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ENVIRONMENTAL MICROBIOLOGY

Community Analysis of Plant Biomass-Degrading Microorganisms from Obsidian Pool, Yellowstone National Park

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Abstract The conversion of lignocellulosic biomass into biofuels can potentially be improved by employing robust microorganisms and enzymes that efficiently deconstruct plant polysaccharides at elevated temperatures. Many of the geothermal features of Yellowstone National Park (YNP) are surrounded by vegetation providing a source of allochthonic material to support heterotrophic microbial communities adapted to utilize plant biomass as a primary carbon and energy source. In this study, a well-known hot spring environment, Obsidian Pool (OBP), was examined for potential biomass-active microorganisms using cultivation-independent and enrichment techniques. Analysis of 33,684 archaeal and 43,784 bacterial quality-filtered 16S rRNA gene pyrosequences revealed that archaeal diversity in the main pool was higher than bacterial; however, in the vegetated area, overall bacterial diversity was significantly higher. Of notable interest was a flooded depression adjacent to OBP supporting a stand of *Juncus tweedyi*, a heat-tolerant rush commonly found growing near geothermal features in YNP. The microbial community from heated sediments surrounding the plants was enriched in members of the Firmicutes including potentially (hemi)cellulolytic bacteria from the genera *Clostridium*, *Anaerobacter*, *Caloramator*, *Caldicellulosiruptor*, and *Thermoanaerobacter*. Enrichment cultures containing model and real biomass substrates were established at a wide range of temperatures (55–85 °C). Microbial activity was observed up to 80 °C on all substrates including Avicel, xylan, switchgrass, and *Populus* sp. Independent of substrate, *Caloramator* was enriched at lower (<65 °C) temperatures while highly active cellulolytic bacteria *Caldicellulosiruptor* were dominant at high (>65 °C) temperatures.

Keywords Thermophiles · Plant biomass utilization · Bioenergy · Microbial communities · Yellowstone National Park · Extremophiles.

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Introduction

Microorganisms that are able to enzymatically deconstruct the complex, heteropolymeric structure of lignocellulosic biomass are of particular interest since these strains or their enzymes can potentially be developed for biofuel production from renewable plant material [1, 2]. The decomposition of

lignocellulosic biomass in nature occurs through the action of complex microbial communities organized into specific metabolic guilds with complementary or synergistic activities. Studies employing metagenomics and/or pyrosequencing of 16S rRNA tags have provided new insights into the diversity and organization of biomass-degrading communities such as within wood-feeding insects [3, 4]; rumen environments [5–7], composting systems [8, 9], and laboratory microcosms [8, 10]. Thermophilic microorganisms with (hemi)cellulolytic capabilities provide additional advantages over mesophiles for utilizing plant biomass such as faster reaction kinetics and greater enzyme stabilities under process conditions [11]; therefore, several model thermophiles are being developed for cost-effective, second-generation biofuel production [12–18]. Despite the intense interest in thermophilic biomass decomposition, microbial ecology-based studies examining the community-wide (hemi)cellulolytic potential of certain terrestrial hot springs have only recently emerged [19]. While many carbohydrate-active isolates have been obtained from hot spring environments, their study in isolation provides little information regarding the potential interactions between other microbes within their niche.

With an interest in rapid thermophilic deconstruction of plant biomass, we examined a well-known terrestrial hot spring environment known for its broad diversity in both cultivated and uncultivated Bacteria [20–24] and Archaea [25–27]. Previous cultivation-based studies from samples collected at Obsidian Pool (OBP), Yellowstone National Park, WY produced highly cellulolytic and xylanolytic strains from the genus *Caldicellulosiruptor* [24, 28] and the isolate *Caldicellulosiruptor obsidiansis* has been shown to effectively colonize cellulose surfaces [29] and deconstruct biomass through a suite of carbohydrate-active enzymes [30–32].

The aim of this study was to get a broader view of the diversity of biomass-active microorganisms inhabiting OBP using both high-throughput (HT) pyrosequencing and laboratory enrichment cultures containing both model and real-world biomass substrates. Enrichment cultures were employed in order to collect metabolite data to verify that active fermentation was occurring on individual insoluble substrates at various temperatures, which would be difficult to control using substrates deployed *in situ* ("bugtraps"). Enrichments established at elevated temperatures (55–85 °C) were designed to select for organisms that were most active on crystalline cellulose (Avicel), xylan, acid-pretreated switchgrass, and dilute acid-pretreated *Populus* biomass. A broad community analyses were performed using 454 FLX pyrosequencing on samples collected from the primary fluid source in the spring itself as well as a flooded depression adjacent to OBP supporting dense ground vegetation (*Juncus tweedyi*). The full-length 16S rRNA gene sequences from clone libraries derived from enriched samples were obtained to provide higher-resolution taxonomic identification of biomass-active organisms at the species or strain level. As expected, much higher overall diversity was encountered in sediment samples collected from the vegetated area where temperatures varied from 57 to 74 °C. Likewise, this environment produced active biomass-fermenting communities dominated by bacteria up to ~80 °C, but activity was inhibited at the higher temperatures tested. Numerous uncultivated organisms were detected in the enrichment samples providing a source of isolation targets for future studies aimed at cooperative biomass deconstruction in the extremely thermophilic temperature range.

Materials and Methods

Sampling Site and Sample Collection

Obsidian Pool is a hot spring in the Mud Volcano Area, which is located within the Yellowstone Caldera (N44°36.603′ W110°26.331′) of Yellowstone National Park (YNP). Sediment and water samples were collected from the main pool (OBP5, OBP3) and a flooded depression (OBP10) on the eastern edge of the spring, which contains both living and decaying *J. tweedyi* (Tweedy's rush) as a source of lignocellulosic material. The temperature and pH of each collection site was measured before sampling. Replicate samples were collected as described earlier [24]. The samples were kept in tightly sealed bottles and placed at 4 °C until they were transported in a cooler (~10 h) to the Oak Ridge National Laboratory (ORNL), Oak Ridge, TN. The samples were stored in the dark at 4 °C until processed within 1 week.

Elemental Analysis

Immediately upon arrival to ORNL, a total of 25 metals were quantified in OBP3 and OBP10 sediments via inductively-coupled plasma mass spectrometry (ICP-MS, PerkinElmer Elan 600) including Li, Be, Na, Mg, Al, K, Ca, Cr, Fe, Mn, Ni, Co, Cu, Zn, Ga, As, Se, Sr, Ag, Cd, Cs, Ba, Pb, Bi, and U. Previous studies have provided water chemistry and hydrogen concentration for OBP: sulfide, 17.6 μM; sulfate, 0.33–0.52 μM; H_2, 133.2–325.3 nM; CH_4, 0.1 μM; CO_2, 12.8–14.9 mM; C_nH_n, 21–63 ng l^{-1}; and Cl, 25–305 mg l^{-1} [33, 34].

