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Effect of Temperature on Microbial Community of a Glucose-Degrading Methanogenic Consortium under Hyperthermophilic Chemostat Cultivation

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We continuously fed an anaerobic chemostat with synthetic wastewater containing glucose as the sole source of carbon and energy to study the effects of temperature on the microbial community under hyperthermophilic (65–80°C) conditions. Methane was produced normally up to 77.5°C at a dilution rate of 0.025 d^{-1}. However, the concentration of microorganisms and the rate of gas production decreased with increasing operation temperature. The microbial community in the chemostat at various temperatures was analyzed based on the 16S rRNA gene using molecular biological techniques including clone library analysis and denaturing gradient gel electrophoresis (DGGE). Aceticlastic methanogens related to *Methanosarcina thermophila* were detected at 65°C and hydrogenotrophilic methanogens related to *Methanothermobacter thermautotrophicus* were the dominant methanogens between 70°C to 77.5°C. Bacteria related to *Clostridium stercorarium* and *Thermoanaerobacter subterraneus* comprised the dominant glucose-fermenting bacteria at temperatures of 65°C and above, respectively. Bacteria related to *Thermacetogenium phaeum* and to *Tepidiphilus margaritifer* and *Petrobacter succinatimandens* were the dominant acetate-oxidizing bacteria at 70°C and at 75–77.5°C, respectively. The results suggested that, at temperatures of 70°C and above, methane production via the aceticlastic pathway was negligible and indirect methanogenesis from acetate was dominant. Since acetate oxidation is a rate limiting step and a higher temperature favors the hydrolysis and acid formation, a two stage fermentation process, acidogenic and methanogenic fermentation stages operated under different temperatures, should be more suitable for the thermophilic anaerobic treatment at temperatures above 65°C.

[**Key words:** hyperthermophilic, methane fermentation, microbial community, glucose]

Methane fermentation is a popular way to treat a wide variety of industrial wastewaters (1). Compared with mesophilic systems (37°C), thermophilic systems (55°C) have become the more widespread choice for treating medium- and high-strength wastewaters since they can handle very high organic loading rates while high treatment efficiency is maintained (2). However, methane fermentation should also be possible under hyperthermophilic conditions at temperatures above 55°C (3, 4). As higher temperatures can result in more hydrolytic activity (3), hyperthermophilic methane fermentation has usually been investigated with respect to treating wastes with high solid contents, such as municipal solid waste and cattle manure (3, 5, 6). In addition, increased demands for pathogen kill during anaerobic digestion increased the interest for digestion at higher temperatures to allow unrestricted use of the digested materials as a fertilizer on farmland. Nevertheless, methane fermentation under hyperthermophilic conditions is reportedly unstable and high loading rates are difficult to achieve (3, 5).

Methane fermentation is the result of cooperativity among many classes of microorganisms that are respectively responsible for hydrolysis, acidogenesis and methanogenesis. Therefore, the performance instability and low loading rates are probably due to an imbalance among these microorganisms. Investigating changes in the microbial community in a methane fermentation reactor operated within the hyperthermophilic range is thus critically important for the optimal application of hyperthermophilic methane fermentation technologies. However, little is understood about the effect of temperature on the composition of the microbial community and information about such communities within the hyperthermophilic range remains limited. Most investigations into the effect of temperature on waste treatment processes within

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the hyperthermophilic range have focused on reactor performance (5–8).

The present study examines process performance and the composition of the microbial community at temperatures above 60°C in terms of methane fermentation. The process was simplified by feeding an anaerobic reactor with a synthetic wastewater containing glucose as the sole source of carbon and energy. To avoid spatial differences in the microbial community, we operated a continuous stirred tank reactor (CSTR) to study the community at different temperatures. The microbial community was analyzed using molecular methods based on the 16S rRNA gene, clone analyses and denaturing gradient gel electrophoresis (DGGE). The effects of temperature on reactor performance, microbial structure and therefore metabolic pathway were elucidated.

MATERIALS AND METHODS

Synthetic wastewater Synthetic wastewater with glucose as the sole carbon and energy source comprised the following (g/*l*): glucose, 21.0; KH_2PO_4, 0.3; $KHCO_3$, 4.0; NH_4Cl, 1.0; NaCl, 0.6; $MgCl_2 \cdot 6H_2O$, 0.82; $CaCl_2 \cdot 2H_2O$, 0.08; cystein–$HCl \cdot H_2O$, 0.1; 10 ml of DSMZ medium 318 trace element solution (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) containing 1.2 mg/*l* of Ni^{2+} and 0.34 mg/*l* of Co^{2+}, and 10 ml of DSMZ medium 318 vitamin solution without B_{12}. The total organic carbon (TOC) concentration of the synthetic wastewater was approximately 8.0 g/*l*.

Operation of glucose-fed chemostat and fixed-bed reactor An anaerobic chemostat was established using a completely stirred tank reactor (CSTR, working volume, 1.8 *l*). A 1.2-*l* portion of thermophilic digested sludge acclimatized with a surplus sewage at an organic matter-loading rate of 1.0 g/*l*·d at 53°C was washed with the synthetic wastewater under anaerobic condition, diluted to 1.8 *l* and then placed in CSTR. The reactor was continuously fed with synthetic wastewater containing glucose at a constant dilution rate of 0.025 d^{-1}. The temperature of the culture broth was incrementally increased from 60°C to 80°C over time by adjusting the thermostatically controlled temperature of the oil that passed through the outer jacket of the reactor (Fig. 1). Culture broth subjected to molecular analysis was sampled at 65°C, 70°C, 75°C and 77.5°C. Under each temperature, multiple samples with different operation times (samples *a* and *b* for 65°C, 70°C and 75°C; samples *a*, *b* and *c* for 77.5°C) were analyzed.

