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Methanogenic pathway and community structure in a thermophilic anaerobic digestion process of organic solid waste

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The methanogenic pathway and microbial community in a thermophilic anaerobic digestion process of organic solid waste were investigated in a continuous-flow stirred-tank reactor using artificial garbage slurry as a feedstock. The decomposition pathway of acetate, a significant precursor of CH_4 and a key intermediate metabolite in the anaerobic digestion process, was analyzed by using stable isotopes. A tracer experiment using ^{13}C-labeled acetate revealed that approximately 80% of the acetate was decomposed via a non-aceticlastic oxidative pathway, whereas the remainder was converted to methane via an aceticlastic pathway. Archaeal 16S rRNA analyses demonstrated that the hydrogenotrophic methanogens *Methanoculleus* spp. accounted for >90% of detected methanogens, and the aceticlastic methanogens *Methanosarcina* spp. were the minor constituents. The clone library targeting bacterial 16S rRNA indicated the predominance of the novel Thermotogales bacterium (relative abundance: ~53%), which is related to anaerobic acetate oxidizer *Thermotoga lettingae* TMO, although the sequence similarity was low. Uncultured bacteria that phylogenetically belong to municipal solid waste cluster I were also predominant in the microflora (~30%). These results imply that the microbial community in the thermophilic degrading process of organic solid waste consists exclusively of unidentified bacteria, which efficiently remove acetate through a non-aceticlastic oxidative pathway.

[**Key words:** Thermophilic methanogenic bioreactor; Organic solid waste; Methanogenic pathway; Microbial community structure]

Reuse and recycling of organic wastes, including garbage and waste from the food industry, have been attracting social attention and concern. Anaerobic digestion is one of the effective technologies for recovering energetic materials from organic waste and is a simple and environmentally acceptable means of reducing and stabilizing organic waste (1).

The thermophilic anaerobic digestion process has advantages over the mesophilic process with respect to digestion efficiency and disinfection of pathogenic organisms (2). Unlike the mesophilic process, the thermophilic process is characterized by limited species of aceticlastic methanogens and simple structure of the bacterial communities (3,4). Several researchers have provided information about microbial composition and distribution in thermophilic reactors treating organic solid wastes (4–7). Their reports have indicated that methanogenesis from solid wastes required microbial members distinct from those in other reactors treating liquid slurry such as industrial wastewater. However, little is known about the methanogenetic pathway in the reactors.

The metabolic pathway to produce methane from organic polymers consists basically of three steps: first, complex polymeric substances such as cellulose and protein are hydrolyzed (hydrolysis), then the hydrolyzed products are degraded to volatile fatty acids (VFAs) and H_2/CO_2 (acidogenesis), and finally methane gas is produced from acetate or H_2/CO_2 (methanogenesis) (8). Acetate has been known as a key intermediate metabolite during methanogenesis, and the decomposition of acetate is considered to be the rate-limiting step of the over-all reaction process (9,10). So far, two methanogenic pathways from acetate have been reported (11–13). One is the direct methanogenesis by aceticlastic methanogenic archaea, such as *Methanosarcina* spp. (aceticlastic cleavage, reaction formula 1). The aceticlastic methanogens convert the methyl and carboxyl groups of acetate to CH_4 and CO_2, respectively. The other is non-aceticlastic oxidation, i.e., the co-metabolism pathway by acetate-oxidizing bacteria (reaction formula 2) and hydrogenotrophic methanogens (reaction formula 3). During the latter pathway, acetate is first oxidized to CO_2 and then the produced CO_2 is reduced to CH_4. The decomposition pathway of acetate is known to be affected by the operation temperature, the composition of organic substances, the types of reactors, and the organic loading rate (14).

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Aceticlastic cleavage

$$^{*}CH_3COO^- + H_2O \rightarrow {}^{*}CH_4 + HCO_3^- \quad (1)$$

Non-aceticlastic oxidation

$$^{*}CH_3COO^- + 4H_2O \rightarrow H^{*}CO_3^- + HCO_3^- + 4H_2 + H^+ \quad (2)$$

$$H^{*}CO_3^- (\text{or } HCO_3^-) + 4H_2 + H^+ \rightarrow {}^{*}CH_4 (\text{or } CH_4) + 3H_2O \quad (3)$$

(Asterisks represent the carbon of methyl group in acetate.)

In the present study, a lab-scale thermophilic continuous-flow stirred-tank reactor (CFSTR) was operated using artificial garbage slurry (AGS) as a model of organic solid waste. After establishing efficient digestion performance for 18 months (organic loading rate [OLR], 6.25 gCODcr l^{-1} day^{-1}), we analyzed the microbial composition of the enriched microflora and the pathway of acetate degradation in the process by using stable isotopes.