Enrichment Culture Experiments

The enrichment culture medium consisted of 4.5 mM KCl, 4.7 mM NH_4Cl, 2.5 mM $MgSO_4$, 1.0 mM NaCl, 0.7 mM $CaCl_2{\cdot}2H_2O$, 0.25 mg/ml Resazurin, 1.4 mM cysteine-$HCl{\cdot}H_2O$, 1 mM $Na_2S{\cdot}9H_2O$, 3.0 mM $NaHCO_3$, 1 mM phosphate buffer, 10 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) pH 6.8, 1× ATCC trace minerals (Manassas, VA), 1× ATCC vitamin supplement (Manassas, VA), 0.02 % (*w*/*v*) yeast extract (Fisher Scientific, Pittsburgh, PA), and 1.0 g/l

carbon source. Carbon and energy sources included (1) high-purity microcrystalline cellulose with ~50-μm particle size (Avicel® PH-101) (Sigma-Aldrich Co., St. Louis, MO), (2) xylan (≥90 % HPLC) from beechwood (Sigma-Aldrich Co., St. Louis, MO), (3) dilute sulfuric acid-pretreated Alamo switchgrass (*Panicum virgatum*), and (4) dilute sulfuric acid-pretreated shavings of *Populus deltoides*. Pretreated biomass substrates were provided by the National Renewable Energy Laboratory, Golden, CO and were processed under the following conditions: 190 °C, 0.050 g sulfuric acid g^{-1} biomass, residence time of 1 min, and 25 % (*w*/*w*) total solids. Media were prepared anaerobically using a modified Hungate technique [35] and sterilized by autoclaving. Enrichment cultures were established by inoculating seven 125-ml serum bottles containing 50 ml of medium with 0.5 ml from environmental sample and incubating at 55, 60, 65, 70, 75, 80, and 85 °C with shaking at 90-rpm. Incubation times varied from 16 to 24 h, after which samples were transferred to a new serum bottle of the same substrate composition. Cultures were transferred three times to ensure enrichment on specified substrates. Growth was monitored by observing direct microscopic cell count, measuring changes in pH, and end-product analyses. Cell densities were determined by using a Thoma cell counting chamber (Blaubrand, Wertheim, Germany) with a Zeiss Axioskop2 Plus microscope with phase-contrast illumination.

Metabolite Measurements

Low Molecular Weight Fatty Acid and Alcohol Analysis

Acetate, lactate, and ethanol production was analyzed via high-performance liquid chromatography (HPLC) by filtering (0.2 μm) 1 ml of supernatant samples and acidifying with 200 mM sulfuric acid (final concentration of 5 mM) before injection into a Waters Breeze 2 HPLC system (Waters Corp., Milford, MA). Metabolites were separated on an Aminex HPX-87H column (BioRad Laboratories, Hercules, CA) at 60 °C using 5 mM sulfuric acid as the mobile phase at 0.6 ml min^{-1} and then passed through a refractive index (RI) detector (Waters 2414). Identification was performed by comparison of retention times with known standards. Quantitation of the metabolites was calculated against linear standard curves. All standards were prepared in uninoculated culture media to account for interference of salts in the RI detector. Samples were run in triplicate.

Gas Analysis

Gases were sampled from the headspace of the serum vial and analyzed immediately on an Agilent 6850 gas chromatograph (Agilent Technologies, USA) equipped with a thermal conductivity detector (TCD). All gas analytes were separated on an HP-PLOT U column (30 m×0.32 mm×0.10 μm film; J&W Scientific, Agilent Technologies, USA). Two HP-PLOT U columns were joined together for a total length of 60 m for optimized separation. Samples for carbon dioxide measurements were injected into a 185 °C split-splitless injector with a split ratio set to 3:1 and isocratic oven (70 °C) and helium carrier flow (5.1 ml min^{-1}). The detector had a helium makeup flow of 10 ml min^{-1} at 185 °C, with the detector filament set for positive polarity. Samples to detect hydrogen concentrations were injected into a 185 °C split-splitless injector with a split ratio of 3:1 and isocratic oven (180 °C) and nitrogen carrier flow (3.5 ml min^{-1}). The detector had a nitrogen makeup flow of 10 ml min^{-1} at 185 °C with the detector filament at negative polarity. Peak identifications were performed by comparison with known standards. Quantification of each compound was calculated against individual linear standard curves.

DNA Extraction from Environmental Samples, 454 Pyrosequencing of 16S rRNA Genes, and Analyses

Total community genomic DNA (gDNA), which contained both prokaryotic and eukaryotic DNA, was extracted from 5 g of original OBP sediment slurry samples using the PowerMax® Soil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA). The theoretical size of the microbial community was estimated based on the total DNA recovered and the assumption that the predicted effective genome size of the individual cell of soil/sediment bacteria or archaea is 4.7 Mb as estimated from metagenomics data [36, 37], and a genome of this size would weigh 4.05 fg [38].

The hypervariable V4 regions of the 16S rRNA gene for Bacteria (~290 bp) and Archaea (~340 bp) were amplified from the total gDNA using the high-fidelity AccuPrime *Pfx* DNA polymerase (Invitrogen, Carlsbad, CA) as described earlier [39, 40]. Sequencing reactions were performed on a 454 Life Sciences Genome Sequencer FLX (Roche Diagnostics, Indianapolis, IN). FASTA files (raw 454 reads) were initially processed through the Ribosomal Database Project (RDP) pyrosequencing pipeline [41], where they were sorted by sample identifiers; the tag and 16S primer sequences were trimmed off, and low-quality sequences (using minimum average quality score of 20, maximum number of N=0, minimum sequence length=100) were removed. For each sample, 9,040 to 18,354 high-quality sequences of size 200–210 bp were obtained. Chimeric sequences were detected by using the Chimera Slayer [42] and were removed from the analyses. The 454 pyrosequences were analyzed using the RDP pyrosequencing pipeline tools for alignment, clustering, rarefaction, and determining Shannon and Chao1 diversity indices from a single sample file. Operational taxonomic units (OTUs) were defined at 97 % identity, and representative sequences were selected using the RDP pipeline. The 16S

rRNA gene sequences were assigned to a set of hierarchical taxa using the RDP Naïve Bayesian rRNA Classifier v.2.0 [43] with a bootstrap cutoff of 50 % for short pyrosequences [44]. Additionally, the 454 pyrosequences processed through the RDP pyrosequencing pipeline were uploaded to MG-RAST [45]. The MG-RAST webserver was used to compare the sequencing data using the "best hit classification" function with RDP as the annotation source, a maximum E value cutoff of 10^{-5}, a minimum percent identity of 97 %, and a minimum alignment length of 50 bp. Phylogenetic data downloaded from MG-RAST were imported into STAMP v2.0.0 (release candidate 6; [46] for additional statistical analyses using ANOVA, and $P<0.05$ was considered significant.

