Desalination and Water Treatment
www.deswater.com
doi: 10.5004/dwt.2019.22965

137 (2019) 11–21
January

Sulfate addition for controlling propionate accumulation in a thermophilic anaerobic codigestion system: methane fermentation process and microbial communities

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Received 11 January 2018; Accepted 15 August 2018

ABSTRACT

The variation of microbial community and population with organic loading rate (OLR) increasing before and after sulfate addition was investigated during thermophilic codigestion of coffee grounds, milk wastes, and activated sludge in an AnMBR. Supplement of sulfate at a low concentration of 500 mg/L was effective for overcoming the propionate accumulation which resulted in the inhibition of AnMBR, since it enhanced the activity of microbes which could convert propionate to CH_4 and shortened the lag time of methanogenesis from propionate. The accumulated propionate of 3.8 g-COD/L started to be degraded after about 20 d with sulfate addition, then maintained at low level even the OLR increased to 15.2 g-COD/L/d which was higher than the maximum OLR for stable performance before sulfate addition. Using DGGE and qPCR analyses, the microbial community was found to vary significantly during the three operational stages. *Methanosarcina* was significantly inhibited by propionate accompanied by a shift of methanogenic pathway from aceticlastic to hydrogenotrophic, but it became dominant rapidly after sulfate adding. The bacterial community was significantly affected by sulfate and the typical bacteria appeared after sulfate addition play an important role in effective degradation of propionate and stable performance of AnMBR.

Keywords: Thermophilic codigestion; Coffee grounds; Dewatered activated sludge; Sulfate addition; Propionate degradation; Microbial community

1. Introduction

Anaerobic digestion is thought to be an optimal technology for energy recovery from organic wastes in form of biogas, such as food waste, sewage sludge, and coffee wastes [1–4]. Compared with mesophilic anaerobic digestion (MAD), thermophilic anaerobic digestion (TAD) is more efficient at removing organic substances and eliminating pathogens and so has been widely used for treating high-strength organic waste[5,6]. However, the imbalance between the generation and degradation of volatile fatty acids (VFAs) would be aggravated because of the fast hydrolysis and

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acidogenesis under thermophilic condition, especially at higher organic loading rate (OLR).

Among the VFAs, propionate is an important intermediate in anaerobic digestion: approximately 30% of the electrons flow through propionic acid for the production of methane [7], it accumulates easily when a perturbation has occurred in anaerobic digesters, but degrades more slowly than other VFAs and the AD system takes longer to stabilize [8]. In addition, it was found that propionic acid accumulates to higher levels during TAD than MAD, especially at higher OLRs [9], and the concentration of propionate-oxidizing bacteria was consistently greater in the mesophilic than in the thermophilic digestion systems [10]. Therefore, syntrophic propionate degradation significantly limits TAD. As known that the oxidation of propionate by acetogens is thermodynamically favorable, it could occur with the cooperation of H_2-utilizing microbe such as methanogens and sulfate-reducing bacteria (SRB) only if the hydrogen partial pressure is kept within a rather low range, between 10^{-6} and 10^{-4} atm [11]. Compared with H_2-utilizing methanogens, SRB exhibit a considerable ecological advantage in anaerobic digestion systems where sulfate is continuously or intermittently available [12,13], since: (1) the degradation of propionate could be accelerated significantly in the presence of sulfate because of the cooperation between SRB and aceticlastic methanogens [14]; (2) certain SRB could oxidize propionate with sulfate reduction if sulfate was available, or act as syntrophic acetogens to degrade propionate syntrophically with H_2-utilizing methanogens if sulfate was not available [13]. It should be noticed that if the ratio of COD/SO_4^{2-} is higher than 10, the methanogenic treatment will not be inhibited [15]. Low concentrations of sulfate and sulfide were required for anaerobic digestion [16]. Therefore, adding sulfate with a higher ratio of COD/SO_4^{2-} could probably overcome the accumulation of propionate without decline of methane yield during the anaerobic digestion.

The effective degradation of propionate not only based on activity of syntrophic partners but also relate to the population of these crucial microbes. To understand the complex interactions between the microorganisms involved in AD, especially those associated with propionate degradation before and after sulfate addition, the analysis of microbial community structures, population and activity are very important. This analysis can help to identify the consortium of dominant microorganism in AD and to reveal the mechanism of how organic substances degrade and the effects of environmental change on microbial succession and activity. Many studies have focused on the microbial community of AD for organic waste treatment [6,17], but the effect of sulfate with a higher ratio of COD/SO_4^{2-} on microbial communities and activity in coffee waste codigestion has not been reported. Meanwhile, the changes of microbial community, population, and activity in different stages of reactor involving stable stage, the stage inhibited by propionate accumulation and stable stage after recovering from inhibition by sulfate adding are also should be analyzed for further understanding.

In this study, the reactor performance and propionate degradation during the stage with and without sulfate addition were investigated in a thermophilic Anaerobic membrane reactor (AnMBR) using coffee residues (coffee grounds and coffee liquid), milk waste, and dewatered activated sludge (DAS) as the cosubstrates. Methanogenic activity test was employed to elucidate the reason why propionate did not accumulate after sulfate addition. The changes in the microbial community and population were compared under different conditions to understand the correlation between propionate degradation and microbial action.

2. Materials and methods

2.1. Feedstocks

The cosubstrate used in this study consisted of coffee residues (coffee grounds and coffee liquid), milk waste, and DAS at a ratio of 14.6:16.2:12.2:7.9 (based on wet weight). All the raw materials were provided by Tokyo Gas Co. Ltd., Japan. The mixture was homogenized using a high-speed blender (WARING LBC-15, USA) at 18,500 rpm for 20 min then stored in the substrate tank at 4°C for subsequent use. The elemental compositions of C, H, O, N, and S of the cosubstrate are 52.07%, 6.98%, 37.21%, 3.42%, and 0.32%, respectively. The physicochemical properties of cosubstrate were 69.6 g-TS/L, 65.1 g-VS/L, 100 g-COD/L, and pH 5.18.

