using DOTUR. The evenness index was calculated within Krebs software as previously described.[26] The H' index considers the equitability of the OTU distribution, I/D also considers both richness and abundance and Chao-1 is a non-parametric estimation of OTU richness.

2.4. Nucleotide sequence accession numbers

The sequences generated in this study were deposited in GenBank. The 16S rRNA sequences retrieved from enrichment cultures were assigned the accession numbers KF018638 to KF018652 (30°C, pH 5 enrichment); KF018606 to KF018637 (30°C, pH 8 enrichment); and KF018572 to KF018605 (60°C, pH 5 enrichment).

2.5. Enzyme assays

The activities β-glucosidase, cellobiosidase and xylosidase, were measured using fluorogenic substrate analogues and multiwells (ZymProfiler®) as described by Vepsalainen et al. [27] Fifty millilitres of the enrichment cultures was mixed with 200 ml of 0.5 M acetate buffer (pH 5.5) and shaken for 3 min in an ice bath. Substrates (100 µl), at a final concentration of 500 µM, were added to 100 µl of diluted enrichment cultures in 96 well-multiwell plates in four replicates. For the background fluorescence measurement, 100 µl of the diluted enrichment culture was pipetted into four blank wells for each enrichment sample and enzyme. Both the 4-methylumbelliferone (MUF) and the 7-aminop-4-pmethylcoumarine (AMC) standards were measured separately for each sample to take the quenching of the fluorescence into account. The standards were diluted into soil slurries to give final concentrations of 0, 0.1, 0.5, 1.0, 5.0, 10, 25 and 50 µM in final volumes of 200 µl. After a 3 h incubation on a multiwell plate shaker (Stat Fax 2200 Incubator Shaker, Bio-Rad, Hercules, CA, USA) at 30°C, 100 µl of 2 M NaOH was added to each well to enhance fluorescence and to stop the reaction. Enzyme substrates were added into blank wells to give a final concentration of 500 µM and the measurement was carried out immediately. The fluorescence was measured using an excitation filter of 355 nm and an emission filter of 460 nm. The quantitative enzyme activities were calculated on the basis of fluorescence measurements. The fluorescence values were compared with the standard curves of each enzyme assayed and the blank measurements were compared with the standard curves; and the MUF and the AMC concentrations were calculated. The mean of tour replicate blanks was subtracted from the enzyme activity measurement and the outcome was corrected to give results on soil fresh weight basis.[27–30]

3. Results and discussion

Degradation of cellulose in switchgrass involves a complex interplay between different enzymes. Among others, it has been widely accepted that three types of cellulases including endoglucanases, exocellulases (cellobiohydrolases), and β-glycosidases act synergistically to convert cellulose to glucose.[31] A broad range of microorganisms secreting these activities either in separate enzymes or in multiprotein complexes have been described in several glycosyl hydrolases have thus been isolated, characterized and classified directly from pure cultures.[32] However, there are many as-yet-unculturable microorganisms in the environment and this unexplored microbial diversity represents a vast untapped source of novel enzyme activities.[33,34] Correspondingly, culture-independent approaches have been developed directly and comprehensively extract microbial DNA from microbial consortia to generate environmental genomic DNA library that yielded novel enzymes with unique biochemical and biophysical characteristics not found for those from cultured microorganisms.[35]

3.1. Bacterial community structures of the enrichment cultures

The bacterial community structures of the enrichment cultures were studied to provide greater knowledge of the mechanisms of effective degradation of switchgrass by cooperation amongst bacteria. A total of 267, 172, and 178 clones were included in the phylogenetic tree analysis that generated 18, 26 and 11 OTUs, respectively, from 30°C, pH5, 30°C, pH 8 and 60°C, pH 5 enrichment cultures (Table 1). The mesophilic enrichments (30°C, pH5 and pH 8) have more species richness compared to the thermophilic enrichments (60°C, pH 5). Diversity indices (Shannon-Weiner index [H'], Simpson's [$1/D$] index, Chao's index and evenness) further pointed

Table 1. Diversity estimates for 16S rRNA gene clones from the enrichments.