DNA Extraction from Enrichment Cultures, Cloning, Sanger Sequencing, and Analyses

The total gDNA was extracted from each out of 24 combinations of growth temperature and substrate using 3 ml of the enrichment culture and the PowerSoil™ DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA). The genomic DNA was amplified using GoTaq Flexi DNA polymerase (Promega, Madison, WI) and universal bacterial primers targeted to *Escherichia coli* 16S rRNA positions 8–27 (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1510 to 1492 (5′-GGT TAC CTT TTA CGA CTT-3′). The resulting PCR product of approximately 1.5 kb contained essentially the complete 16S rRNA gene allowing better taxonomic resolution. PCR products were purified from UltraPure™ Agarose (Invitrogen, Carlsbad, CA) using QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, CA). PCR products were ligated in pCR 2.1-TOPO vectors (Invitrogen), transformed into TOP10 chemically competent *E. coli*, and plated onto LB agar containing 50 μg ml^{-1} kanamycin and X-gal. Transformants were incubated overnight at 37 °C, and white colonies were selected. The clones were sequenced in both directions at The Genome Center of Washington University using Applied Biosystems 3730 Platform (unfortunately clones from 70 °C were lost during transportation). The Sanger raw sequences were run via the Joint Genome Institute's 16S GeneLib pipeline yielding 1,509 high-quality sequences. The 16S rRNA gene sequences were assigned to a set of hierarchical taxa using the RDP Naïve Bayesian rRNA Classifier v.2.0 [43] with confidence threshold of 80 %. The sequences were aligned using the Needleman alignment method [47] and the greengenes alignment database within the program Mothur [48]. To determine variations between combinations of substrate and temperature, the enrichments were compared at the sequence level using an unweighted UniFrac algorithm [49, 50] and visualized through principal component analysis (PCA). For UniFrac analysis, the neighbor-joining tree generated using MEGA4 software [51] was rooted with the *Methanosarcina mazei* 16S rRNA gene sequence (EF452664). The environmental input file for the UniFrac analysis contained the count of how many times the representative sequences appeared in the clone library. P values were corrected for multiple comparisons by multiplying the calculated P value by the number of comparisons made (Bonferroni correction) [49, 50].

Nucleotide Accession Numbers

The Sanger sequences obtained during this study were deposited in GenBank under accession numbers KJ479277–KJ480727. The 454 pyrosequences were uploaded to MG-RAST and made publically available with the following accession numbers: 4535428.3, 4535429.3, 4535430.3, 4535431.3, 4535432.3, and 4535433.3.

Results

Sampling Site Description

The Mud Volcano area (encompassing OBP) is generally categorized as a vapor-dominated (acid-sulfate) system, which is fed via fractures by reduced gases (e.g., H_2 and H_2S) from the 3–10-km-deep magma body [52, 53]. Approaching the surface, the sulfides are oxidized resulting in acidic fluids of elevated sulfate concentrations capable of considerable chemical weathering, which releases cations into solution [54]. Elevated concentrations of metals (except Al, Be, K, Se, Ag, Cs, Bi) were found in the peripheral area in comparison to the main pool (Table S1). Obsidian Pool is relatively shallow with a maximum depth of ~1 m and varies in size throughout the year [34]. Algal mats and both living and decaying *J. tweedyi* (Tweedy's rush) plants were present, especially at the eastern and southern edge of OBP, which is flooded with heated water (Fig. 1).

Microbial Community Size Estimation

The gDNA yield ranged from 0.6 to 2.0 μg g^{-1} of water-sediment slurry. The theoretical size of the microbial community based on the yield of gDNA ranged from 1.5×10^8 to 5.0×10^8 cells g^{-1} (Table 1). However, the theoretical estimation of microbial cells was significantly higher than DAPI whole cell counts of 2.3×10^6 cells g^{-1} determined previously from OBP sediments [55]. This overestimation is potentially due to the eukaryotic component of the total extracted gDNA.

Bacterial and archaeal diversity in the main OBP varied somewhat between the October and July sampling times. Archaeal diversity in the main pool was higher than bacterial; however, in the vegetated area, bacteria were more diverse

