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RAPID COMMUNICATION

Quantitative analysis of microbial community structure in two-phase anaerobic digesters treating food wastewater

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Abstract−An acidogenic reactor with a 0.5-L working volume and a methanogenic digester with a 5-L of working volume were operated for 150 days on a continuous mode to investigate the structure of a microbial community during food wastewater treatment. During the steady state of anaerobic digestion, volatile solids (VS) removal efficiency in the pilot plant was approximately 65%. The bacterial population was higher than any other methanogens detected during the entire anaerobic process and treatment of raw food wastewater. *Methanomicrobiales* (MMB), *Methanosarcinales* (MSL), and *Methanobacteriales* (MBT) were detected during digestion. The methanogenic population present in the acidogenic reactor was directly affected by the archaeal community in raw food wastewater. However, the shift of microbial community in the methanogenic digester was relatively gradual. The performance of methanogenic digester might be more related to the change of microbial metabolism affected by the physicochemical properties of the input substrate.

Keywords: Food Wastewater, Anaerobic Digestion, *Methanobacteriales*, *Methanomicrobiales*, *Methanosarcinales*

INTRODUCTION

Most Korean food waste is collected separately from other wastes and recycled as feed or compost. During the recycling, food wastewater is leached out, causing serious environmental pollution due to its high organic content [up to 150 g chemical oxygen demand (COD)/L] [1]. In the past, food wastewater was dumped into the ocean, but such dumping has been banned since 2012 and thus an alternative treatment method of food wastewater is warranted.

Anaerobic digestion has been of interest to engineers and scientists as a treatment method for food wastewater based on several advantages, such as minimal sludge production, combustible biogas generation, and negligible release of odors [2]. Anaerobic digestion can be divided into two biochemical steps: acidogenesis and methanogenesis. In the acidogenic process, acidogenic bacteria degrade raw organics into hydrogen or volatile fatty acids (VFAs). In the methanogenic process, these products are then utilized by methanogens to generate methane. The behavior of methanogens has been extensively studied because of their major role in the treatment of organic loads and production of biogas in the rate-limiting step of methanogenesis [3]. They are also a matter of primary concern to engineers due to their unique physiological properties such as low growth rate, high susceptibility to external conditions, and limited substrate utilization range, which make the anaerobic processes more sensitive to the external environments [4].

Previous culture-based molecular approaches have been limited to the examination of the behavior of microbial populations in anaerobic systems [5]. Culture-independent molecular techniques such as 16S rRNA gene sequences have given valuable insight into the understanding of microbial community structure and population without cultivation [6]. Thus, the microbial population was analyzed using quantitative polymerase chain reaction (qPCR) of archaeal 16S rRNA genes.

To date, field operation of anaerobic digestion is performed on the basis of empirical knowledge and existing technology, often resulting in unsuccessful processing or miscalculations in digester volumes [7]. Coupling of microbial information with process performance data and their concerted activity is necessary for the improvement of anaerobic digestion efficiency. Therefore, this study aimed to investigate microbial communities based on their population and then to liaise their profiles with process performance in two-phase anaerobic digesters for the treatment of food wastewater.

MATERIAL AND METHODS

1. Reactor Configuration and Operation

Experiments were conducted using a two-stage anaerobic digestion system. The two-stage anaerobic process consisted of an acidification digester, which is a continuously stirred tank reactor (CSTR) with a working volume of 0.5 L. This was directly connected to other CSTRs with 5-L working volumes for the methanation of the acidogenic effluent. All digesters were equipped with temperature controllers and operated continuously throughout the entire experimental period. The operating temperature was kept at 35±1 °C. Stir-

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‡This paper is dedicated to commemorate Prof. Ji-Won Yang (KAIST) for his retirement.