Enrichments	Number of clones[a]	OTU[b]	H'[c]	$1/D$[d]	Chao-1[e]	Evenness
30°C, pH 5	172	18	0.2683	27	1.75	0.606
30°C, pH 8	178	26	0.3010	20	2.00	0.684
60°C, pH 5	267	11	0.1588	71	1.55	0.548

[a]Number of clones sequenced from each library.
[b]OTU based on partial 16S rRNA gene sequences (≥97% nucleotide sequence similarity).
[c]Shannon-Weiner index; higher number represents higher diversity.
[d]Reciprocal of Simpson's index; higher number represents lower diversity.
[e]Chao diversity index.

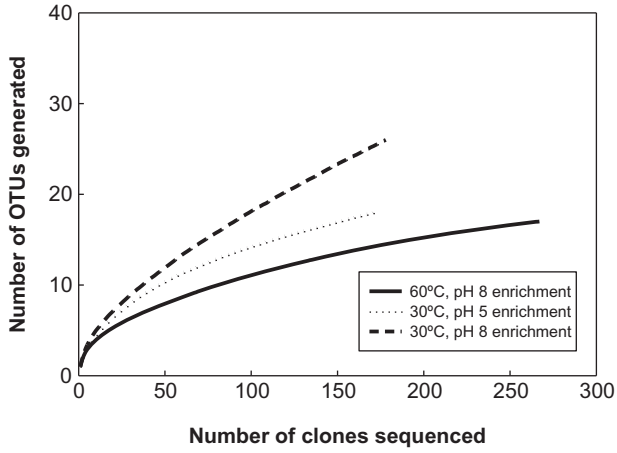

Figure 1. Rarefaction curves of 16S rRNA gene libraries of three clone libraries: ([1] 30°C, pH 5 [2] 30°C, pH 8 and [3] 60°C, pH 5), illustrating the relationship between number of clones sequenced and the phylotype richness. Sequences were grouped into OTUs based on 97% sequences similarity and the curves were established by DOTUR.

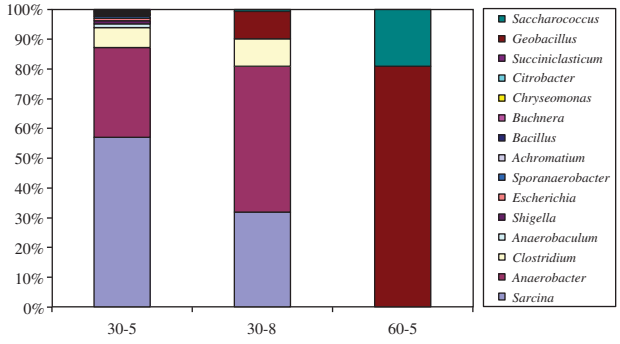

Figure 2. Distribution and abundance of bacterial populations of the clone libraries based on the RDP classifier. 30–5 (30°C pH 5 enrichment); 30–8 (30°C pH 8 enrichment); 60–5 (60°C pH 5 enrichment).

to relatively higher mesophilic enrichments ($H' = 0.2683$ and 0.3010; $1D = 27$ and 20; Chao-1 = 1.75 and 2.00; evenness = 0.606 and 0.684) compared with the thermophilic enrichment ($H' = 0.1588$; $1D = 71$; Chao-1 = 1.55; evenness = 0.548) (Table 1). The lower diversity in the thermophilic enrichment was most likely due to higher temperature (60°C) of incubation that selected only specialized bacterial populations adapted to tolerate high temperatures. Rarefaction analysis which plots the number of clones versus the number of OTUs generated showed that in the 30°C pH 8 (mesophilic enrichment) library, the diversity was less exhaustively captured compared to the 30°C pH 5 and (60°C, pH 5 clone libraries). The rarefaction curve of the thermophilic clone library was more non-asymptotic than the mesophilic clone libraries (Figure 1). The mesophilic enrichments (30°C, pH 5 and 30°C, pH 8) were dominated by the genera *Sarcina* and *Anaerobacter*, while the thermophilic enrichment (60°C, pH 5) was dominated by *Geobacillus* and *Saccharococcus* (Figure 2).