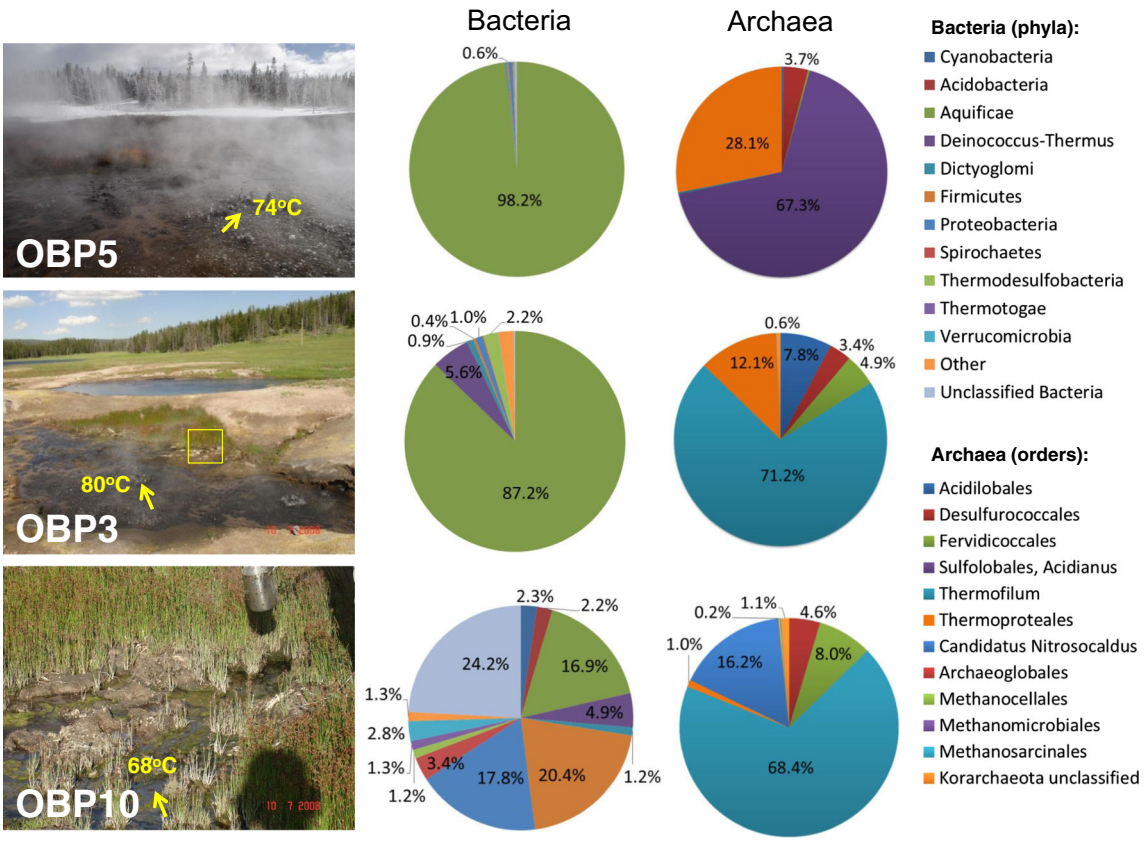

Fig. 1 Relative abundance of Bacteria and Archaea in Obsidian Pool (OBP5, OBP3) and adjacent vegetated area (OBP10). Samples were collected in October 2007 (OBP5) and July 2008 (OBP3, OBP10). Increased steam due to low air temperatures partially obscures the OBP5 image. Collection sites (*arrows*) and water temperatures are indicated. The *yellow box* indicates the OBP10 sample area in relation to Obsidian Pool. Clones that could not be assigned with the >50 % confidence threshold to any phylum were referred as "unclassified." The bacterial group "Other" includes the phyla Actinobacteria, Bacteroidetes, Chlorobi, Chloroflexi, Gemmatimonadetes, Nitrospirae, Synergistetes, and Tenericutes, which were present at <0.4 %

(Table 1). Rarefaction analyses show that a number of individuals sampled in the main pool reached saturation and more intensive sampling was not likely to yield new additional species (Fig. S1, Table 1). The steep slope of the curve for the OBP10 Bacteria (Fig. S1) reflects significantly higher phylotype diversity in the bordering area supporting *J. tweedyi* (OBP10) than in the main pool (OBP5 and OBP3). The OBP10 sample containing live and dead plant material was selected as an inoculum for enrichment cultures to further investigate the presence of putative cellulolytic and xylanolytic microorganisms.

Changes in Microbial Community Structure with Varying Growth Substrate and Incubation Temperature

Based on the diversity analysis results, the OBP10 sample was selected as an inoculum for laboratory enrichment culture experiments with crystalline cellulose (Avicel), xylan, acid pretreated switchgrass, or *Populus* as the dominant carbon and energy source. Incubation temperatures ranged from 55 to 85 °C in 5 °C increments to examine temperature-dependent changes in microbial community activity and structure. Regardless of the substrate used, cell counts after the third passage on the respective substrate were higher at 55–65 °C (ca. 10^8 cells ml^{-1}) than at 75–80 °C (ca. 10^7 cells ml^{-1}), and no cells were detected at 85 °C. An increase in incubation temperature caused a shift in bacterial community structure from a *Caloramator*-dominated community at 55 °C to a *Caldicellulosiruptor*-dominated community at higher temperatures (Fig. 2).

The metabolite measurements showed that the primary product regardless of substrate and temperature combinations was acetate (Table 2). A maximum acetate concentration of 7.6 mM was detected on *Populus* at 65 °C; low acetate concentrations (1.2–1.3 mM) were measured on xylan at 55–70 °C, and no acetate was detected at 85 °C. The production of lactate was observed at temperatures of 75 and 80 °C on Avicel (2.3–2.6 mM) or *Populus* (1.8–2.0 mM), whereas ethanol at a concentration of 1.1 mM was detected only on Avicel at 80 °C (Table 2). Gases including H_2 (0.4–8.24 mM) and CO_2 (0.5–3.6 mM) were detected in all enrichments (Table 2).

Table 1 Characteristics of 454 FLX pyrosequencing libraries derived from OBP for bacterial and archaeal community analysis

Sample ID	Sample description, collection date	Temp., °C	pH	Theoretical estimation of cell number, $\times 10^8$ cells ml^{-1}	Bacteria				Archaea			
					Total number of sequences (Bacteria)[a]	OTUs[b] (singletons) [predicted OTUs]	Shannon[c]	Coverage[d]%	Total number of sequences (Archaea)[a]	OTUs[b] (singletons) [predicted OTUs]	Shannon[c]	Coverage[d]%
OBP5	Main pool, October 2007	74	5	1.5	18,360 (17,975)	55 (19) [66]	0.30	0.997	14,283 (14,121)	41 (10) [41]	1.29	0.997
OBP3	Main pool, July 2008	80	5	5.0	12,726 (12,534)	45 (8) [48]	0.83	0.996	7,700 (7,464)	36 (5) [36]	1.76	0.995
OBP10	Vegetated area, July 2008	68	5	3.1	9,048 (9,014)	621 (250) [801]	4.35	0.931	6,525 (6,235)	79 (24) [83]	3.00	0.987

[a] Total number of 454 FLX pyrosequences obtained per sample. In brackets is shown the number of sequences assigned to Bacteria or Archaea using the RDP Classifier at 50 % confidence threshold. These sequences were used to calculate OTUs, Shannon index, and coverage

[b] The total number of operational taxonomic units (OTUs) was defined at the 97 % identity level of the partial 16S rRNA gene sequences. Number of singletons in each library is indicated in parenthesis. The predicted number of OTUs is given in brackets if we continue to sample the population. The prediction was done using nonlinear regression $y=ax/(b+x)$ where $x=\infty$ and $b<<x$ at the software DataFit 9.0 (Oakdale Engineering, Oakdale, PA)

[c] Shannon index, with higher number represents greater diversity

[d] The equation for calculating the coverage for a single sample (X) is, $C_X=1-(N_X/n)$ where N_X is the number of OTUs in the sample and n is the total number of sequences. The lower the value of the homologous coverage, the higher is the number of OTUs or unique sequences in the population

A relationship between bacterial communities derived from individual enrichments was analyzed using an unweigthed UniFrac algorithm [49]. PCA revealed that 36.5 % of the total variance could be explained by the first two principal coordinates (Fig. 3). Both factors correlated with temperature. Axis 1 accounted for 24.6 % of the variation between communities grown at lower (55–65 °C) temperatures and communities from higher (75–80 °C) temperatures. Axis 2 explained 11.9 % of the variation and corresponded with changes in lignocellulosic substrate within any given temperature set. Regardless of the substrate, 16S rRNA gene sequences from 55 °C enrichments were placed in close proximity to each other and separately from others indicating that temperature, not substrate, was the main parameter affecting community development.