2.2. AnMBR reactor

A submerged AnMBR with a working volume of 7 L was used in the present study. A flat sheet microfiltration membrane module made of chlorinated polyethylene was immersed in the AnMBR reactor. The pore size and total area of this membrane (Kubota Membrane Cartridge, Osaka, Japan) were 0.2 μm and 0.116 m^2, respectively. The temperature of the AnMBR reactor was maintained in a range from 55°C to 57°C using a water jacket and a thermostatically controlled water bath. The AnMBR system is shown in Fig. 1 as described by Li et al. [18]. After a successful start-up, the long-term experiment was conducted in three stages: stage I (with no sulfate addition), stage II (inhibition stage), and stage III (with sulfate addition).

2.3. Methanogenic activity test

The methanogenic activity was determined using a 120-mL serum bottle with 50 mL of seed sludge to evaluate the acetate- and propionate-utilizing kinetics. Sodium acetate and sodium propionate as the sole substrates were mixed with the seed sludge. Two concentrations (1,500 and 3,000 mg-COD/L) were chosen to investigate the effects of substrate concentration on the methanogenic activity and lag time. Seed sludge was taken from the AnMBR reactor on the 24^{th} day (HRT 30 d), the 103^{th} day (HRT 15 d), and the 122^{th} day (HRT 10 d) after sulfate addition. After the substrate was bottled with seed sludge, nitrogen gas was used to purge the oxygen for 2 min. The bottles were then put into a water bath at a temperature of 55°C. After each bottle had reached the set temperature, the headspace was vented using a syringe to release the pressure caused by the thermal expansion. Biogas production was measured by the amount collected in the syringe. The kinetic of methanogenesis from acetate and propionate was obtained by Gompertz model as described by Isa et al. [19].

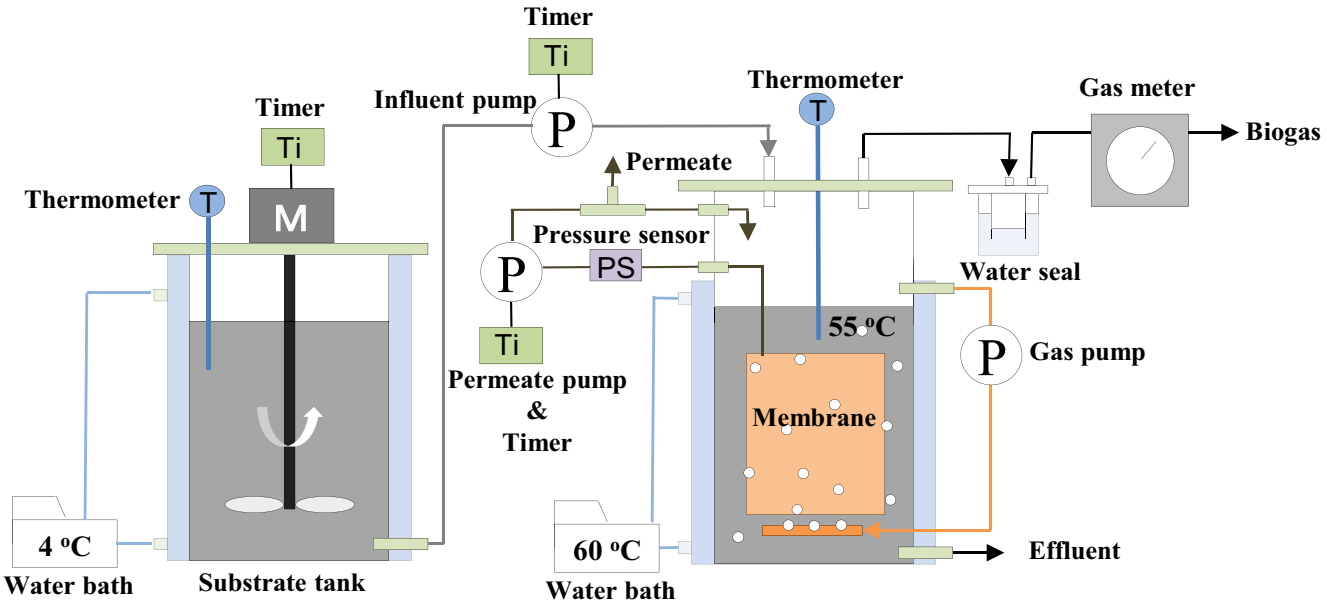

Fig. 1. Thermophilic AnMBR system used in this study.

2.4. Chemical analysis

The daily biogas production was recorded using a wet gas meter, and its composition (CH_4, CO_2, N_2, and H_2) was measured using a Shimadzu GC-8A gas chromatograph (Kyoto, Japan). The pH, COD, TS, VS, VSS, and alkalinity were determined using the Japan Standard Testing Method for Wastewater [20]. VFAs were assayed using an Agilent 6890 gas chromatograph (Santa Clara, CA, USA). SO_4^{2-} was determined by ion chromatography (GC, Agilent 6890).

2.5. Microbial community

2.5.1. DNA extraction

Before DNA extraction, the samples 2 mL sludge samples collected from the AnMBR at stage I (HRT 10), stage II (inhibition stage), and stage III (HRT 30, 15, 10, and 8 d) were centrifuged at 13,000 rpm for 10 min, then the sediment was washed with phosphate-buffered saline twice by resuspension and centrifugation. Then, the DNA was extracted using a PowerSoil® DNA Isolation Kit (MO BIO, USA) following the manufacturer's instructions. The extracted DNA was stored at −20°C until analysis.

2.5.2. Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis

Analyzing the bacterial and archaeal microbial community was performed by targeting the 16S rRNA gene. The bacterial (GC-338F, 805R) and archaeal (GC-787F, 1059R) primers and touch down PCR protocol used in this study are described by Shin et al. [21]. DGGE was performed using a DCode Universal Mutation Detection system (Bio-Rad, USA). The PCR products were run on acrylamide gels (6% w/v) containing a 40%–60% denaturant gradient for 12 h at 70 V and 60°C in 1 × TAE buffer. The gel was then stained with Gel Red for 30 min then photographed using an ultraviolet transilluminator.

