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Stable performance and microbial community dynamics under low HRT conditions in a propionate-fed UASB reactor

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ABSTRACT

The propionate removal was achieved above 94.1% at hydraulic retention time (HRT) of 10–4 h in a propionate-fed upflow anaerobic sludge blanket. The microbial community characteristics under different HRT conditions were investigated by 454 pyrosequencing. The result showed that the microbial diversity showed a decreasing trend as the HRT decreases. When the HRT was lowered to 4 h from 10 h by stepwise, the dominant propionate-oxidizing bacteria were rather stable in composition and comprised relatives of *Syntrophobacter*. Their relative abundance was increased to 18.5% (HRT 6 h) and 23.0% (HRT 4 h) from 9.2% (HRT 10 h). Simultaneously, low abundance (0.1%–1.2%) of *Smithella* was observed in this system and its portion also was increased with HRT decreases. In addition, some syntrophic fatty acid-oxidizing bacteria (*Syntrophomonas* and *Syntrophus*) and fermentative acidogenic bacteria (*Petrimonas*, *Kosmotoga* and *Aminiphilus*) could be detected in three samples. Methanogens from four genera (*Methanosaeta*, *Methanoculleus*, *Methanospirillum* and *Methanobacterium*) were observed in three detected samples. *Methanosaeta* was the major acetotrophic methanogens with the relative abundance of 3.5%–7.1%. The hydrogenotrophic methanogens were *Methanobacterium* (0.4%) and *Methanoculleus* (0.1%) at HRT 10 h. When the HRT decreased to 6 and 4 h, the predominant hydrogenotrophic methanogens were shifted to *Methanobacterium*/*Methanospirillum* (HRT 6 h) and *Methanospirillum* (HRT 4 h).

Keywords: Upflow anaerobic sludge blanket; Hydraulic retention time; Pyrosequencing; Propionate-oxidizing bacteria; Methanogens

1. Introduction

Anaerobic digestion technology is widely used for treating all kinds of organic wastes including municipal sewage, organic wastewater, animal waste and agricultural wastes, and simultaneously generates methane as an energy source [1–3]. This process involves four steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis. When the system is shocked by temperature fluctuation, toxic substances or high organic load rate (OLR), aforementioned four steps would become unbalanced, which in turn leads to the accumulation of volatile fatty acids (VFAs) [4,5]. Propionate is a common VFA during the degradation of biomass to biogas. It is mainly derived from odd-numbered fatty acids, which were generated by degrading fat, oil and carbohydrate [6]. Propionate is easily accumulated, due to propionate oxidation is highly endergonic (ΔG° = +76.1 kJ/mol under standard conditions) [4,7]. Its accumulation would decrease the system pH and then lead to process failure and instability [8]. In addition, high concentration

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of propionate may inhibit the activity of propionate-oxidizing bacteria and methanogens, even if pH is maintained near neutral [9]. Therefore, propionate degradation is considered as a rate-limiting step during the anaerobic digestion [10].

Under methanogenic conditions, propionate is converted by syntrophic cooperation of propionate-oxidizing bacteria and methanogens, which rely on each other for maintaining energy and growth [11]. The syntrophic association between them is completed by interspecies electron (H_2 or formate) transfer [12]. Syntrophic propionate-degrading consortia are extensively present in several anaerobic ecosystems, including bioreactors, freshwater sediments, landfills, etc. [13–15]. Their metabolic level is influenced by various factors, including temperature, hydraulic retention time (HRT), hydrogen partial pressure and micronutrients [8,16–18]. In addition, some studies have investigated propionate degraders in quantitative and qualitative by molecular techniques based on 16S rRNA genes, such as denaturing gradient gel electrophoresis, fluorescence in situ hybridization, quantitative real-time polymerase chain reaction (PCR). For instance, *P. schinkii* was recognized as the dominant propionate-oxidizing bacteria in an upflow anaerobic sludge blanket (UASB) reactor when the temperature was lower than 20°C [19]. Worm et al. [17] reported that *Syntrophobacter* spp. was the dominant propionate-oxidizing bacteria in a propionate-fed UASB reactor. Ariesyady et al. [20] found high proportions of *Smithella* spp. and low proportions of *Syntrophobacter* spp. in anaerobic sludge, but they did not determine *Pelotomaculum* spp. Thus, the species distributions of propionate-oxidizing bacteria had vary in different systems.

Although some researches about propionate degrading community have been done, there was still not much information about the effects of HRT on the diversity and structure of syntrophic propionate-degrading consortia. High throughput sequencing can provide an insight for the diversity and richness of microbial groups at a fine scale and coverage. Therefore, the object of this study is to investigate the shift of syntrophic propionate-degrading consortia as decreasing HRT in a propionate-fed UASB reactor by 454 pyrosequencing. The characterization would focus on determination of propionate-degrading and methanogenic communities in detected samples.

2. Materials and methods

2.1. Bioreactor operation

A previously described UASB reactor was operated under 35°C condition [16]. The inoculated sludge was originally collected from a lab-scale anaerobic baffled reactor dealing with molasses wastewater [21]. A synthetic propionate wastewater (propionate as a sole carbon source) was used as substrate from startup. The basic media used in the reactor was described previously [16]. The propionate concentration in influent was maintained at 2,000 mg/L. The pH of the reactor was remained at around 7.5 by $NaHCO_3$. After a stable performance was achieved, the HRT decreased in four stages, 10, 8, 6 and 4 h.

