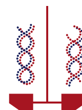
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# Reorganization of the bacterial and archaeal populations associated with organic loading conditions in a thermophilic anaerobic digester


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**Organic loading conditions are an important factor influencing reactor performances in methanogenic bioreactors. Yet the underlying microbiological basis of the process stability, deterioration, and recovery remains to be understood. Here, structural responses of the bacterial and archaeal populations to the change of organic loading conditions in a thermophilic anaerobic digester were investigated by process analyses and 16S rRNA gene-based molecular approaches. The biogas was produced stably without the accumulation of volatile fatty acids (VFAs) at low organic loading rates (OLRs) in the beginning of reactor operation. Increasing OLR in stages disrupted the stable reactor performance, and high OLR conditions continued the deteriorated performance with slight biogas production and high accumulation of VFAs. Thereafter, the gradual decrease of OLR resulted in the recovery from the deterioration, giving rise to the stable performance again. The stable performances before and after the high OLR conditions conducted were associated with compositionally similar but not identical methanogenic consortia. The bacterial and archaeal populations were synchronously changed at both the transient phases toward the deteriorated performance and in recovery process, during which the dynamic shift of aceticlastic and hydrogenotrophic methanogens including the recently identified *Methanomassiliicoccus* might contribute to the maintenance of the methanogenic activity. The distinctive bacterial population with a high predominance of *Methanobacterium formicicum* as archaeal member was found for the deteriorated performance. The results in this study indicate the coordinated reorganization of the bacterial and archaeal populations in response to functional states induced by the change of organic loading conditions in the anaerobic digester.**


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[**Key words:** Methanogenic population; Organic loading condition; Anaerobic digester; Reorganization; 16S rRNA gene-based molecular approach]


Anaerobic digesters have been widely used for the treatment of industrial and municipal wastewaters (1,2). In the treatment process, organic waste was converted to biogas (mainly composed of methane and carbon dioxide) that would serve as a sustainable energy source. The methanogenic degradation of organic matter is catalyzed by the concerted activity of physiologically distinct microorganisms, such as hydrizers, acidogens, syntrophs, and methanogens (3,4). The population imbalance of these microbial groups causes the process deterioration, resulting in the accumulation of degradation intermediates, e.g., volatile fatty acids (VFAs) and ethanol, in the formation of biogas from organic matter (2,5–7).

Organic loading conditions are one of the most significant factors determining functional states such as the process stability, deterioration, and recovery in anaerobic digesters (2). The biogas production decreases when high-strength wastewaters unexpectedly flow into reactors, which induces most seriously the breakdown of reactor performances. So far, there have been investigations focusing on reactor conditions artificially overloaded in laboratory-scale anaerobic digesters (8–12). Organic loading rate (OLR) has been increased by the shortened hydraulic retention times (HRTs) and/or pulse dosage of concentrated wastewaters. However, HRT is generally fixed in the actual operation of full-scale anaerobic digesters and a temporal exposure of anaerobic digesters to high OLRs provokes the unsteady state in reactor performances, which makes it difficult to clarify the underlying microbiological basis of the change in reactor performance. Given that one reactor condition is changed to another, at least tripled operational period of the HRT has been reported to require the establishment of the steady state condition (13). In order to investigate overloaded conditions that are relatively close to those found practically in full-scale anaerobic digesters, it is favored that the OLRs are controlled by changing the strength of wastewaters supplied at a constant HRT in laboratory-scale anaerobic digesters.


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The culture-independent molecular methods are available for studies on the relationship between reactor performance and microbial community structure (3,4). For instance, the microbial populations in different runs of anaerobic digesters have been investigated to determine the microorganisms potentially responsible for the functional states of reactor operations (14–16). However, with a special focus on an operation of an anaerobic digester, only little is known about when and how the methanogenic consortium responds to environmental change and functional perturbation, and whether the once functionally disrupted methanogenic consortium reorganizes again to recover the methanogenic degradation ability.

The objective in this study was to investigate the succession of the bacterial and archaeal populations in response to functional states (i.e., the stable, deteriorative, and recovery phases) associated with organic loading conditions in a thermophilic anaerobic digester using a combination of process analyses and molecular fingerprinting tools, i.e., single-strand conformation polymorphism (SSCP) and clone library analyses based on the 16S rRNA gene.