All selected bands were excised directly and washed twice using sterilized water, then eluted with 50 µL sterilized water at 4°C for 24 h. The eluted DNA was amplified using the bacterial and archaeal primers with no GC clamps. The PCR products were purified and cloned using the pMD19-T vector (TaKaRa Code: D102A, Japan) and sequenced by a commercial biotechnological company. These sequences were identified by comparison with the reference database in GenBank using the BLAST program. Neighbor-joining trees were constructed using MEGA 6.

2.5.3. Quantitative polymerase chain reaction (qPCR)

qPCR was conducted using the same primer set with no GC clamp for bacteria and archaea as described earlier. The qPCR mixtures (25 µL) contained 12.5 µL of SYBR Premix Dimer Eraser™ (Takara Bio, Kusatsu, Japan), 1 µL of each primer (10 µmol), 2 µL of DNA, and 8.5 µL of sterilized water. The objective genes were quantified using a 7500 qPCR system (Applied Biosystems, USA) as follows: initial denaturation at 95°C for 30 s, followed by 40 cycles at 95°C for 15 s, annealing at 60°C for 30 s, and 72°C for 30 s. The plasmids of *Escherichia coli* K12 (DSM 1607) and *methanomicrobium mobile* BP (DSM 1539) were used to create a standard curve, the concentration of plasmids was 2.14×10^{10} and 2.60×10^{10} GEC/µL, respectively.

3. Results and discussion

3.1. Effect of sulfate addition on methane fermentation

3.1.1. AnMBR performance

The long-term experiment was divided into three stages (Fig. 2). During stage I, although the biogas production

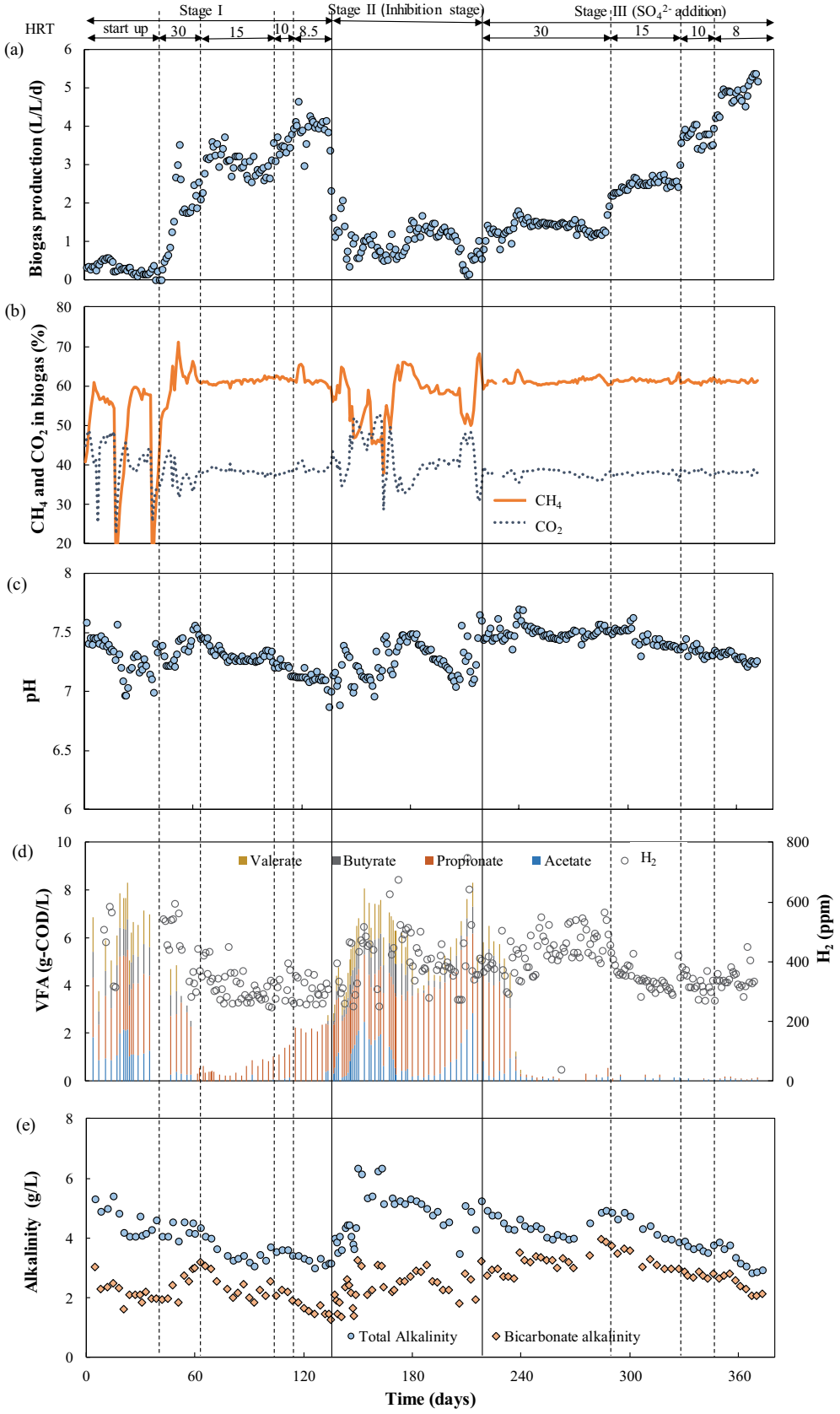

Fig. 2. Performance of the AnMBR reactor during three stages: (a) biogas production; (b) biogas composition; (c) variation in pH; (d) concentration of VFAs and H_2; and (e) variation in alkalinity and ammonium.

increased as the OLR increased from 3.98 to 14.6 g-COD/L d by shortening HRT from 30 to 8.5 d, the methane yield decreased from 0.23 to 0.17 L/g-COD_{added} (Table 1). Meanwhile, the pH decreased linearly accompanied by the accumulation of VFAs. After 20 d of stable performance at an OLR of 14.6 g-COD/L d, the pH, biogas production, percentage methane in the biogas, and bicarbonate sharply decreased as the concentration of total volatile fatty acids (TVFA) increased to 2,417 mg-COD/L, indicating that the performance of the AnMBR deteriorated because of overloading. As shown in Fig. 2(d), at the end of stage I, propionate accounted for a higher proportion (more than 90%) of the accumulated TVFA. Its concentration increased to 2,237 mg-COD/L while other VFAs were still present at a very low level, indicating propionate is easier to accumulate than other VFAs.