Characterization of Bacterial and Archaeal 16S rRNA Pyrosequences

A total of 40,134 and 28,508 high-quality sequences were obtained after processing of 454 pyrosequencing data generated for the V4 region of bacterial and archaeal 16S rRNA genes, respectively. A small fraction of sequences (0.4–2.1 %) amplified with Bacteria-specific primers were affiliated with Archaea, and 1.1–4.4 % of sequences amplified with Archaea-specific primers were affiliated with Bacteria (Table 1). These sequences were removed from further analyses.

In the main OBP, the phylum Aquificae accounted for up to 98.2 and 87.2 % of the bacterial population in the October and July samples ($P<1e{-}15$), respectively (Figs. 1 and S2). The relative abundance of other bacteria in the October sample was as low as 1.8 % of the total sequences with Proteobacteria (0.6 %) being the highest. While the phylum Crenarchaeota represented the majority of Archaea in both October (100.0 %) and July (99.3 %) main pool samples (Fig. 1), a change in sulfur-metabolizing members of the community from sulfur-reducing *Acidianus* dominating in October to organotrophic *Thermofilum* dominating in July was detected. In July, the temperature in the main pool was 6 °C higher and elemental sulfur deposits were observed on the surrounding banks. An increase of sulfur-dependent Crenarchaeota (*Thermofilum*, *Pyrobaculum*, *Caldisphaera*, *Staphylothermus*; $P<1e{-}15$; Fig. S3), sulfate-reducing bacteria *Thermodesulfobacteria* ($P<1e{-}15$), organotrophs such as *Deinococcus-Thermus* ($P<1e{-}15$) and *Dictyoglomi* ($P<1e{-}15$; Fig. S2), plus organotrophic archaea including *Fervidicoccus* ($P<1e{-}15$, Fig. S3) was observed. The phyla Thaumarchaeota and Korarchaeota were found in July main pool samples in low (<0.6 %) abundance.

The flooded marsh-like area contained high bacterial diversity with 24.2 % of the sequences assigned to unclassified bacteria. Between the vegetated sediments and the main pool,

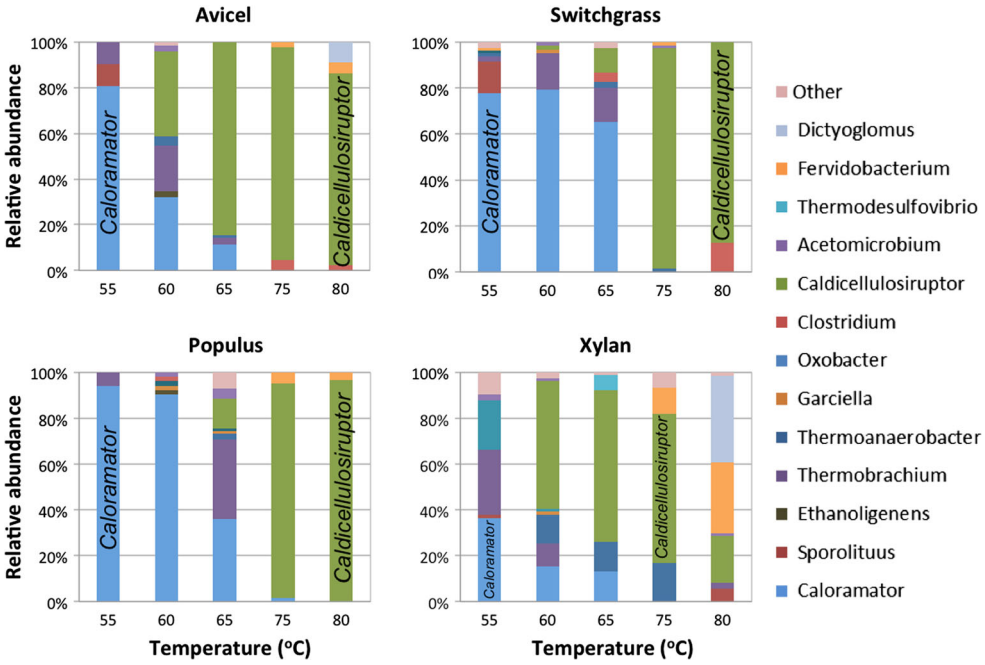

Fig. 2 Changes in bacterial community structure when grown on Avicel, switchgrass, *Populus*, and xylan with increasing incubation temperature. The data are derived from a total of 1,509 high-quality, full-length 16S rRNA gene sequences obtained by the Sanger method. The sequences were assigned to taxa using the Ribosomal Database Project Naïve Bayesian rRNA Classifier v.2.0 [43] with a confidence threshold of 80 %

a decrease in the relative abundance of Aquificae ($P<1e{-}15$), and Thermodesulfobacteria ($P=3.06e{-}5$) was observed, while changes in *Deinococcus-Thermus* ($P=0.789$) and *Dictyoglomi* ($P=3.949$) were not significant (Figs. 1 and S4). The members of the phyla Proteobacteria, Firmicutes, Verrucomicrobia, Acidobacteria, Spirochaetes, and Thermotogae were significantly higher ($P<1e{-}15$) in abundance in OBP10 (Figs. 1 and S4). The Firmicutes comprised 20.4 %, the majority of which belonged to ten families (21 genera) of the class Clostridia including potentially cellulolytic bacteria from the genera *Clostridium*, *Anaerobacter*, *Caloramator*, *Thermoanaerobacter*, and *Caldicellulosiruptor*. The phyla Cyanobacteria, Chloroflexi, Gemmatimonadetes, Chlorobi, and Nitrospirae were only found in the peripheral sample (OBP10). With respect to Archaea, the peripheral area showed a decrease in the total abundance of Crenarchaeota ($P<1e{-}15$), especially *Caldisphaera* ($P<1e{-}15$), *Pyrobaculum* ($P<1e{-}15$), and *Staphylothermus* ($P=0.019$). Nevertheless, increases in crenarchaeal representatives such as *Fervidicoccus* ($P=2.08e{-}5$) and *Thermosphaera* ($P<1e{-}15$) were observed. A relative abundance of *Candidatus Nitrosocaldus*, a member of the phylum Thaumarchaeota, increased substantially up to 16.2 % ($P<1e{-}15$) in OBP10. The methanogenic euryarchaeote, *Methanocella*, was also abundant ($P=0.04$) in the peripheral area sediments (Figs. 1 and S5).