Table 1. Physicochemical operation parameters of anaerobic digesters treating food wastewater

Parameter	Raw food wastewater	Acidogenic effluent	Methanogenic effluent
pH	4.2±0.1	4.1±0.1	7.5±0.2
COD (g/L)	177.7±2.5	173.2±4.2	50.2±3.3
sCOD (g/L)	45.6±3.3	44.1±2.1	5.6±0.5
TS (g/L)	101.0±1.2	93.2±3.7	35.0±1.1
VS (g/L)	84.0±0.7	80.3±3.3	29.5±1.2
Ethanol (g/L)	9.4±1.1	8.9±0.9	N. D.[a]
Acetate (g/L)	5.5±1.3	5.9±1.0	0.21±0.03
Propionate (g/L)	0.01±0.00	N.D.[a]	N.D.[a]
Butyrate (g/L)	N.D.[a]	N.D.[a]	N.D.[a]
Methane production (Nm^3/kg $VS_{rem.}$)	-	-	0.30±0.05

[a]N.D.: not detected

ring involved using a stirring bar. After the anaerobic sludge, which was collected from a local municipal wastewater treatment plant, was fully seeded in the methanogenic digester, stabilization was performed through sedimentation for a month in batch-mode. As start-up of anaerobic digestion, a stepwise increase in organic loading rate (OLR) of food wastewater feeding was carried out from 0.85-4 g VS/(L·d) in a continuous-run for an additional month, and then lastly, the OLR value was maintained throughout the continuous process for four months. The pH value of the acidification and methanation reactors was not regulated; nevertheless, it was maintained above 7.0 through regular monitoring. Samples were collected at the steady state when the variations in chemical oxygen demand (COD) and the daily biogas production were <5% after three volumetric turnovers operation.

2. Source of Wastewater and Physicochemical Analysis

Food wastewater was obtained from a nearby metropolitan area (Table 1). Physicochemical parameters were periodically analyzed throughout the operation of the reactors. Chemical properties such as COD, soluble chemical oxygen demand (sCOD), and protein concentrations, as well as physical properties including total solids (TS), and volatile solids (VS) were determined according to the procedures in Standard Methods [8]. A gas chromatograph (6890 plus, Agilent, Palo Alto, CA) equipped with an Innowax capillary column and a flame ionization detector was used to determine the concentrations of volatile fatty acids (VFAs). Helium was used as the carrier gas at a flow rate of 2.5 mL/min with a split ratio of 10 : 1. Another identical gas chromatograph with an HP-5 capillary column and a thermal conductivity detector was used to analyze gas composition in the biogas. Helium was the carrier gas at a flow rate of 8 mL/min with a split ratio of 70 : 1. All analyses were duplicated, and the results quoted as mean values. Steady states were assumed when both the effluent COD concentration and the methane production rate did not vary by more than ±5%. Samples taken at each steady state were analyzed to evaluate the process performance.

3. DNA Extraction

Extraction of DNA from anaerobic sludge and bioreactor samples was performed as described elsewhere [9]. Cells from samples were harvested by centrifuging at 14,000 rpm for 5 min, then decanting the supernatant and resuspending the residual pellet in 1 mL of double-distilled water (DDW), centrifuging again in the same manner to ensure maximal removal of the residual medium. The supernatant was carefully removed, and the pellet was resuspended in 200 μL of DDW before DNA extraction. A fully-automated nucleic acid extractor (Magtration System 6GC, PSS Co., Chiba, Japan) employing the magnetic bead technology was used to extract and purify genomic DNA in 100 μL DDW [10]. The automated instrumentation extracts DNA with a consistent efficiency and high purity by eliminating manual preparation steps that may cause cross-contamination [11]. Genomic DNA extracted from the samples was stored at −20 °C until analysis.