To encourage the system to recover from the inhibitory state during stage II, feeding was stopped or fed at a low OLR intermittently. Meanwhile, $NaHCO_3$ and NH_4HCO_3 were added into the AnMBR to ensure a pH above 6.8. During AD, the ammonium nitrogen (NH_4^+-N) generates from the decomposition of nitrogen-containing organics and combines with CO_2 to form NH_4HCO_3 which is an alkali chemical and can be used to neutralize VFAs. With the accumulation of VFAs, NH_4^+-N was "consumed" excessively resulted in the low buffer capacity of AD system as described by Qiao et al. [22]. Therefore, the extra alkali chemical was needed to maintain the pH at suitable range, but the accumulation of VFAs was not stopped probably due to the damage of microbes. Between the 156th and 185th days, the concentration of acetate declined from 2.48 to 0.23 g-COD/L, with the accumulated butyrate and valerate concentrations also falling from 1.28 to 1.10 g-COD/L and from 1.47 to 0.22 g-COD/L, respectively. However, the concentration of the problematic propionate still increased from 2.82 to 3.44 g-COD/L. When the feeding restarted from the 178th to the 205th day, all the VFAs accumulated rapidly. This indicated that propionate was difficult to degrade compared with the other VFAs as reported by Shin et al. [23], due to the thermodynamically unfavorable property [Eq. (1)]. The high concentration of propionate also hindered the recovery of the AnMBR from the inhibition state after pH and alkalinity adjustment. Therefore, to promote the fast degradation of accumulated propionate should be the crucial strategy for stable performance of AD in this study.

$$C_2H_5COO^- + 3H_2O = CH_3COO^- + HCO_3^- + H^+ + 3H_2 \quad \Delta G^0 = +76.1 \text{ KJ/mol} \tag{1}$$

$$C_2H_5COO^- + 0.75H_2O = CH_3COO^- + 0.25HCO_3^- + 0.25H^+ + 0.75CH_4 \quad \Delta G^0 = -25.6 \text{ KJ/mol} \tag{2}$$

$$C_2H_5COO^- + 0.75SO_4^{2-} = CH_3COO^- + HCO_3^- + 0.75HS^- + 0.25H^+ \quad \Delta G^0 = -37.8 \text{ KJ/mol} \tag{3}$$

As known that, propionate could be degraded under the cooperation between propionate-oxidizing bacteria and H_2-utilizing methanogens only if the H_2 partial pressure is in the low range (10^{-4} and 10^{-6}). Compared with acetogens and H_2-utilizing methanogens, SRB has the thermodynamic advantages [Eqs. (2) and (3)], thus regarded as a promising mechanism for propionate degradation. Therefore, during stage III, Na_2SO_4 was added to the AnMBR at a concentration of 500 mg/L with a COD/SO_4^{2-} ratio of 200 from the 219th to the 254th day (HRT 30 d). A significant drop in the concentration of propionate was observed on 20th days after adding sulfate. Meanwhile, the AnMBR completely recovered from the inhibition stage. From the 255th day, the concentration of SO_4^{2-} was decreased to 300 mg/L, but the AnMBR still performed well and no VFAs accumulated even when the OLR increased to 15.2 g-COD/L d which was the limiting OLR in stage I. The concentration of effluent SO_4^{2-} was around 10–30 mg/L, indicating the sulfate-reducing reaction occurred in this stage.

Table 1
Reactor performance under different conditions

		Stage I (without SO_4^{2-} addition)				Stage III (SO_4^{2-} addition)			
		HRT 30d	HRT 15 d	HRT 10 d	HRT 8.5 d	HRT 30 d	HRT 15 d	HRT 10 d	HRT 8 d
SRT	Days	60	30	20	17	60	30	20	16
Duration	Days	40–62	63–102	103–113	114–134	219–288	289–327	328–345	346–373
OLR	g-COD/L d	3.98	8.17	11.9	14.6	4.06	7.16	11.7	15.2
Biogas production	L/L d	1.51 ± 0.52	2.96 ± 0.33	3.49 ± 0.14	3.91 ± 0.27	1.39 ± 0.15	2.52 ± 0.16	3.76 ± 0.20	4.68 ± 0.42
CH_4 in biogas	%	61.5 ± 1.05	61.0 ± 0.84	61.9 ± 0.42	61.8 ± 1.59	61.2 ± 0.92	61.3 ± 0.45	61.1 ± 0.65	61.2 ± 0.34
CO_2 in biogas	%	37.5 ± 1.47	38.4 ± 0.64	37.6 ± 0.48	38.9 ± 0.99	37.8 ± 0.97	37.8 ± 0.37	38.1 ± 0.67	38.0 ± 0.35
CH_4 yield	L/g-COD added	0.23 ± 0.08	0.22 ± 0.02	0.18 ± 0.01	0.17 ± 0.01	0.20 ± 0.02	0.21 ± 0.01	0.20 ± 0.01	0.19 ± 0.02
pH		7.36 ± 0.11	7.32 ± 0.07	7.24 ± 0.04	7.12 ± 0.03	7.50 ± 0.06	7.46 ± 0.07	7.35 ± 0.05	7.29 ± 0.04
Effluent TVFA	mg-COD/L	–	482 ± 220	1,173 ± 207	2,134 ± 279	148 ± 101	177 ± 150	107 ± 57	130 ± 47
Effluent HPr	mg-COD/L	–	468 ± 183	1,093 ± 189	2,070 ± 265	73.6 ± 72.1	87.1 ± 69.3	75.3 ± 28.9	64.9 ± 36.2
Alkalinity	g-$CaCO_3$/L	4.28 ± 0.27	3.54 ± 0.39	3.58 ± 0.03	3.24 ± 0.14	4.40 ± 0.32	4.37 ± 0.37	3.67 ± 0.14	3.36 ± 0.41
Bicarbonate alkalinity	g-$CaCO_3$/L	2.37 ± 0.46	2.41 ± 0.43	2.16 ± 0.10	1.63 ± 0.19	3.18 ± 0.36	3.27 ± 0.31	2.75 ± 0.09	2.48 ± 0.30