16S rRNA gene clone library analysis We studied the effect of temperature on the microbial community in the chemostat by constructing 16S rRNA gene clone libraries (9) based on DNA extracted from the microbial community (10). The 16S rRNA genes for the archaeal and bacterial libraries were amplified using the primer sets PRA46F (5′-YTAAGCCATGCRAGT-3′) and PREA1100R (5′-YGGGTCTCGCTCGTTRCC-3′) (11), as well as Eu27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1490R (5′-GGTTACCT TGTTACGACTT-3′), respectively. Four archaeal (HTA1 to HTA4) and 4 bacterial (HTB1 to HTB4) 16S rRNA gene libraries were constructed using DNA extracted from the communities fed with glucose at 65°C (sample *b*, 48 d of operation), 70°C (sample *b*, 80 d of operation), 75°C (sample *a*, 22 d of operation) and 77.5°C (sample *c*, 170 d of operation) under steady-state conditions. Twenty clones from each archaeal library and 50 clones from each bacterial library were sequenced using a CEQ8000 genetic analysis system (Beckman Coulter, Fullerton, CA, USA). All 16S rRNA sequences were manually checked for chimeric artifacts using CHIMERA_CHECK ver. 2.7 of the Ribosomal Database Project II (RDP-II) (12). Similar sequences were searched using the BLASTN program (13). Multiple alignments were generated using the Clustal X program ver. 1.8 (14) and phylogenetic trees were constructed using MEGA ver. 3.0 (15). Bootstrap resampling analysis (16) for 500 replicates estimated the degree of confidence in tree topologies. Sequences with >99% similarity were defined as identical and used for further phylogenetic analysis as operational taxonomic units (OTUs). The OTUs were designated as clones of the following libraries shown in parentheses: HTA1-A1 to HTA1-A4 (HTA1), HTA2-A1 and HTA2-A2 (HTA2), HTA3-A1 and HTA3-A2 (HTA3), HTA4-A1 and HTA4-A2 (HTA4), HTB1-B1 to HTB1-B13 (HTB1), HTB2-B1 to HTB2-B12 (HTB2), HTB3-B1 to HTB3-B11 (HTB3) and HTB4-B1 to HTB4-B13 (HTB4).

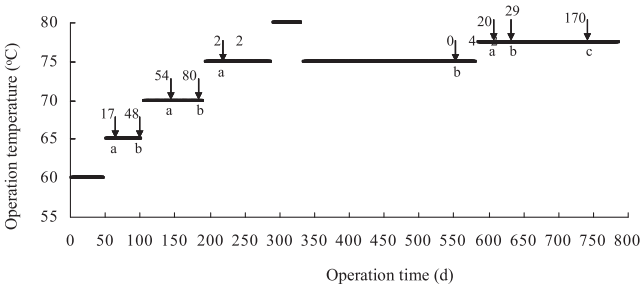

FIG. 1. Operation schedule for hyperthermophilic CSTR. Arrows, times of DNA extraction for 16S rRNA gene analysis; numbers beside arrows, operation time under operation temperature; letters, different samples at same operation temperature.

Denaturing gradient gel electrophoresis (DGGE) We performed DGGE as described (17) using the DCode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Fragments of approximately 200 bp in the V3 region of the archaeal and bacterial-16S rRNA gene were amplified from DNA extracted from the microbial community at each operation temperature and represent clones obtained by analysis of the rRNA gene library. The position of bands derived from the community DNA and rRNA gene clones were compared to consider the correlation. Archaeal and bacterial rRNA genes were resolved using 30% to 60%, and 35% to 65% denaturing gradients, respectively. The electrophoretic conditions comprised 20 min at 25 V for both the archaeal and bacterial rRNA genes, followed by 130 V for 8 h at 65°C, and for 14 h at 63°C, respectively for each.

Other analytical methods Relative concentrations of coenzyme F_{420} in the culture broth at each operation temperature were determined as described (18). The fluorescent intensity of the culture broth from an acetate-fed CSTR at a dilution rate of 0.025 d^{-1} was defined as 1 (18).

The methane content of the biogas and concentrations of volatile suspended solids (VSS), soluble total organic carbon (TOC) and volatile fatty acids (VFA) in the culture broth were measured as described (9).

Nucleotide sequence accession numbers The DDBJ/EMBL/GenBank accession numbers for the sequences of OTUs HTA1-A1 to HTA4-A2, HTB1-B1 to HTB4-B13 are AB374100 to AB374139 and AB434891 to AB434905.

RESULTS

Continuous degradation of glucose in an anaerobic chemostat under hyperthermophilic conditions Continuous anaerobic degradation of synthetic wastewater containing glucose as the sole carbon and energy source proceeded for almost three years under hyperthermophilic conditions. The dilution rate of the chemostat was maintained at 0.025 d^{-1} (TOC loading rate of 0.2 g/*l*·d) throughout the en-