MATERIALS AND METHODS

Operation of thermophilic CFSTR Two liters of seed sludge collected from a thermophilic anaerobic digester treating garbage slurry (15) was cultivated at 55 °C in a 3 L jar fermenter (MDL-301s; B.E. Marubishi, Tokyo, Japan) with agitation at 100 rpm. The initial anaerobic condition in the bioreactor was established by replacing the gas phase with argon gas. AGS was prepared from 20 g of a commercial dog food (Vita-One; Nihon Pet Food, Tokyo, Japan) dissolved in 1 L of sterilized water. The physicochemical characteristics of the AGS are as follows: total chemical oxygen demand (COD), 25 gCODcr l^{-1}; soluble COD concentration, 8.5 gCODcr l^{-1}; suspended solid (SS), 16.5 g l^{-1}; volatile suspended solid (VSS), 15.5 g l^{-1}. The supply of AGS to the reactor was accompanied by the concomitant removal of an equal amount of broth from the reactor. The hydraulic retention time (HRT) was controlled using timer-controlled peristaltic pumps at a constant value. The reactor was initially operated at HRT of 10 days (OLR, 2.5 gCODcr l^{-1} day^{-1}) for 150 days. Thereafter, the OLR was stepwise increased to 3.13 gCODcr l^{-1} day^{-1} (for 30 days at HRT of 8.0 days), 3.75 gCODcr l^{-1} day^{-1} (for 30 days at HRT of 6.7 days), and 5.0 gCODcr l^{-1} day^{-1} (for 15 days at HRT of 5.0 days). Finally, the reactor was stably operated at OLR of 6.25 gCODcr l^{-1} day^{-1} (i.e., HRT of 4.0 days) for 600 days. The pH value in the reactor was maintained at 7.2 by automatic titration with 5 N NaOH.

Analyses of the reactor performance The gas production rate was measured periodically by water displacement with a graduated cylinder. The broth in the reactor was collected for analyses of physicochemical parameters at 3-day intervals. The COD concentration was determined by using the dichromate method with a COD analyzer (DR-300; Hach, Loveland, CO, USA). To determine the concentration of total SS, 2 ml of the broth was passed through the membrane (Cellulose Acetate Membrane Filter; 0.2 μm–47 mm, Toyo Roshi Kaisha, Ltd., Tokyo, Japan) after which the membrane was weighed after drying at 105 °C for 3 h. The concentration of VFAs was determined using a liquid chromatograph (L6300; Hitachi, Tokyo, Japan) equipped with a TSKgel OAPak-A column (Tohso, Tokyo, Japan) and a UV spectrophotometric detector (SPD-7A; Shimadzu, Kyoto, Japan). The generated gas composition was analyzed by gas chromatography-mass spectrometry (GC-MS) (GC17A-QP5050; Shimadzu) equipped with a CP-PoraPLOT Q (L: 25 m, ID: 0.32 mm, *df*: 10 μm; GL Science Inc., Tokyo, Japan).

Analysis of isotope distribution in the generated gas with ^{13}C-labeled acetate First, 10 ml of broth (600 days, HRT of 4.0 days) was taken from the reactor and transferred to 25 ml vials. The vials were sealed with a butyl rubber stopper and an aluminum cap. The gas phase was replaced with nitrogen gas using a Deoxygenized Gas Pressure Injector (IP-8; Sanshin, Yokohama, Japan). In order to equalize the degradation activity among the vials and to reduce the unlabeled acetate originally presented in the broth, the vials were pre-incubated for 6 days at 55 °C with shaking. After the pre-cultivation, the gas phase was replaced with nitrogen gas. Then, 4 mM of sodium acetate-2-^{13}C, sodium acetate-1-^{13}C, or sodium acetate-1,2-^{13}C (99 atom %; Sigma-Aldrich, Tokyo, Japan) were added to the pre-cultured vials. After 24 h of incubation with shaking, the gaseous products were analyzed using the selected ion monitoring (SIM) method by a GC-MS (GC17A-QP5050; Shimadzu) equipped with a CP-PoraPLOT Q (L: 25 m, ID: 0.32 mm, *df*: 10 μm; GL Science Inc.,). Helium was used as a carrier gas at a flow rate of 1.5 ml min^{-1}, and the column temperature was 35 °C. The peaks at *m/z* 15 and 17 were regarded as the fragment ion for $^{12}CH_4$ and the molecular ion for $^{13}CH_4$, respectively. The peaks at *m/z* 44 and 45 were regarded as the molecular ion for $^{12}CO_2$ and $^{13}CO_2$, respectively.