Phylogenetic Comparison of Bacterial Communities from Hot Spring Samples and Lignocellulose Enrichments

Bacterial sequences from sample OBP10 obtained through 454 pyrosequencing upon amplification with bacterial primers (9,014 sequences), archaeal primers (290 sequences), and Sanger sequences derived from enrichments (1,545 sequences) comprised a total of 10,848 sequences. These sequences were aligned and clustered using the RDP pyrosequencing pipeline resulting in 740 OTUs at 0.03 distance cutoff with 333 OTUs containing a single sequence. The remaining 407 OTUs were divided into five groups: 350 (86.0 %) OTUs contained only 454 pyrosequences obtained with bacterial primers, 30 (7.4 %) OTUs contained only Sanger sequences derived from enrichments, 21 (5.2 %) OTUs contained bacterial 454 pyrosequences obtained with both bacterial and archaeal primers, 5 (1.2 %) OTUs contained 454 pyrosequences and Sanger sequences, and 1 (0.2 %) OTU contained bacterial 454 pyrosequences obtained only with archaeal primers. Taxonomic identification showed that 108 OTUs were affiliated with potential cellulolytic bacteria such as *Clostridia* (103 OTUs), *Dictyoglomi* (3 OTUs), and *Thermotogae* (2 OTUs). In spite of the fact that bacteria from the genus *Caldicellulosiruptor* were abundant in the enrichments (668 sequences, 43.2 %) and there were no priming mismatches found, only 9.8 % of the total pyrosequences were annotated in the MG-RAST analysis as belonging to *Caldicellulosiruptor saccharolyticus* at an average identity of 98 % over 50 % of the alignment length. These *Caldicellulosiruptor*-like sequences were detected in the peripheral OBP10 area but not in water and sediment samples from the primary pool. A member of the genus *Thermoanaerobacter*, which could potentially utilize cellodextrins or xylan, was detected in laboratory enrichments (43 sequences, 2.8 %) with 37 sequences detected in xylan enrichments at 60, 65, and 75 °C. In the peripheral OBP10 area, the pyrosequences affiliated with the family

Table 2 Fermentation end-products from enrichment cultures

Substrate	Temp. (°C)	Acetate	Lactate	Ethanol	CO_2	H_2
Avicel	55	6.0±0.1	0.0	0.0	3.1	6.3
	60	5.5±0.1	0.0	0.0	2.9	5.5
	65	3.6±0.1	0.0	0.0	3.2	7.3
	70	5.5±0.1	0.0	0.0	3.2	7.1
	75	6.4	2.6	0.0	2.5	5.0
	80	6.7	2.3	1.1±0.9	0.7	0.4
	85	0.0	0.0	0.0	0.5	0.6
Populus	55	6.1±0.1	0.0	0.0	1.1	1.3
	60	6.4±0.2	0.0	0.0	1.2	1.7
	65	7.6	0.0	0.0	1.7	3.5
	70	6.7±0.1	0.0	0.0	3.6	8.2
	75	4.6	2.0	0.0	0.8	0.9
	80	5.8	1.8	0.0	1.3	2.8
	85	0.0	0.0	0.0	0.6	0.5
SWG	55	5.4±0.1	0.0	0.0	1.5	2.2
	60	4.6	0.0	0.0	1.4	2.1
	65	2.8	0.0	0.0	1.3	0.4
	70	3.0	0.0	0.0	1.8	4.1
	75	2.2	0.0	0.0	0.7	0.4
	80	2.3±2.2	0.0	0.0	0.8	0.4
	85	0.0	0.0	0.0	0.6	0.5
Xylan	55	1.3	0.0	0.0	1.6	2.5
	60	1.3	0.0	0.0	1.4	2.2
	65	1.2	0.0	0.0	1.5	2.3
	70	1.3	0.0	0.0	0.9	0.9
	75	5.9	0.0	0.0	0.9	1.0
	80	6.3	0.0	0.0	0.8	0.5
	85	0.8±0.7	0.0	0.0	0.6	0.6

Mean and standard deviation from three technical and two biological replicates are shown. Standard deviation <0.05 is not shown. End-products are expressed in millimolar concentrations.

SWG switchgrass

Thermoanaerobacteraceae comprised only 0.7 % and were related to *Carboxydothermus* sp., *Thermanaeromonas* sp., and *Thermoanaerobacter* sp. Bacteria involved in the breakdown of lignocellulosic material such as *Dictyoglomus*, *Fervidobacterium* (*Thermotogae*), *Caloramator*, and *Clostridium* were detected in environmental OBP samples and in enrichments (Fig. 4).

Discussion

This study demonstrated that (1) bacterial diversity was significantly higher in the adjacent vegetated area of OBP versus the main spring likely in response to temperature gradients and higher organic nutrients, (2) microbial community structure and activity changed with respect to incubation

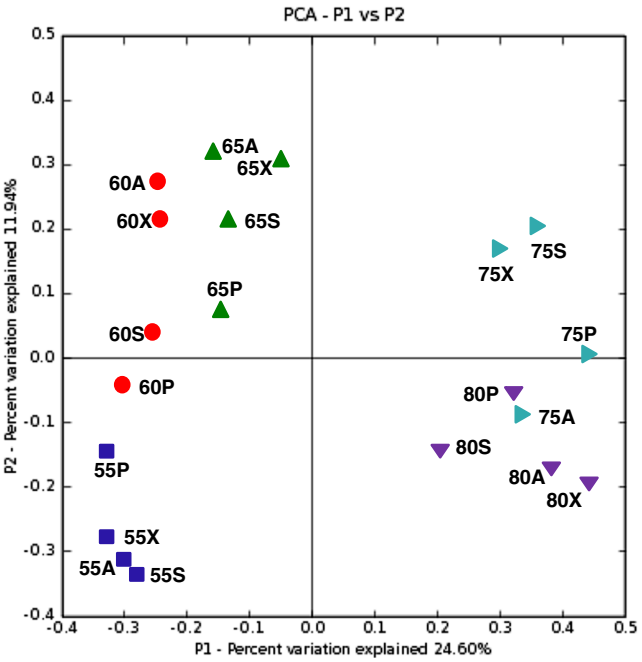

Fig. 3 PCA analysis of 16S rRNA gene sequences derived from different enrichments with unweighted UniFrac. Neighbor-joining tree was rooted with an archaeal outgroup. The ends of the alignment were trimmed prior to the analysis so that the aligned sequences were all the same length. The enrichment names contain incubation temperature from 55 to 80 °C with interval of 5 °C, and letter stands for substrate: *A* Avicel, *P Populus*, *S* switchgrass, *X* xylan

temperature and biomass substrate in enrichment culture experiments but was limited by temperatures above 80 °C, and (3) the most active cellulolytic microorganisms were detected through enrichment culture techniques.