3.2. *Phylogenetic relationships of 16S rRNA sequences recovered from the enrichments*

In the 30°C, pH 5 enrichment (Figure 3), the OTUs were distributed into three clusters belonging to *Clostridiales* (96.24% of the total clones), *Bacillales* (0.38% of the total clones), *Enterobacteriales* (2.64% of the total clones), and *Pseudomonadales* (0.38% of the total clones) and *Thiotrichales* (0.38% of the total clones), spanning within the phyla *Firmicutes* and *Preotebacteria*. The genus *Clostridium* includes obligately anaerobic bacteria; however, most clostridia are aerotolerant and do not resume growth if oxygen is present.[36] The presence of *Clostridium*-related OTUs in the 30°C, pH 5 clone library, indicated the enrichment of bacteria in media bottles sealed with a rubber stopper, which cause the deprivation of oxygen after 7–10 days of growth period. During the initial stage of growth, the dissolved oxygen in the culture medium and the oxygen in the headspace favoured growth of aerobic and facultative anaerobes (*Achromatium, Bacillus, Buchnera, Chryseomonas, Citrobacter, Escherichia*, and *Shigella* spp). In the 30°C, pH enrichment (Figure 4), the OTUs were affiliated with genera found the 30°C, pH enrichment (e.g. *Anaerobacter*, *Sarcina*, and *Clostridium*).

The enrichments incubated at 30°C (pH 5 and pH 8) were dominated by *Sarcina*, *Anaerobacter*, and *Clostrium*. These microbial groups were not found in the enrichment