Extraction and purification of DNA The broth (300 days, HRT of 4.0 days) from the reactor was divided into two fractions by filtration using a nylon net filter (pore size: 41 μm) (NY41; Millipore, Tokyo, Japan). The fraction remaining on the net filter was defined as the solid fraction. The fraction passing through the net filter was defined as the liquid fraction. The genomic DNA was extracted from the total fraction, solid fraction, and liquid fraction by the benzyl-chloride method (16). The concentration of genomic DNA was measured by a UV spectrophotometer (DU® 7400; Beckman-Coulter Co., Fullerton, CA, USA) at 260 and 280 nm, and checked by 0.8% agarose gel electrophoresis. For clone analyses, 10 ng of the genomic DNA was used as the template of PCR amplification.

Cloning and phylogenetic analysis Clone libraries of bacterial and archaeal 16S rRNA gene sequences were constructed from genomic DNA. PCR amplification of the partial 16S rRNA genes was carried out by using AmpliTaqGold® with GeneAmp® (Applied Biosystems, Tokyo, Japan) according to the manufacturer's instructions. PCR was performed with a thermal cycler (PTC-200 DNA Engine; MJ Japan, Tokyo, Japan). The 16S rRNA genes were amplified using the bacterial primers Ba27f/Ba907r (17,18) or the archaeal primers Ar109f/Ar912rt (19,20). The thermal cycle condition was started with an initial denaturation at 94 °C for 10 min, followed by 25 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 45 s, extension at 72 °C for 90 s, and the final extension step at 72 °C for 5 min. The PCR products purified using the QIAquick Gel Extraction kit (Qiagen, Tokyo, Japan) were ligated to the pGEM-T Easy Vector (Promega, Tokyo, Japan) according to the manufacturer's instructions. The ligation products were transformed into *Escherichia coli* JM109 (Toyobo, Tokyo, Japan). Plasmids were extracted using a GenElute plasmid Miniprep Kit (Sigma-Aldrich). Sequencing was performed by using a 3130*xl* Genetic Analyzer (Applied Biosystems) with a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The sequences were checked for chimeric artifacts by using the CHIMERA_CHECK program from the Ribosomal Database Project (21). Homology searches were performed with the BLAST program at the web site of the National Center for Biotechnology Information. Sequences with more than 99.5% identity were treated as the same operational taxonomy unit (OTU) (22,23). Phylogenetic analyses were performed using the ARB software package (24). A phylogenetic tree was calculated and represented by using the neighbor-joining, maximum-parsimony, and maximum-likelihood methods. Bootstrap values were obtained from 1000 replications.

Nucleotide sequence accession numbers The nucleotide sequence data obtained in this study have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases under the following accession numbers: bacterial sequences, AB428524, AB428526 to AB428539, and AB428540 to AB428543; archaeal sequences, AB428544 to AB428549.

RESULTS

Reactor operation and performance Table 1 summarizes the average values of reactor performance from 60 to 600 days when the reactor was operated at HRT of 4.0 days (OLR, 6.25 gCODcr l^{-1} day^{-1}). Gas production was stable during this period, and the accumulation of acetate and propionate was negligible at concentrations of 1.85 (±1.81) mM and 0.86 (±0.83) mM, respectively. More than 60% of SS supplied to the reactor was solubilized, and COD removal efficiency was over 65%. The gas produced contained approximately 80% CH_4, and the remainder was CO_2. Low content of CO_2 could be due to increase of alkalinity caused by NaOH titration. It was calculated that more than 80% of decomposed COD equivalent was recovered as methane gas.

These reactor performance values approximated those of previous thermophilic CFSTRs treating organic solid wastes at similar OLR (6,25,26). Our reactor's operation and performance also stably digested AGS at the short HRT, whereas the previous reactors were operated at HRT of 10 days or more.

Isotope distribution in the generated gas from ^{13}C-labeled acetate Broth was taken from the reactor after 600 days of

TABLE 1. Operational parameters and reactor performance during stable operation at the shortest HRT in this study.

Operational parameters			Reactor performance[a]				
HRT (day)	OLR (gCODcr l^{-1} day^{-1})	Controlled pH	Gas production rate (ml l^{-1} day^{-1})	COD removal ratio (%)	SS removal ratio (%)	Acetate (mM)	Propionate (mM)
4.0	6.25	7.2	1810 (±118)	65.8 (±2.80)	61.8 (±5.59)	1.85 (±1.81)	0.86 (±0.83)

[a] Data are average values obtained between the 60th and 600th days of operation at HRT of 4.0 days.