Comparing the COD mass balances during stage I (with no sulfate addition) with stage III (with sulfate addition) (Table 2), the methane conversion efficiency declined rapidly from 60.5% to 48.1% when the OLR increased beyond 8.17 g-COD/L d with no sulfate addition, resulted by the significant accumulation of propionate which not only decreased the methane conversion rate but also inhibited activity of methanoges. However, methane conversion efficiency remained at around 58% with sulfate addition even if the OLR increased to 15.2 g-COD/L d. It indicated that adding sulfate was an effective method for overcoming the accumulation of propionic acid and stabilizing the performance of the AnMBR under a higher OLR, and it was coincident with the result that propionate degradation could be strongly accelerated by the presence of sulfate [24].

3.1.2. Methanogenic activity from acetate and propionate

To understand the effect of sulfate on the degradation activity of propionate, the methanogenic activity test was conducted using acetate and propionate as substrates. It was clear that acetate was easily degraded with no lag time and its methanogenic activity was significantly higher than propionate (Fig. 3), similar to other findings [23,25].

Table 2
Effect of sulfate addition on COD mass balance during stages I and III

	OLR (g-COD/L d)	Methane (%)	Sludge (%)	Permeate (%)
Stage I	3.98	63.3	30.9	5.88
	8.17	60.5	34.8	4.67
	11.9	53.6	40.8	5.67
	14.6	48.1	45.8	6.06
Stage III	4.06	59.2	34.7	6.09
	7.16	58.6	35.9	5.56
	11.7	57.4	37.9	4.69
	15.2	58.7	37.1	4.18

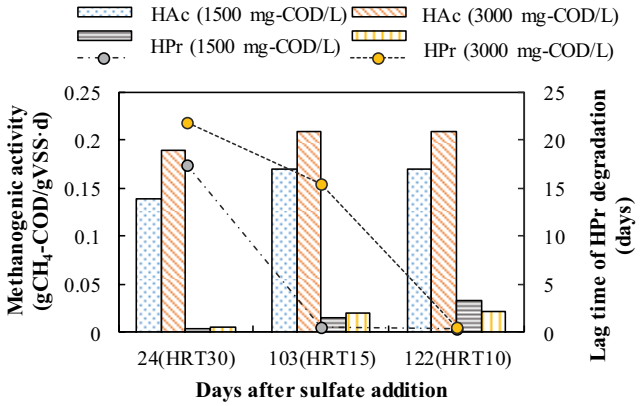

Fig. 3. Methanogenic activity and the lag time of acetate and propionate degradation during stage III (sulfate addition) at various acclimation periods.

Regarding acetate, after 122 d of acclimation with sulfate, its methanogenic activity only increased from 0.14 and 0.19 gCH_4-COD/gVSS d to 0.17 and 0.21 gCH_4-COD/gVSS d at lower (1,500 mg-COD/L) and higher (3,000 mg-COD/L) concentrations, respectively. For propionate, on the 24th day (HRT 30 d) after sulfate addition, the methanogenic activity was less than 0.005 gCH_4-COD/gVSS d. The lag times for methane generation from propionate were 17.5 and 21.9 d at concentrations of 1,500 and 3,000 mg-COD/L, respectively. After 103 d of acclimation (HRT 15 d), the methanogenic activity increased by a factor of approximately 4, while the lag time for methanogenesis from propionate decreased to 0.532 and 15.5 d for lower (1,500 mg-COD/L) and higher (3,000 mg-COD/L) concentrations, respectively. This probably indicates that propionate-oxidizing bacteria gradually adapt to a sulfate environment. After 122 d of acclimation with sulfate, the methanogenic activity from propionate increased to over 0.02 gCH_4-COD/gVSS d, and the lag time decreased to less than 0.6 d, indicating that the propionate-oxidizing microorganisms adapted well to the AnMBR conditions. Comparing methanogenic activity using acetate and propionate as substrates, it was clear that the lower efficiency of acetogenesis of propionate might be the main reason for it to accumulate.

After sulfate addition, the propionate accumulation was overcome completely even though the OLR had risen to 15.2 g-COD/L d. This was probably because of the adaption of microorganisms, which could metabolize propionate alone or with their syntrophic partner in the sulfate environment. Therefore, investigating the microbial community is very important for understanding microbial actions during propionate degradation.

3.2. Variation in microbial community at different stages

3.2.1. Succession of microbial community

Microbial diversity and community succession were revealed using PCR-DGGE and subsequent phylogenetic identification. In Figs. 4 and 5, the archaeal and bacterial communities showed significant difference in different stages, and were probably affected by the accumulated propionate which resulted in the deterioration of AnMBR.

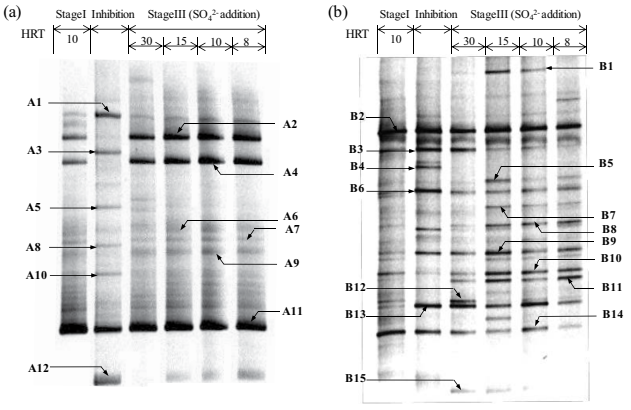

Fig. 4. DGGE profile of the (a) archaeal and (b) bacterial community during stages I, II, and III at various HRTs.