## MATERIALS AND METHODS

**Operation of a thermophilic anaerobic digester** A seed culture was obtained from a thermophilic anaerobic digester treating garbage wastewater (17). The digester microorganisms were cultivated at 55°C in a 2.0-l stirred tank reactor (MDL-8L; B. E. Marubishi, Tokyo, Japan) agitated with a magnetic stirrer at 100 rpm. The culture volume in the anaerobic digester was 1.4 l. Synthetic wastewater was supplemented to the reactor at an HRT of 10 days (dilution rate was 0.1 $day^{-1}$). The synthetic wastewater contained glucose as a sole carbon and energy source, and the composition was as follows (g $l^{-1}$): $KH_2PO_4$, 0.908; $Na_2HPO_4 \cdot 12H_2O$, 2.39; $NH_4Cl$, 0.5; $MgCl_2 \cdot 6H_2O$, 0.18; yeast extract (Difco, Detroit, MI, USA), 2.0; and glucose, 10; as well as 10 ml of a vitamin solution containing (mg $l^{-1}$): biotin, 2.0; folic acid, 2.0; pyridoxine HCl, 10.0; thiamine HCl, 5.0; riboflavin, 5.0; nicotinic acid, 5.0; DL-calcium pantothenate, 5.0; vitamin $B_{12}$, 0.1; *p*-aminobenzoic acid, 5.0; and lipoic acid, 5.0. The minerals were injected directly into the reactor once per 2 days at the following final concentrations (mg $l^{-1}$): $FeSO_4 \cdot 7H_2O$, 5.53; $CoCl_2 \cdot 6H_2O$, 0.48; $ZnCl_2$, 0.67; $CaCl_2 \cdot 2H_2O$, 0.59; $CuCl_2 \cdot 2H_2O$, 0.16; $MnCl_2 \cdot 4H_2O$, 2.02; $H_3BO_4$, 0.063; $Na_2MoO_4 \cdot 2H_2O$, 0.0045; $NiCl_2 \cdot 6H_2O$, 0.65. The pH was kept at 7.1 using an automatic titration with 5 M NaOH. Before starting a run of the anaerobic digester perturbed with high organic loading conditions, the reactor was operated stably for more than 30 days to acclimatize the digester microorganisms to the reactor condition mentioned above. The organic loading condition was controlled by the concentration of glucose dissolved in the synthetic wastewater (the concentration range was 1–6% [wt/vol]). Firstly, the OLR of reactor operation was increased in stages from day 0 to 156. The operational durations with the OLRs of 0.4 g TOC $l^{-1}$ $day^{-1}$ (days 0–12), 0.8 g TOC $l^{-1}$ $day^{-1}$ (days 13–48), 1.6 g TOC $l^{-1}$ $day^{-1}$ (days 49–96), and 2.4 g TOC $l^{-1}$ $day^{-1}$ (days 97–156) were defined as periods of P1, P2, P3, and P4, respectively. The OLR was then gradually decreased from day 157 to 255. The durations with the decreased OLR of 1.6 g TOC $l^{-1}$ $day^{-1}$ (days 157–204) and 0.8 g TOC $l^{-1}$ $day^{-1}$ (days 205–255) were defined as the periods P5 and P6, respectively. In order to stabilize the activity of the digester microorganism, the reactor was operated under each organic loading condition for more than 30 days, which is equal to a tripled period of the HRT (13). The analytical methods for monitoring reactor performances were described previously (6). Briefly, the biogas production was measured daily with a gas meter based on the liquid displacement. The concentration of VFAs was determined using an HPLC (Alliance 2695; Waters, Tokyo, Japan) equipped with an OApak-A column (Tosoh, Tokyo, Japan) and a photodiode array (2996; Waters). The ethanol and glucose concentrations were measured enzymatically with F-kits (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. Total carbon (TC) and inorganic carbon (IC) were determined using a total carbon analyzer (TOC-V CSN; Shimadzu, Tokyo, Japan). Total suspended solid (SS) was determined as described previously (7). The total SS was used for an indicator of microbial cell in the reactor, because the synthetic wastewater contained no solid matter. Culture samples were taken at 3–5 time points under each organic loading condition; a total of 26 time points were sampled during the operation as shown by arrows in Fig. 1 for physicochemical analysis and molecular ecological analysis.