4. Real-time PCR Analysis

Quantitative real-time PCR was carried out using the Light-Cycler 1.2 system (Roche, Mannheim, Germany) with four primer and probe sets for the quantification of bacteria and methanogens (Table 2). Most bacteria (BAC) and methanogens in anaerobic bioreactors could be analyzed using these primers and probe sets [3,12]. Consequently, three methanogenic groups - *Methanobacteriales* (MBT), *Methanomicrobialses* (MMB), and *Methanosarcinales* (MSL) - at the order level, whole archaea, and bacteria were monitored with real-time PCR with the corresponding primer and probe sets. Each reaction mixture was prepared by using the LightCycler FastStart DNA master Hybridization Probes kit (Roche) as follows: 9.6 mL of PCR-grade pure water, 2.4 mL of $MgCl_2$ stock solution (final concentration, 4 mM), 1 mL of each primer (final concentration 500 nM), 2 ml of the TaqMan probe (final concentration, 200 nM), 2 mL of the 10X reaction mix solution, and 2 mL of the template DNA. Amplification was performed in a two-step thermal cycling procedure as follows: pre-denaturation for 10 min at 94 °C followed by 40 cycles at 94 °C for 10 s and at 60 °C for 30 s (except 63 °C for MMB-set). All DNA templates were analyzed in duplicate. The standard curves for the primer and probe sets used were constructed as previously described [9], using the representative strains listed in Table 2. The target rRNA gene sequences were amplified from each strain by PCR with the corresponding primer sets (Table 2) and cloned into pGEM-T vectors (Promega, Mannheim, Germany). For each plasmid, a 10-fold serial dilution series ranging from 10^2-10^9 copies/mL was generated and amplified in triplicate using real-time PCR with the corresponding primer and probe sets. The determined threshold cycle (C_T) values were plotted against the logarithm of their initial copy concentrations. The standard curves constructed using different strains for a primer and probe set showed no significant differences in their slopes at a 1% α-level. Thus, the average values of

Table 2. The characteristics of the primer and probe sets for real-time PCR in this study[a]

Name	Function[b]	Target group	Sequence (5′→3′)	Representative strains[c]
BAC338F	F primer	*Methanobacteriales*	ACTCC TACGG GAGGC AG	Universal bacteria[d]
BAC516F	TaqMan		TGCCA GCAGC CGCGG TAATA C	
BAC805R	R primer		GACTA CCAGG GTATC TAATC C	
MBT857F	F primer	*Methanobacteriales*	CGWAG GGAAG CTGTT AAGT	*Methanobacterium thermoautotrophicum*
MBT929F	TaqMan		AGCAC CACAA CGCGT GGA	(DSM1053)
MBT1196R	R primer		TACCG TCGTC CACTC CTT	*Methanobrevibacter arboriphilicus* (DSM 1536)
MMB282F	F primer	*Methanomicrobiales*	ATCGR TACGG GTTGT GGG	*Methanocorpusculum parvum* (DSM 3823)
MMB749F	TaqMan		TYCGA CAGTG AGGRA CGAAA GCTG	*Methanomicrobium mobile* (DSM 1539)
MMB832R	R primer		CACCT AACGC RCATH GTTTA C	*Methanospirillum hungatei* (DSM 864)
MSL812F	F primer	*Methanosarcinales*	GTAAA CGATR YTCGC TAGGT	*Methanosarcina barkeri* (DSM 800)
MSL860F	TaqMan		AGGGA AGCCG TGAAG CGARC C	
MSL1159R	R primer		GGTCC CCACA GWGTA CC	

[a][22]
[b]F primer, Taqman, and R primer indicate forward primer, Taqman probe, and reverse primer, respectively
[c]The number in parentheses indicates culture collection number
[d][3]

slope and intercept for each set were used to quantify methanogenic rRNA genes.

RESULTS AND DISCUSSION

1. Reactor Performance

The performance of the overall anaerobic processes is shown in Table 1. The methanogenic digester was operated along with the stepwise decrease of hydraulic retention time (HRT) from 70 to 15 days for a month and the HRT was kept constant during four turnovers. VS removal efficiency was observed at ca. 65%. Ethanol was not detected in the methanogenic effluent because it is a major intermediate during anaerobic digestion. It can be readily converted to VFAs such as acetate by anaerobic or syntrophic oxidation of methanogens or bacteria [13]. In these anaerobic processes, acetate was the predominant VFAs, and more than 90% of these appear to be reduced in the methanogenic digester because no other VFAs, including ethanol, were detected (Table 1).