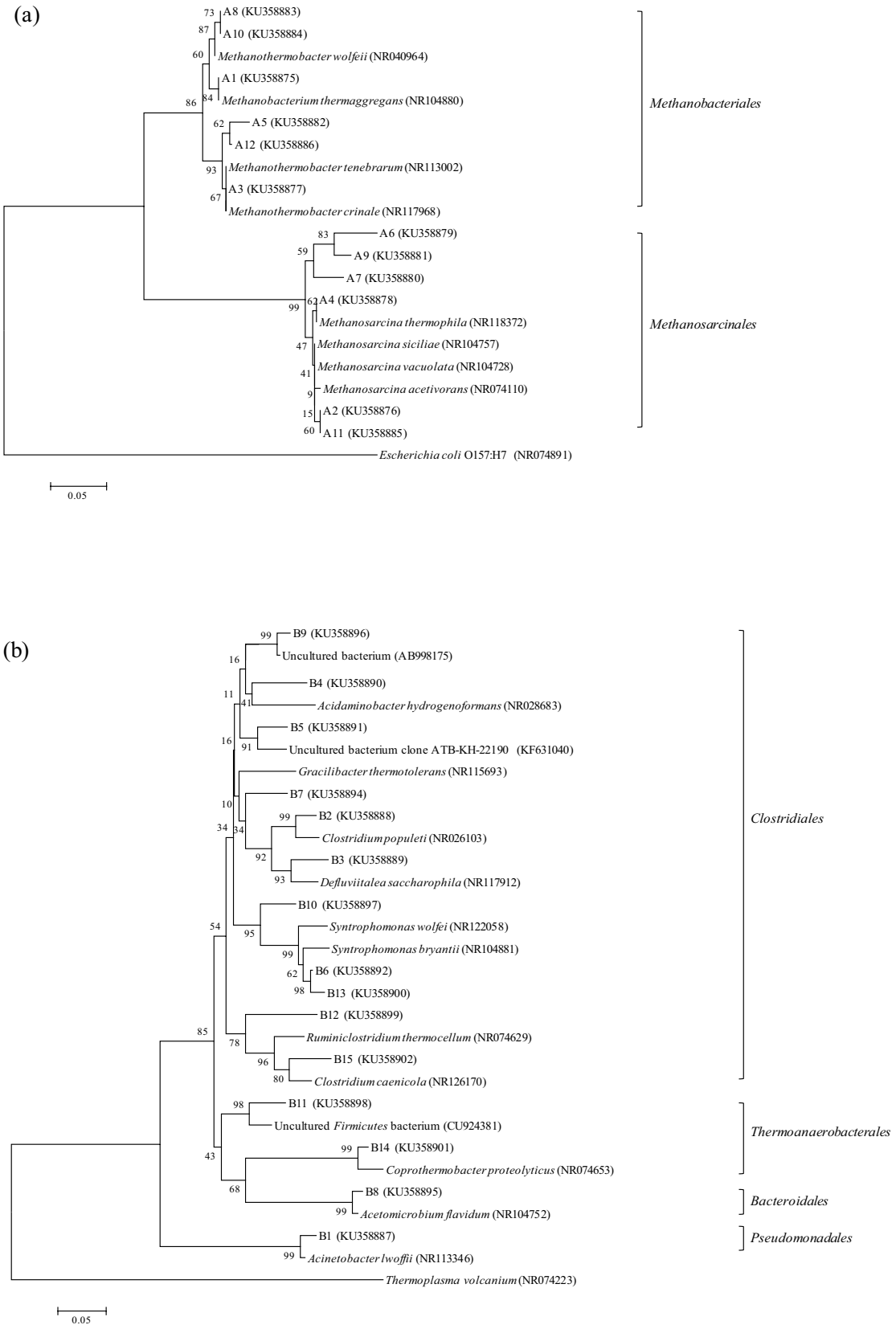

Fig. 5. Neighbor-joining tree presenting the (a) archaeal and (b) bacterial phylogenetic affinity to the DGGE band sequences.

The redundancy analysis revealed that the archaeal and bacterial communities during the inhibition stage were very different compared with those in the stable states during stages I and III, with the microbial community tending to become stable after acclimation to sulfate addition (Fig. 6). Meanwhile, all the typical environmental factors showed significant effect on the succession of microbial community, except pH.

In Fig. 4(a), bands A2, A4, and A11, appearing during the stable state of stages I and III, exhibited strong signals in the DGGE profile, indicating that the archaea related to these bands were dominant. As shown in Table 3 and Fig. 5, these band sequences are all aceticlastic strains of *Methanosarcina*, they can use acetate as electron acceptors, indicating that the aceticlastic methanogenic pathway dominated the methane production [26]. Therefore, acetate did not accumulate during the stable state of stages I and III. Significant changes in the archaeal communities occurred when the AnMBR deteriorated because of propionate accumulation: bands A2 and A4 with strong signals during the stable state disappeared, accompanied by the appearance of bands A1, A3, A5, A8, and A10. These newly appearing band sequences were closely related to *Methanobacterium* and *Methanothermobacter*, which are obligate autotrophs and grow in H_2/CO_2, indicating that the methanogenic pathway shifted from aceticlastic to hydrogenotrophic resulted by the accumulation of propionate. The survival of these two hydrogenotrophic methanogens probably due to the high tolerance to propionate compared with *Methanosarcina* which almost disappeared in inhibition stage thus lead to the accumulation of acetate.