**Quantification of coenzyme M** Coenzyme M (CoM) is a coenzyme of the methyl-coenzyme M reductase catalyzing methanogenesis and is contained in all known methanogens (18,19). To evaluate roughly the change of the methanogen biomass, the CoM concentration was determined one day prior to the sampling times according to the procedure of Elias et al. (20). The cultured cells were suspended in 1% tri-(*N*)-butylphosphine (TBP) dissolved in 2-isopropanol, and the mixture was incubated for 1 h at room temperature. After centrifugation, the

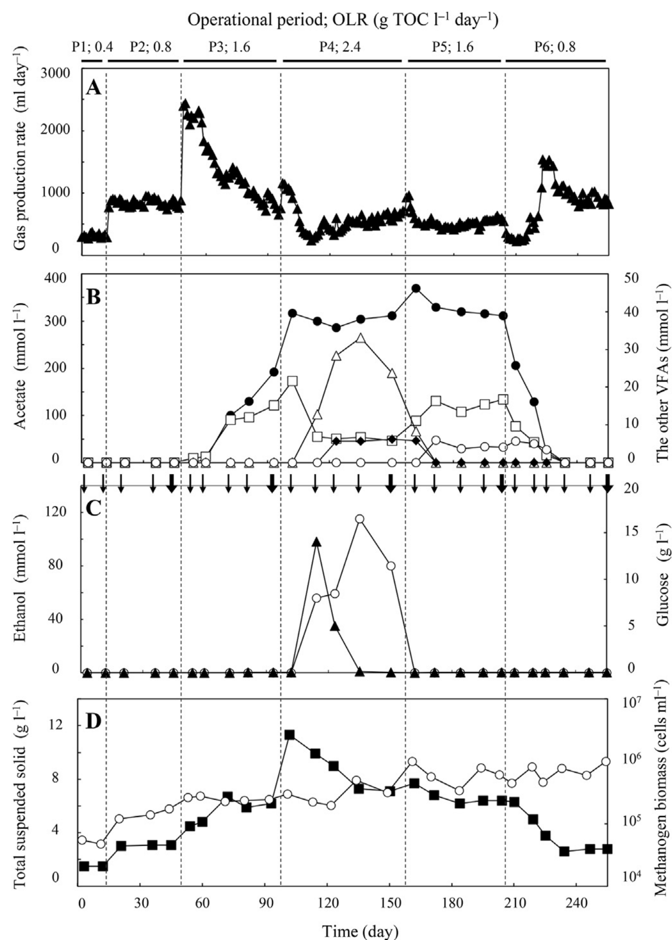


FIG. 1. Change in physicochemical parameters and methanogen biomass during the operation of the anaerobic digester. (A) Gas production rate (closed triangles). (B) Concentrations of formate (closed diamonds), acetate (closed circles), propionate (open squares), butyrate (open circles), and lactate (open triangles). (C) Concentrations of glucose (closed triangles), and ethanol (open circles). (D) Total SS concentration (closed squares) and methanogen biomass (open circles). The defined operational periods and their OLR were presented in the upper part. Arrows in lightface and boldface indicate the sampling points for SSCP and clone library analyses, respectively. The cell number of methanogen was estimated from the concentration of coenzyme M.

supernatant was derivatized with *o*-phthalaldehyde (20 mg $ml^{-1}$ of methanol) and ethanolamine (20 μl $ml^{-1}$ of boric acid buffer, pH 9.0). The CoM concentration was determined using an HPLC (alliance 2695; Waters) equipped with an ODS column (Zorbax; Agilent Technologies, Tokyo, Japan) and a multi λ fluorescence detector (2475; Waters). In the fluorescence detection, wavelength of excitation was 338 nm and that of emission was 445 nm. The mobile phase in chromatography was the 50 mM sodium acetate buffer (pH 5.7)-acetonitrile mixture (the ratio of 70:30) flowing at a rate of 1 ml $min^{-1}$. The reported analytical formula (0.41 fmol of CoM $cell^{-1}$-methanogen) was utilized for the estimation of the methanogen biomass (20).

**DNA extraction and PCR amplification** The genomic DNAs were extracted from the culture samples by benzyl chloride method and were used as PCR templates (21). PCR amplification was carried out with AmpliTaqGold (Applied Biosystems, Tokyo, Japan) for SSCP and clone library analyses. For the SSCP, a primer set of B342If/U806Ir was used to amplify the bacterial 16S rRNA gene (6). The archaeal 16S rRNA gene was amplified by nested PCR. Primer sets U341If/A1399r and A348If/U806Ir were used for the first and second PCR, respectively. Reverse primer of U806Ir was phosphorylated at the 5′ for the single-strand digestion. The thermal cycle programs of PCR were described previously (6). For the clone library analysis, primer sets of B27f/B907r and A109f/A912rt were used for PCR amplification of the bacterial and archaeal 16S rRNA genes, respectively (5). The thermal cycle programs of PCR consisted of 20 cycles for *Bacteria* and 25 cycles for *Archaea*, each including 30 s at 94°C, 45 s at 52°C, and 90 s at 72°C, followed by a final extension step of 5 min at 72°C.

**SSCP fingerprinting and subsequent statistical analyses** The PCR products were purified with a QIAquick PCR purification kit (Qiagen, Tokyo, Japan). The

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