2. Quantitative Analysis of the Microbial Community

The methanogenic community dynamics in the anaerobic digesters was analyzed through qPCR. Methanogens mainly detected in field are divided into two groups based on substrates utilized: one is hydrogenotrophs using H_2/CO_2, and the other is aceticlasts using acetate for the methane production [14]. Fig. 1 shows the quantitative profile of microbial populations and each methanogenic fraction in raw food wastewater. As displayed in Fig. 1, the average copy numbers of bacteria and methanogens were approximately 3.4×10^8 and 1.3×10^5 copy/mL, respectively, indicating that bacteria was dominant over archaea by about 1,000 times. However, the copy number of methanogens in this study is relatively low compared to other raw food or swine wastewater [15]. In this study, the methogenic archaea consisted of MMB, MBT belonging to hydrogenotrophs, and MSL, the aceticlastic groups. *Methanococcales* (MCC) was not detected in our system, which was ascribed to the requirement of a high salt concentration for their growth [0.3-9.4% (w/v) NaCl] [16]. Of all these things, MMB and MSL predominated over MBT in the raw food wastewater because MSL had the most widespread substrate availability, and MMB were also tolerant to high salinity levels of up to 1 M NaCl in food wastewater

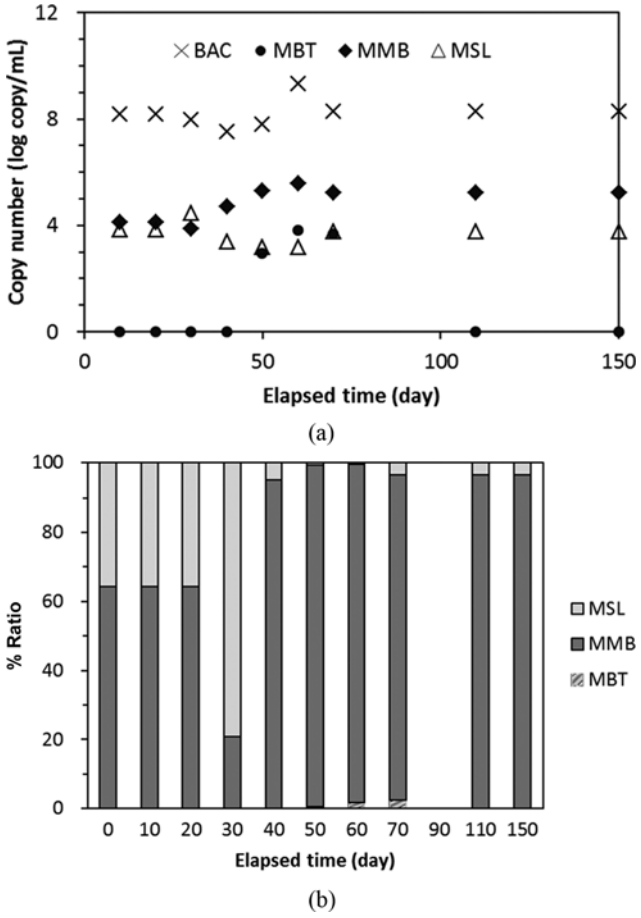

Fig. 1. Quantitative profile of the microbial population (a) and each methanogenic fraction (b) in raw food wastewater.