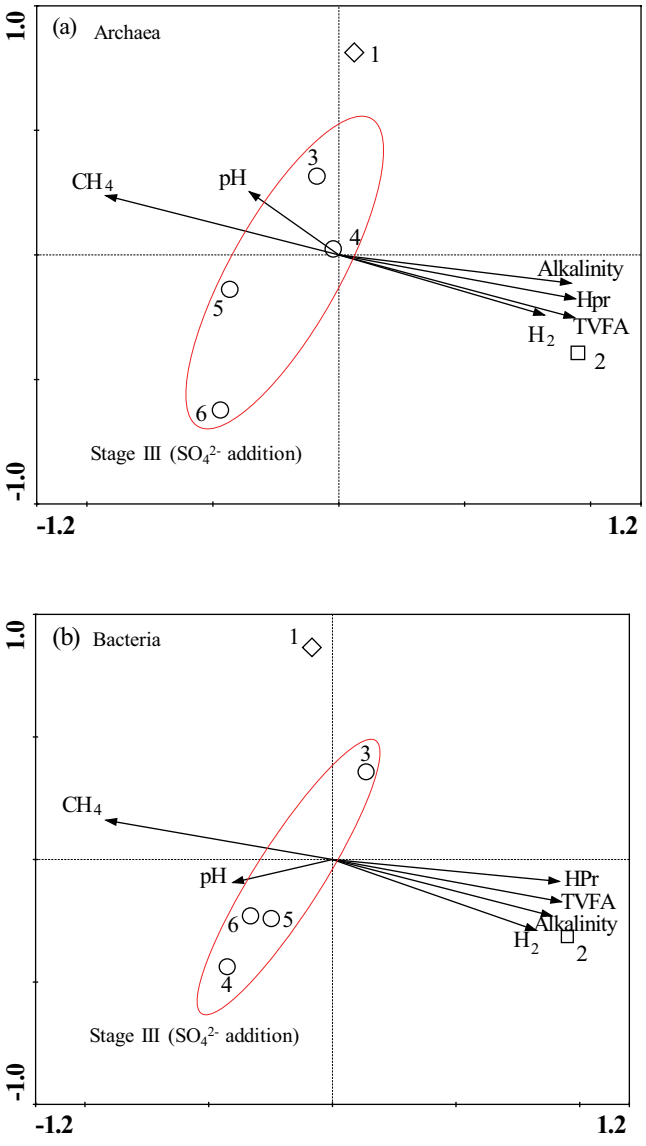

Fig. 6. Redundant analysis of the datasets of (a) archaeal and (b) bacterial communities and environmental factors. Empty circles, diamonds, and squares indicate the microbial community at stages I, II, and III, respectively; numbers beside the symbols indicate the order of DGGE lanes.

The variation in bacterial profiles was shown in Fig. 4(b). Phylogenetic identification revealed four orders: *clostridiales*, *thermoanaerobacterales*, *bacteroidales*, and *pseudomonadales*, but *bacteroidales* and *pseudomonadales* only existed in stage III after sulfate was added. Of the bands excised, B2, B3, B6, B9, B13, and B14 appeared in all samples. B2 linked to *Clostridium populeti*, which can achieve higher H_2 production from cellulose [27], showed an intense signal under all conditions. This might be a reason for the higher level of H_2 production even under the stable state (Fig. 2(d)). Bands B6 and B13, belonging to *Syntrophomonas* which was a syntrophic fatty-acid-oxidizing bacteria and could degrade VFAs to acetate and H_2 exhibited strong signals in inhibition stage. The accumulation of butyrate and valerate might stimulate the growth of *Syntrophomonas*, and resulted in the subsequent degradation with the cooperation of H_2-utilizing *Methanobacterium* and *Methanothermobacter*. Band B4, only present during the inhibition stage, is close to *Acidaminobacter hydrogenoformans*, it can produce acetate, propionate as major products in the mixed culture with methanogens, it might aggravate the accumulation of acetate and propionate in inhibition stage [28]. Before adding sulfate, no typical syntrophic propionate-oxidation acetogens could be found in the DGGE bands excised from samples, the lack of syntrophic partners for propionate degradation should be the main reason for the low efficiency of acetogenesis. That was why even if the butyrate and valerate were degraded by the syntrophic partners during stage II, propionate still accumulated to a level of 3.8 g-COD/L (Fig. 2(d)). When the AnMBR recovered from inhibition after adding sulfate, several DGGE bands appeared during stage III, such as B1, B5, B7, B11, and B15. These related bacteria may play an important role to help the AnMBR to overcome perform well after sulfate addition. Band B7 belongs to *Gracilibacter thermotolerans*, which has been isolated from wetland constructed to treat acid sulfate containing wastewater [29]. Band B11 is close to an uncultured bacterium, with the most similar cultured bacterium being *Thermoanaerobacter sulfurophilu*, it is a typical thermophilic SRB able to reduce elemental sulfur to hydrogen sulfide. Band B8 close to *Acetomicrobium flavidum*, a thermophilic acetogen, existed during the stable stage with no propionate accumulation. Its signal became stronger accompanied by an increase in propionate methanogenic activity after sulfate addition. Although there was no direct evidence to demonstrate that these bacteria could metabolize propionate effectively in syntrophic or direct pathway,