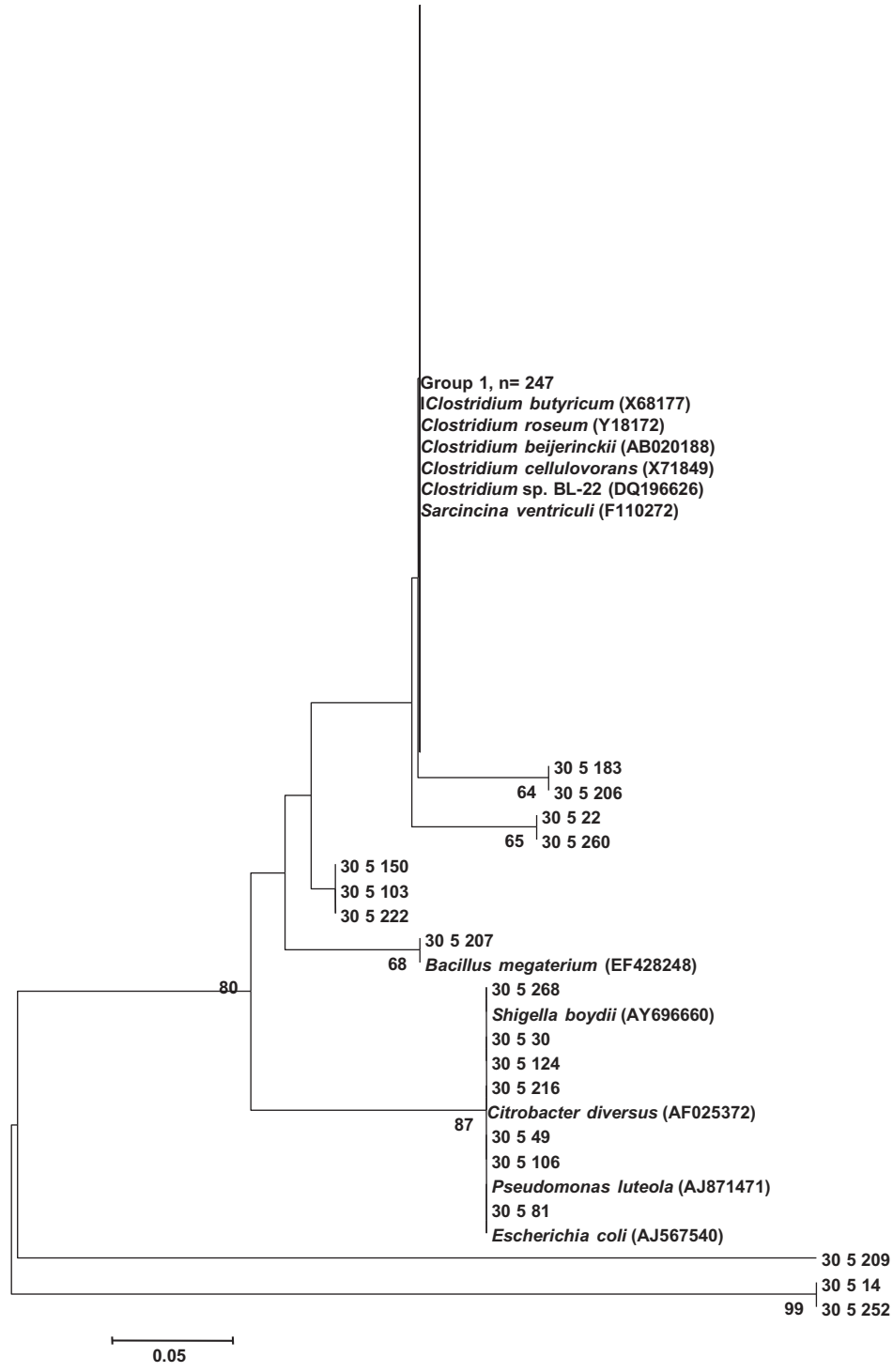

Figure 3. Phylogenetic dendogram showing the relationship between 16S rRNA gene sequences retrieved from 30°C, pH 5 enrichment with references from Genbank. For OTUs representing multiple clones, the number of additional clones is given in parentheses. The tree was constructed under phylogenetic and statistical analysis of clone libraries section in text. The scale bar represents 0.05 substitutions per nucleotide position. Numbers on the node are bootstrap values (%). Boot strap values <50% were not shown.

incubated at 60°C (pH 5). The 60°C, pH 5 enrichment (Figure 5) was selected for two types of bacteria belonging to the class Bacilli (*Geobacillus* and *Saccharococcus*), both of which exhibit thermophilic characteristics with some cellulolytic members. It has been reported that members belonging to genus *Geobacillus* are rod-shaped, chemoorganotrophic, aerobic or facultative anaerobic and obligatorily thermophilic with an optimum growth