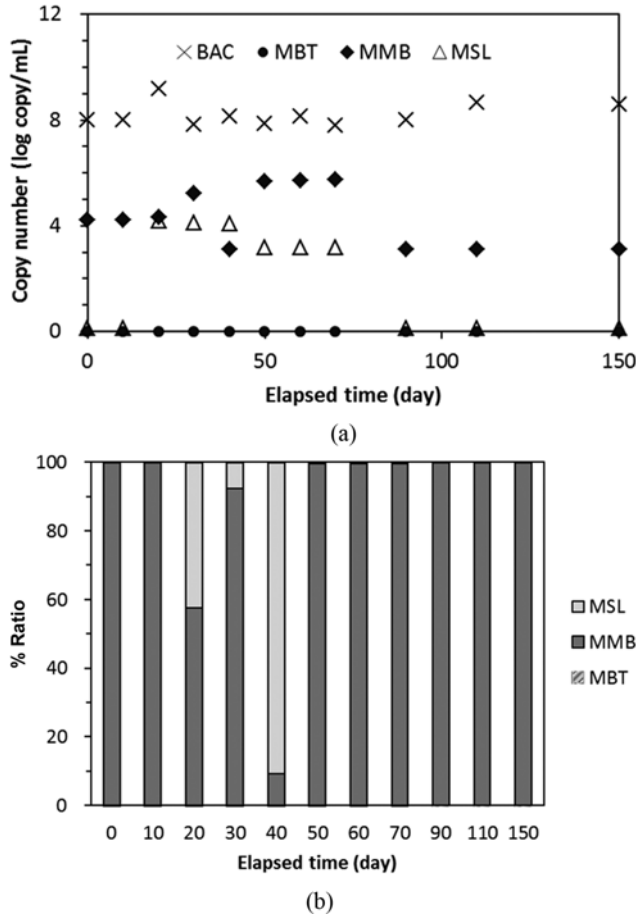

Fig. 2. Quantitative profile of the microbial population (a) and each methanogenic fraction (b) in the acidogenic effluent.

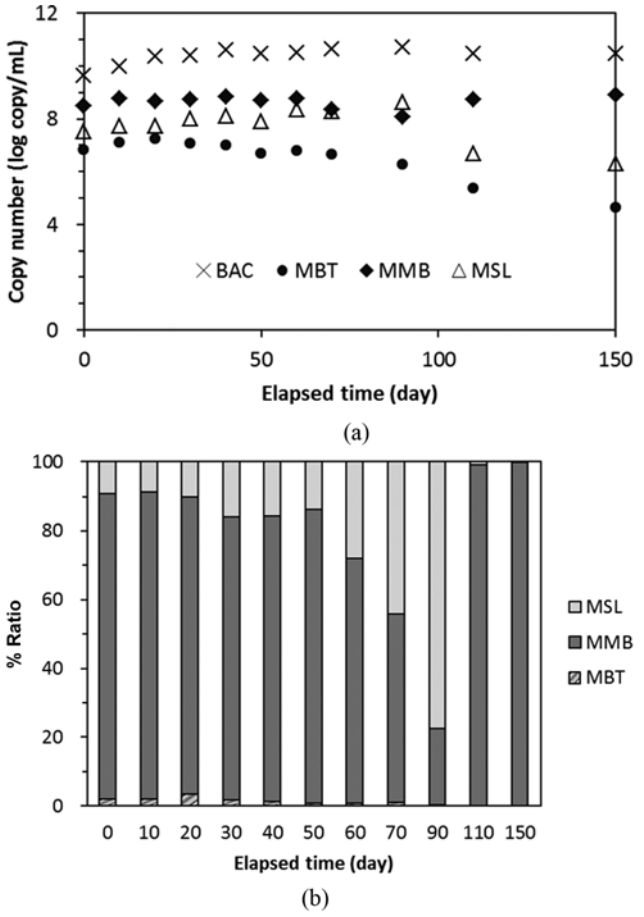

Fig. 3. Quantitative profile of the microbial population (a) and each methanogenic fraction (b) in the methanogenic effluent.

[17]. MBT is almost not detected in the input substrate.

Fig. 2 shows the quantitative profile of the microbial population and each methanogenic fraction in acidogenic effluent. The bacterial population was still dominant throughout the entire acidogenic reaction. MMB was dominant in most acidogenic periods at an average of 1.6×10^5 copies/mL. MBT was almost not observed during the entire acidogenic operation. MSL was not detected until the day 10, but observed with an average of 7.6×10^3 copies/mL from day 20-70. It was also not observed after 90 days of digestion. The bacterial population in the acidogenic bioreactor was larger than that in the raw food wastewater, which was probably caused by bacterial proliferation for hydrolysis and acidogenesis in the reactor. Meanwhile, the methanogenic population was estimated not more than 1.7×10^5 copies/mL, which was calculated from the summation of MMB, MSL, and MBT. These results indicate that methanogenesis almost did not occur in the acidogenic process because the bacterial population was much larger than archaeal one by approximately 1,000 times.