In OBP, members of the thermophilic phyla Aquificae and Crenarchaeota, which are capable of using carbon dioxide as the sole carbon source and gain energy by the oxidation of inorganic substances like hydrogen and sulfur [34], dominated over other microorganisms. Both bacteria and archaea in OBP showed variations in abundance, which may reflect changes in geochemical activity. Previous studies of OBP showed fluctuations in H_2 (133.2–325.3 nM), CO_2 (12.8–14.9 mM), and SO_4^{2-} (0.33–0.52 μM) [33]; the SO_4^{2-} concentration can range from 51.8 μM to greater than 10.4 mM [55]. Increases in elemental sulfur and SO_4^{2-} concentrations potentially led to increases in the abundance of potentially sulfur-dependent Crenarchaeota and sulfate-reducing bacteria *Thermodesulfobacteria* and *Thermodesulforhabdus*. The abundance of Aquificae, which exclusively or preferentially use molecular H_2 as an energy source [34], changed between 98.2 % in October and 87.2 % in July. Crenarchaeota of the family *Sulfolobaceae* was detected only in the October sample with *Acidianus* spp. being dominant. High abundance of the Aquificae and Crenarchaeota suggested that primary production in OBP is likely driven by chemoautotrophic hydrogen or sulfur oxidation. Cole et al. [56] used pyrosequencing tags to examine the microbial diversity from a number of samples collected from Great Boiling Spring (GBS)

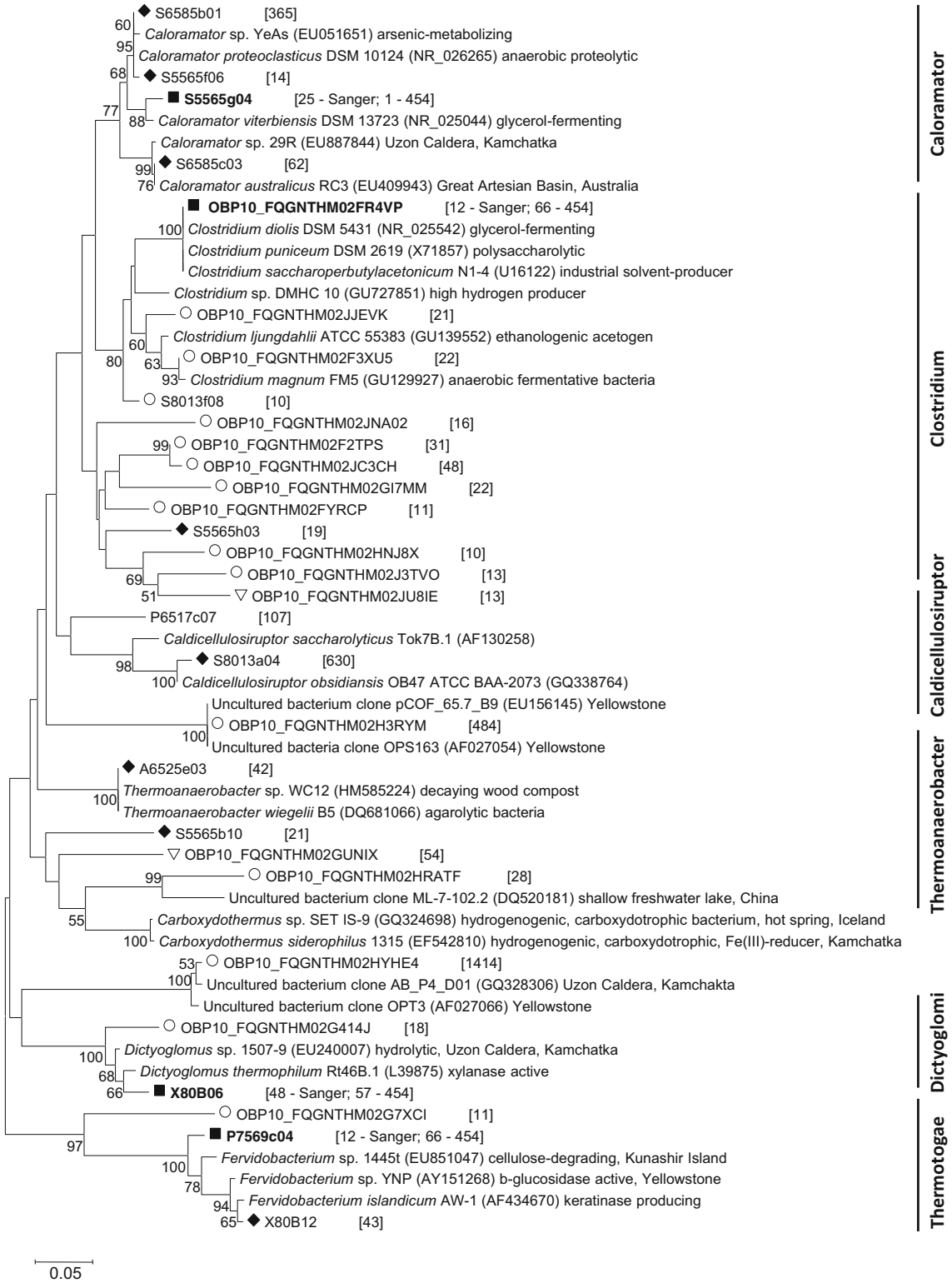

Fig. 4 Phylogenetic analysis of 16S rRNA gene sequences obtained by FLX 454 pyrosequencing of the V4 region amplified from OBP10 environmental gDNA and full-length Sanger sequences derived from OBP10 batch enrichments. The clusters with >10 sequences and those affiliated with potential cellulolytic bacteria are presented. The number of clones in each cluster is indicated in brackets. Symbols indicate clusters with sequences detected by 454 pyrosequencing using bacterial primers (*open circles*), or bacterial and archaeal primers (*open triangles*), clusters with Sanger sequences only (*filled diamonds*), and clusters which contain sequences obtained by 454 pyrosequencing and Sanger sequencing (*filled squares*). Additionally, the clusters with 454 pyrosequences and Sanger sequences are in bold, and the number of sequences obtained by each approach is indicated. The tree was produced by a neighbor-joining method. Bootstrap values were based on 1,000 replicates. The scale bar represents 0.05 changes per nucleotide position

in Nevada. They compared communities from the spring water to those inhabiting sediments, some of which were collected near the edge of the spring, which is also surrounded by grasses. Similar to OBP, the main source water was dominated by Aquificae and crenarachaeotes (*Thermoprotei*); however, sediment samples were much more diverse with archaea dominating sediments at higher temperatures (82–87 °C) and bacterial groups representing the most abundant OTUs between 80 and 62 °C [56]. While this study did not examine as many samples as Cole et al., the overall observations were similar in that saturated OBP sediments occurring at lower temperatures than the hydrothermal source also harbor a much greater diversity of thermophilic microorganisms which are predominantly bacterial.