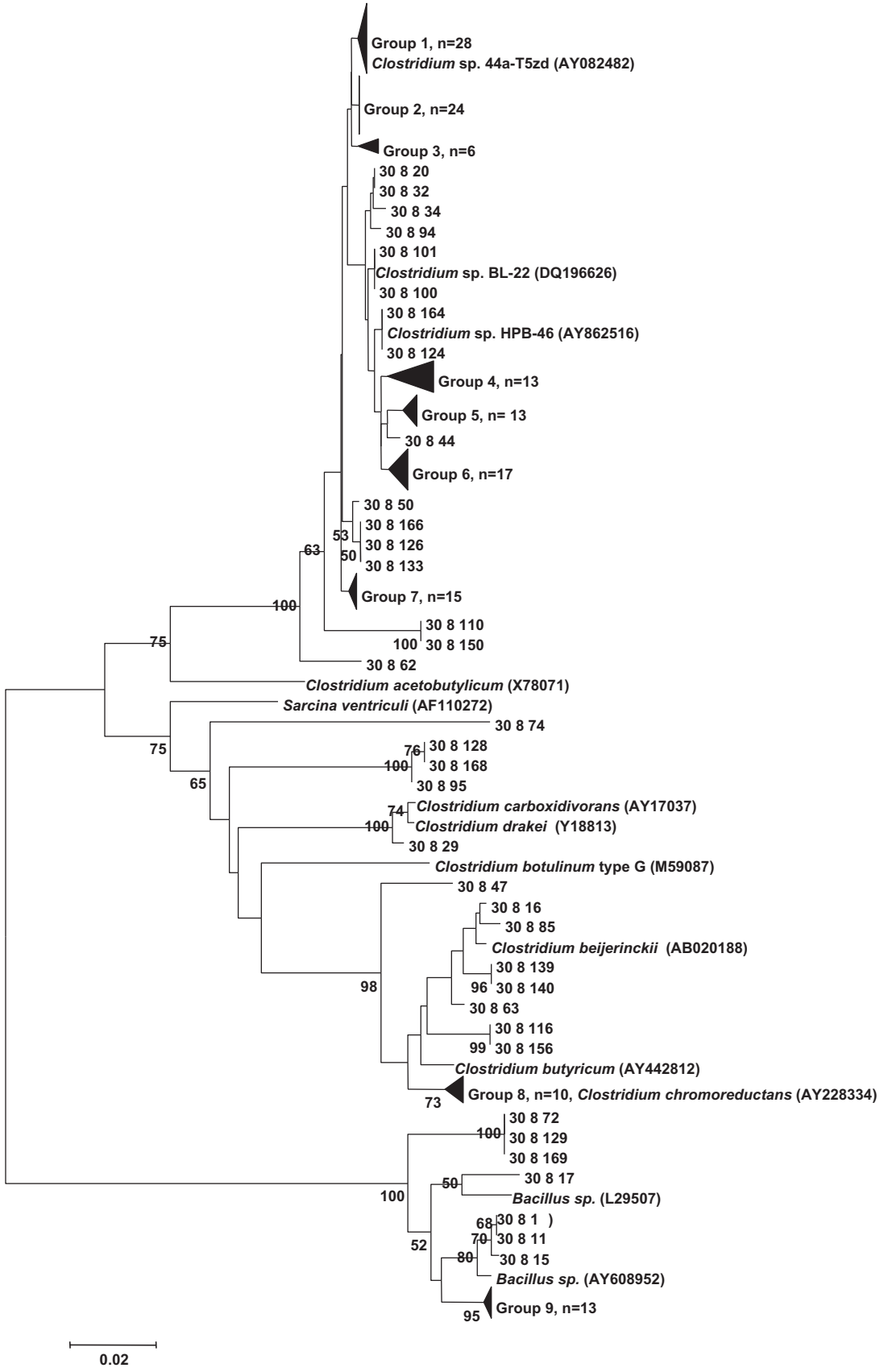

Figure 4. Phylogenetic dendogram showing the relationship between 16S rRNA gene sequences retrieved from 30°C, pH 8 enrichment with references from Genbank. For OTUs representing multiple clones, the number of additional clones is given in parentheses. The tree was constructed under phylogenetic and statistical analysis of clone libraries section in text. The scale bar represents 0.02 substitutions per nucleotide position. Numbers on the node are bootstrap values (%). Boot strap values <50% were not shown.