Fig. 3 shows the quantitative profile of the microbial population and each methanogenic fraction in the methanogenic effluent. Bacterial and methanogenic populations in the methanogenic digester were at an average of 2.9×10^{10} and 6.0×10^8 copies/mL, respectively. Compared to the acidogenic reactor, the bacterial population was 100-fold higher, and methanogenic population proliferated 3,000-fold, which suggested that methane production was substantially activated in the methanogenic digester. The bacterial population also increased with methanogenic proliferation in the methanogenic digester because acidogens transfer hydrogens to methanogens syntrophically for the steady production of methane [18]. For the analysis of the methanogenic community population in the methanogenic digester, MBT was almost not detected, showing the maximal coverage fraction no more than 2.4%. This phenomenon is consistent with the predominance in an anaerobic process treating swine wastewater at the HRTs shorter than 10 d [6]. Preference of the short HRT and high temperature more than 45 °C might be the factors hindering the MBT growth in this system [19]. MMB population was kept at an average of 4.8×10^8 copies/mL during the entire methanogenic process, but its dominance gradually decreased from 88.8 to 22.3% after 90 days of digestion. MSL was estimated at 3.2×10^7 copies/mL in the initial stage, and then reached a peak at 4.2×10^8 copies/mL after 90 days, when it predominated over MMB. At this point, MSL occupied more than three quarters of whole archaeal community. However, MSL drastically decreased to 2.0×10^6 copies/mL after 110-150 days.

To summarize, bacteria were the predominant microbes in the entire anaerobic digesters as well as in the raw input substrate. In view of methanogens, their population was smallest in the raw food wastewater, and largest in the methanogenic digester. When MSL

was temporarily dominant in the raw food wastewater at 30th days (Fig. 1), it was also subsequently dominant in the acidogenic reactor at 40 days (Fig. 2), which indicated that the dominant pattern of raw food wastewater could directly affect the acidogenic archaeal dominance pattern. Gradual increase of MSL community in the methanogenic digester until 90 days might be related to the physicochemical characteristics of food wastewater affecting the methanogenic metabolism [20]. The cause for drastic decline of MSL afterwards is not clear, but probably due to the minor interruption of substrate supply for 48 hours after 90 days elapse. MMB finally comprised more than 99% of the whole archaeal population after 110 days of digestion, which was likely attributed to the larger specific growth rate of hydrogenotrophs than that of aceticlasts [21].

CONCLUSIONS

Bacteria and methanogen populations were measured using quantitative PCR analysis based on their 16S rRNA sequences. In both acidogenic and methanogenic digesters, bacteria were dominant. For the methanogenic population, MMB, MSL, and MBT were detected in the system, but MBT was almost not observed. MMB was dominant in the raw food wastewater, acidogenic and methanogenic reactors in most periods. Temporal dominance of MSL in the raw food wastewater directly affected the microbial community in the acidogenic process. MSL increase in the methanogenic digester was relatively gradual.

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ABBREVIATIONS

BAC : most bacteria
COD : chemical oxygen demand
CSTR : continuously stirred tank reactor
C_T : threshold cycle
DGGE : denaturing gradient gel electrophoresis
HRT : hydraulic retention time
MBT : *Methanobacteriales*
MCC : *Methanococcales*
MMB : *Methanomicrobiales*
MSL : *Methanosarcinales*
OLR : organic loading rate
qPCR : quantitative polymerase chain reaction
sCOD : soluble COD
TS : total solids
TVFAs : total volatile fatty acids
VFAs : volatile fatty acids
VS : volatile solids

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