Cellulosic detritus from heat-tolerant *J. tweedyi* [57] is a possible source of carbon for heterotrophic microorganisms at least in localized niches, where the dilute acid-sulfate water of OBP [55] and elevated temperatures likely aid in solubilizing C5 sugars and possibly lignin providing a mild pretreatment of plant material for further enzymatic decomposition. The phylogenetic analysis of 16S rRNA gene pyrosequences from the OBP10 sample showed a diverse community represented by 3 archaeal and 18 bacterial phyla and many unclassified bacteria. High taxonomical and functional diversity driven by the abundance of micronutrients and organic material creates the potential for discovering novel bacterial species with robust cellulolytic capabilities. The OBP10 sample contained a high abundance of Firmicutes, for example *Clostridium*, *Anaerobacter*, *Caloramator*, *Thermoanaerobacter*, and *Caldicellulosiruptor*, which could participate in different stages of cellulose degradation. Interestingly, *Caldicellulosiruptor*-like sequences detected by 16S rRNA gene pyrosequencing had low percent identity to *Caldicellulosiruptor* spp. detected in the OBP10 sample enrichments, while *Caldicellulosiruptor* sequences from the OBP10 enrichments were 96.6 % identical to *C. obsidiansis* OB47 which was previously isolated from OBP [24]. This possibly suggests that in the natural environment, *Caldicellulosiruptor* spp. are highly diverse with only a small portion (below 0.005 %) of *Caldicellulosiruptor* population able to grow under laboratory enrichment conditions tested in this study. The same conclusion may be true for another potentially hemicellulose-degrading bacterium, *Thermoanaerobacter*, which was detected at low abundance (<0.7 %) in the OBP10 sample, and these sequences had an average of 80 % identity to *Thermoanaerobacter* spp. detected in xylan enrichments.

Different combinations of substrate and temperature stimulated the formation of distinct microbial communities with varying levels of metabolic activity. Metabolite measurements showed that acetate was the main product with any combination of substrate and temperature, whereas lactate and ethanol were produced at 75–80 °C on Avicel and *Populus*. Phylogenetic analyses of each enriched microbial community detected significant changes bound to temperature-dependent distribution of *Caloramator* and *Caldicellulosiruptor*. *Caloramator* spp. dominated Avicel, switchgrass, and *Populus* enrichments at 55 and 60 °C. Crystalline cellulose utilization is not widespread in the genus *Caloramator*; however, the ability to grow on cellulose has been reported for *Caloramator australicus* [58] and other strains have dominated cellulose enrichments under thermophilic conditions [59]. At higher incubation temperatures, *Caldicellulosiruptor* spp. clearly outcompeted other organisms, especially for Avicel, and pretreated switchgrass and *Populus* were crystalline and amorphous cellulose would be the primary carbon source. Many *Caldicellulosiruptor* spp. express a diverse set of glycoside hydrolases and can utilize polymeric components of plant cell walls including cellulose and hemicellulose [15, 60, 61], plus the 70–80 °C temperature range is optimal for their growth [24, 62]. A *Dictyoglomus* sp., represented by a single OTU, was also detected in 80 °C enrichments, especially when xylan was added as the carbon and energy source which is consistent with the known ability of these organisms to efficiently breakdown C5 polymers [63, 64]. *Fervidobacterium* spp. were also present in cultures with xylan incubated at 75–80 °C, and members of this genus have been reported to readily ferment xylan at temperatures up to 82 °C [65].

Other recent attempts to characterize lignocellulose degraders at extreme to hyperthermophilic temperature ranges *in situ* at GBS [19] produced quite different results compared to our laboratory enrichments. *Thermotoga* spp., while present in low abundance in the natural background, were highly enriched on deployed aspen and corn stover samples at 85 °C while our enrichments produced virtually no growth at these temperatures. However, enrichment of *Caldicellulosiruptor* spp. was not detected by Peacock et al. [19] at lower temperatures (77 °C), which contrasts our results suggesting that these organisms are not ubiquitous in freshwater hot spring environments. The temperature limit for cultivable plant biomass-degrading communities inhabiting Obsidian Pool appears to be ~85 °C, where no production of acetate, lactate, or ethanol was observed with the exception of a trace amount of acetate (1 mM) in 85 °C xylan enrichments. Interestingly, no lactate was produced from enrichments with pretreated switchgrass or xylan at 75 and 80 °C, while lactate was produced from Avicel and *Populus* at these temperatures. Similar end-product profiles were also observed with the isolate *C. obsidiansis* when grown on these substrates [24], and highly related organisms dominated the Avicel and *Populus* enrichments at 75 and 80 °C. While this potentially provides an explanation for the difference in end-products at the higher temperature enrichments between substrates, factors controlling substrate-dependent (i.e., Avicel vs. switchgrass) carbon flux to either acetate or lactate are not well understood in *Caldicellulosiruptor* spp. Although acetate production was observed across all temperatures and substrates,

the highest combined concentrations of acetate, lactate, and ethanol were always found to be at temperatures of 75 and 80 °C.

Characterizing and understanding natural microbial communities that are able to deconstruct plant biomass at elevated temperatures is highly relevant for improving future lignocellulosic bioconversion technologies. Obsidian Pool in YNP remains a "hot spot" for revealing diverse microbial communities with a wide range of phenotypic characteristics. In this study, we have pinpointed an area of the spring that harbors an excellent source of cellulolytic and xylanolytic thermophiles due to the unique ecology of the OBP10 sample environment. Deep-dive metagenomics/proteomics and identification of the specific glycoside hydrolases involved in plant cell wall deconstruction (studies in progress) will shed more light on the microbial ecology of thermophilic biomass decomposition in nature with future applications for biotechnology.

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