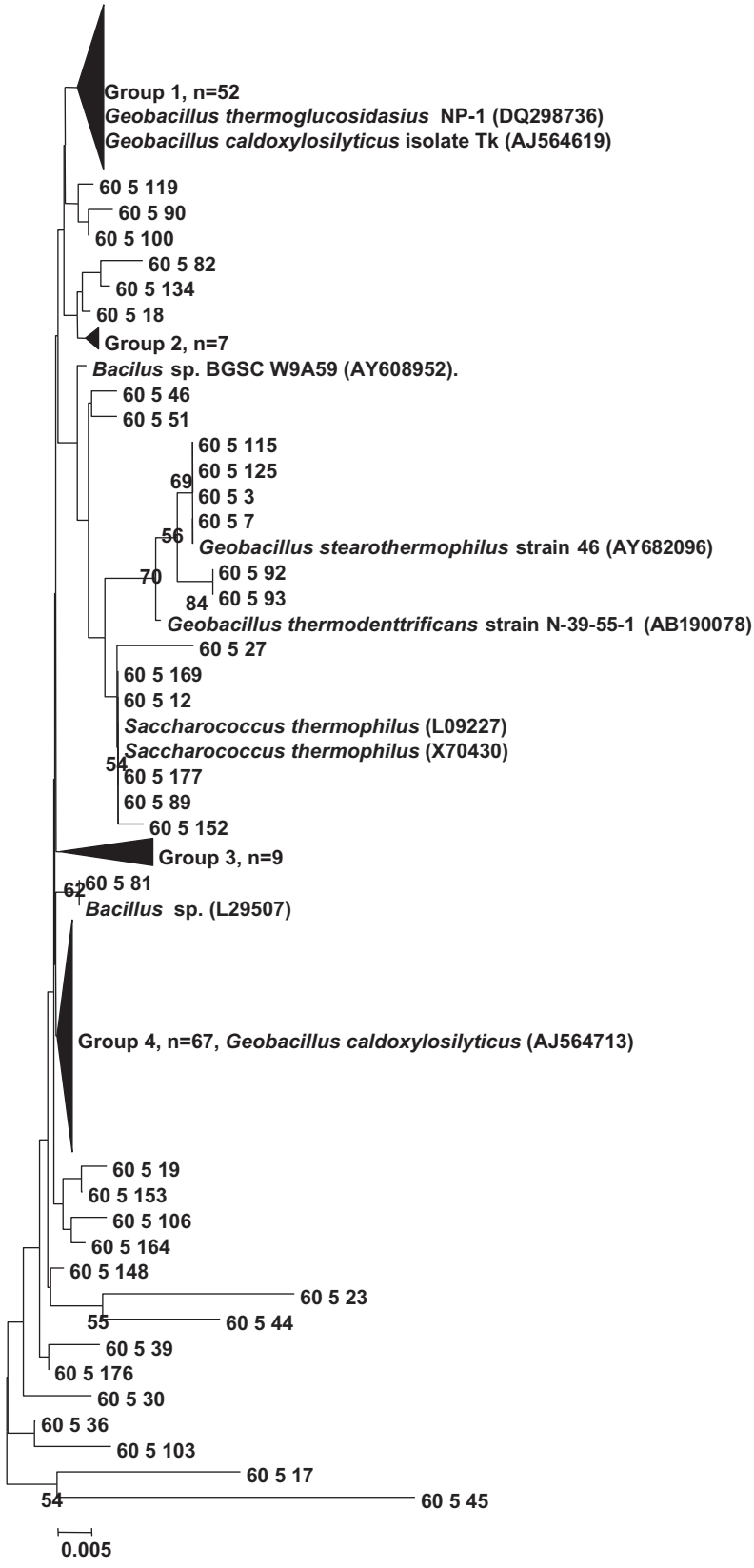

Figure 5. Phylogenetic dendogram showing the relationship between 16S rRNA gene sequences retrieved from 60°C, pH 5 enrichment with references from Genbank. For OTUs representing multiple clones, the number of additional clones is given in parentheses. The tree was constructed under phylogenetic and statistical analysis of clone libraries section in text. The scale bar represents 0.005 substitutions per nucleotide position. Numbers on the node are bootstrap values (%). Boot strap values <50% were not shown.

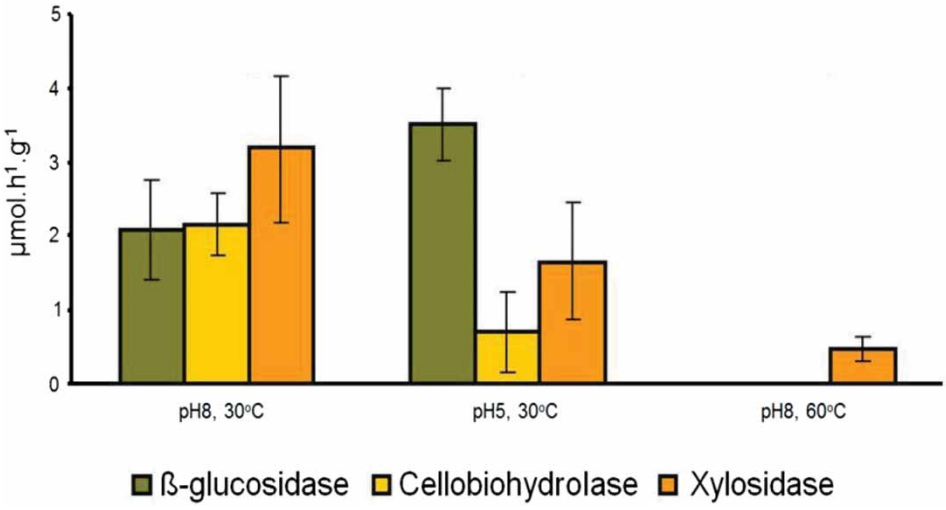

Figure 6. Enzymatic assays for the enrichments.