Table 3
Archaea and bacteria identification using bands excised from DGGE gels

Band name	Affiliation	Identity	Order
Archaea			
A1	*Methanobacterium thermaggregans*	100	*Methanobacteriales*
A2	*Methanosarcina acetivoran*s	99	*Methanosarcinales*
A3	*Methanothermobacter crinale*	99	*Methanobacteriales*
A4	*Methanosarcina thermophila*	100	*Methanosarcinales*
A5	Methanothermobacter tenebrarum	99	*Methanobacteriale*s
A6	*Methanosarcina siciliae*	99	*Methanosarcinales*
A7	*Methanosarcina siciliae*	99	*Methanosarcinales*
A8	*Methanothermobacter wolfeii*	100	*Methanobacteriales*
A9	*Methanosarcina siciliae*	98	*Methanosarcinales*
A10	*Methanothermobacter wolfeii*	100	*Methanobacteriales*
A11	*Methanosarcina acetivoran*s	100	*Methanosarcinales*
A12	*Methanothermobacter tenebrarum*	99	*Methanobacteriales*
Bacteria			
B1	*Acinetobacter lw*offii	98	*Pseudomonadale*s
B2	*Clostridium pop*uleti	96	*Clostridiale*s
B3	*Defluviitalea saccharop*hila	94	*Clostridiales*
B4	*Acidaminobacter hydrogenoforma*ns	91	*Clostridiales*
B5	Uncultured bacterium clone ATB-KH-22190	94	*Clostridiales*
B6	*Syntrophomonas brya*ntii	94	*Clostridiales*
B7	*Gracilibacter thermo*tolerans	91	*Clostridiales*
B8	*Acetomicrobium flav*idum	99	*Bacteroidales*
B9	Uncultured bacterium	98	*Firmicutes*
B10	*Syntrophomonas wolfei*	90	*Clostridiales*
B11	Uncultured *Firmicutes* bacterium	96	*Thermoanaerobacterales*
B12	*Ruminiclostridium thermocell*um	89	*Clostridiales*
B13	*Syntrophomonas bryantii*	93	*Clostridiales*
B14	*Coprothermobacter proteolyt*icus	97	*Thermoanaerobacterales*
B15	*Clostridium caenicola*	94	*Clostridiales*

the relationship between their population and the efficiency of propionate degradation indicated that they may play an important role in propionate degradation after adding sulfate. This may have ensured the stable performance at an OLR of 15.2 g-COD/L d, which was the threshold for AnMBR failure without sulfate addition.

3.2.2. Microbial quantitative analysis by qPCR

The quantitative analysis for different operational conditions of the bacterial and archaeal population was assessed by qPCR. It was found that archaea were more sensitive to environmental change than bacteria. During stage II, when the concentration of propionate rose above 3 g-COD/L, the population of archaea decreased from 1.87×10^8 to 0.26×10^8 copies/mg. Although the dominant archaea shifted from *Methanosarcinales* to *Methanobacteriales*, which can use H_2 for CH_4 generation, the lower population of archaea could not lower the H_2 concentration effectively as shown in Fig. 1(d). During stage III, after the propionate was completely degraded, the inhibition on *Methanosarcinales* was eased and it became dominant again accompanied with the increase of archaeal population. The population of archaea was maintained around 2.16×10^8 copies/mg at different OLR of stage III. In contrast, the bacterial population remained within the range between 2×10^9 and 3×10^9 copies/mg during each stage even the AD system had deteriorated. Compared with the change of population of archaea and bacteria, it was clear that methanogens was very sensitive to environmental change and would be inhibited when the system deteriorated especially for aceticlastic methanogens. In AnMBR, even the HRT was shortened to 8 d, the SRT was still maintained at 16 days which was sufficient for the growth of methanogens, that should be the main reason for keeping the microbial population at high level, but it did not work for the loss of methanogens caused by inhibition.

3.2.3. Relationship between microbial characterization and propionate degradation

Regarding the AnMBR performance and microbial characteristics before and after sulfate addition, a possible reason for propionate accumulation and degradation needs to be provided. The Gibbs free energy for the oxidation of

propionate to acetate is positive (+76.1 kJ/mol) [Eq. (1)]. This process can occur with the cooperation of syntrophic hydrogen-using bacteria and acetogens, only when the hydrogen partial pressure is low enough [11]. Microbial community analysis showed that no typical syntrophic propionate-oxidation acetogen could be found in the DGGE bands excised from samples before sulfate was added. This might be the main reason for the long lag time and low efficiency of methanogenesis of propionate. With increasing OLR, more propionate was produced during substrate degradation so that the imbalance between its production and degradation resulted in a large accumulation and thus deterioration in the AnMBR. When the AnMBR was inhibited, the dominant archaea shifted from *Methanosarcina* to *Methanobacterium* and *Methanothermobacter*. The significant decrease in total archaea may have caused the increase in H_2 concentration during stage II and made it difficult to lower the hydrogen partial pressure below the theoretical level during the inhibition stage.

To enhance the propionate degradation, sulfate was added to the TAD system. The effects of adding sulfate on propionate degradation involved two aspects: first, the hydrogen-using SRB could lower the hydrogen partial pressure and consequently promote the syntrophic reaction; and second, certain propionate-oxidizing SRB could degrade propionate directly. During this stage, a typical thermophilic acetogen *Acetomicrobium flavidum*, a typical SRB *Thermoanaerobacter sulfurophilus*, and a bacterium *Gracilibacter thermotolerans* isolated from sulfate-containing wastewater-treated wetland, exhibited strong signals during the second phase of stage III, which corresponded with the increase in propionate methanogenic activity. Although there was no direct evidence to demonstrate that these bacteria could metabolize propionate, the relationship between their population and the efficiency of propionate degradation indicated that they may play an important role in propionate degradation during stage III. Meanwhile, *Methanosarcina* recovered from the inhibition stage and became the dominant archaea again so no acetate accumulated. This may have ensured the stable performance at an OLR of 15.2 g-COD/L d, which was the threshold for AnMBR failure without sulfate addition.

4. Conclusions

Adding sulfate helped the thermophilic codigestion of coffee grounds, milk wastes, and activated sludge to overcome the accumulation of propionate in AnMBR, and achieve a stable and efficient performance at OLR of 15.2 g-COD/L d which was the threshold for AnMBR failure without sulfate addition. The lack of syntrophic partners should be the main reason for the low efficiency of acetogenesis of propionate resulting in its accumulation. The toxicity of propionate on *Methanosarcina* resulted in the sharp decrease of archaeal population and the shift of methanogenic pathway from aceticlastic to hydrogenotrophic. Compared with archaea, the community of bacteria was significantly affected by sulfate adding, some typical bacteria which may relate to propionate degradation were detected with strong signal, such as *A. flavidum* and *T. sulfurophilus*. These bacteria corresponded with the increase in propionate methanogenic activity probably play an important role in propionate degradation.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Grant No. 51608430), the Scientific Research Program Funded by the Shaanxi Provincial Education Department (Grant No. 17JS077), and the Natural Science Foundation for Young Scientists of Xi'an University of Architecture and Technology, China (Grant No. QN1615).

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