temperature range of 55–65°C.[37] It has been isolated from wastewater, soil, composts, hot spring and goldmine [38–44] and has been shown to produce β-xylosidase.[45] *Saccharococcus* are xylanolytic, sporulating, Gram-positive, rod-shaped bacterium with optimum temperature for growth at 65°C. It is a facultative anaerobe that grew on a wide range of carbon sources including glucose, lactose, starch and xylose,[46] and has been shown to produce xylose isomerase [46] and D-xylulokinase.[47]

3.3. *Enzymatic assays of the enrichments*

Cellulases are responsible for the hydrolysis of cellulose and can be divided into three major enzyme activity classes.[48–50] These are endoglucanases or endo-1-4-β-glucanase, cellobiohydrolase and β-glucosidase. Endoglucanases, often called carboxymethylcellulases (because of the artificial substrate used for their detection), are thought to initiate attack randomly at multiple internal sites in the amorphous regions of the cellulose fibre which opens up sites for subsequent attack by the cellobiohydrolases.[51] Cellobiohydrolase, often called exoglucanase, is the major component of the fungal cellulase system accounting for 40–70% of the total cellulase proteins, and can hydrolyse highly crystalline cellulose.[52,53] Cellobiohydrolases remove monomers and dimers from the end of the glucan chain. β-glucosidase hydrolyses glucose dimers and in some cases cellulose-oligosaccharides to glucose, hemicelluloses, on the other hand, are biodegraded to monomeric sugars and acetic acid. Xylan is the main carbohydrate found in hemicellulose. Hemicellulases are frequently classified according to their action on distinct substrates, endo-1,4-β-xylanase generates oligosaccharides from the cleavage of xylan and xylan 1,4-β-xylosidase produces xylose from oligosaccharides.[54] In the present study, the presence and the activities of the enzymes involved in cellulose and (β-glucosidase and cellobiohydrolase) and hemicellulose (β-xylosidase) degradations were determined (Figure 6). Both β-glucosidase and cellobiohydrolase were detected in the mesophilic enrichment but were absent in the thermophilic enrichment. β-xylosidase degradation was detected in both mesophilic and thermophilic enrichments but the activity tends to be higher in the mesophilic enrichments than in the thermophilic enrichments. Although thermophilic bacteria are a potentially rich source of glycoside hydrolases for biomass deconstruction, these bacteria generally secrete low levels of glycoside hydrolases, especially cellulolytic enzymes.[31,55] The cellulolytic activities were evident in mesophilic enrichments, however, the level of activities were relatively low (Figure 6). Cultivations on pretreated switchgrass needed to be explored to enhance the cellulolytic activity of the recovered supernatants.

In conclusion, we found that growing soil microbial communities on extracted switchgrass under mesophilic and thermophilic conditions generated simplified bacterial consortia that produced enzymes involved in cellulose and hemicellulose degradations. Adaptive cultivation on switchgrass indicates that this method is a useful tool for developing simplified biomass-degrading consortia tailored to deconstruct a designated feedstock under defined conditions, such as temperature or pH. Microbial community analysis has demonstrated that these consortia are composed of a few dominant phylotypes that consist of both well-studied and novel biomass deconstructing bacteria. Therefore, these consortia are amenable to detailed genomic and proteomic investigations, which will reveal the suite of cellulose and hemicellulose degraders used to deconstruct complex biomass. This approach will allow the characterization of new bacterial celluloses and hemicellulases and accessory proteins from uncultivated organisms that can enhance biomass deconstruction.

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