2.2. Analytical methods

VFAs and methane content were determined by two gas chromatographs (Shandong Lunan Instrument Factory, China) as described in previous study [16]. The biogas volume was measured daily by wet gas meters (Changchun Filter Company, China). The pH and mixed liquor volatile suspended solid (MLVSS) were measured as described in the standard methods [22].

2.3. Nucleic acid extraction

Microbial samples were obtained on 56, 96 and 140 d with HRT of 10, 6 and 4 h, respectively. Anaerobic sludge was collected from the sludge blanket of UASB reactor. After gentle rinses with deionized water, 0.15 g sludge was weighted to extract DNA by a Powersoil DNA Isolation Kit (MO Bio Laboratories, Carlsbad, CA, USA). The DNA concentration was determined by a spectrophotometer (Thermo Fisher Scientific Inc., USA).

2.4. Pyroquencing analysis

16S rRNA gene libraries based on 454 pyrosequencing were constructed using the degenerate primers of bacteria and archaea 341F (5′-CCTACGGGRBGCAGCAG-3′) and 789R (5′-GGACTACMVGGGTATCTA-3′) for the V3–V5 region of the 16S rRNA gene [23]. There is a 10-nucleotide barcode between the Life Sciences primer A and 341F primer. The barcode was used for assorting multitudinous samples in a single 454 GS-FLX run. Raw pyrosequencing data were deposited to the NCBI Sequence Read Archive database with accession numbers SRR3471431, SRR3471435 and SRR3471436. In order to minimize the impact caused by random sequencing errors, we eliminated the low-quality sequences containing any base calls (Ns), eight or more consecutive identical bases, length shorter than 200 nucleotides or longer than 1,000 nucleotides. Pyrosequencing produced 5,318 (HRT 10 h), 3,957 (HRT 6 h) and 7,273 (HRT 4 h) high-quality V3–V5 tags of the 16S rRNA gene.

2.5. Microbial diversity and phylogenetic classification

All effective sequences in each sample were clustered into operational taxonomic units (OTUs) through setting a 97% similarity by the Muthur program [24]. The effective sequences were designated to taxonomic classifications by a ribosomal database project database (http://www.mothur.org/wiki/Silva_reference_files) [25]. The phylogenetic location of the sequences from per sample was performed at phylum, class and genus levels. The relative abundance represented that the sequences of a specified phylogenetic group made up a percentage of all sequences per sample. Rarefaction curves, Shannon diversity index, Simpson diversity indices and species richness estimator of Chao1 were generated in quantitative insights into microbial ecology (QIIME) for each sample. Hierarchical cluster analysis was performed using gplots package of R in Linux. Venn diagram and principal component analysis (PCA) were conducted by QIIME [26].

3. Results and discussion

3.1. Bioreactor performance

UASB process was recognized as one of the most feasible method for treating various pollutants [27,28]. Therefore,

an UASB reactor was used as a carrier for investigating the effect of short HRT on propionate removal. The result showed that the UASB system achieved high propionate removal (96.9%–94.1%) at HRT 10–4 h (Table 1). The specific COD removal rate of anaerobic sludge increased to 1.7 kg COD/(kg VSS d) at HRT 4 h from 1.1 kg COD/(kg VSS d) at HRT 10 h. The specific methane production rate was also improved by 18.1%–69.4% with a maximum of 338.9 L CH_4/(kg $COD_{removed}$ d) at HRT 6 h. Therefore, short HRT is not a limiting factor for propionate degradation in UASB reactor.

3.2. Richness and diversity of microbial phylotypes

High-rate methanogenesis is essential for transforming organic pollutions to methane. In this complex process, syntrophic propionate degraders and methanogens play an indispensable role. Monitoring microbial community dynamics is very helpful for predicting and explaining functional changes in treating propionate under different HRT conditions. In current study, three 16S rRNA gene libraries (named HRT 10 h, HRT 6 h and HRT 4 h) were generated by 454 pyrosequencing. HRT 10h, HRT 6 h and HRT 4 h communities contained 5,318, 3,957 and 7,273 high-quality V3–V5 tags, respectively (Table 2). These effective reads had an average read length of 328 bp. Bacteria were the dominant microbial group in these three samples, accounting for 90.7%–95.3%. The relative abundance of archaea in the anaerobic sludge was 9.3%, 4.2%, 4.7% for HRT 10 h, HRT 6 h and HRT 4 h, respectively. These effective reads were clustered into 226 (HRT 10 h), 198 (HRT 6 h) and 198 (HRT 4 h) OTUs by setting a 97% similarity [24]. It is seemingly suggested from the number of OTUs that the diversity of microbial communities in HRT 10 h is much higher than those in HRT 6 h and HRT 4 h.

As shown in Table 2, Shannon and Simpson diversity indices showed a similar result with observed OTUs. The Shannon diversity index not only shows species richness, but also reveals the abundance of each species in the community [24]. HRT 10 h had the highest diversity (Shannon = 6.80) in these communities. The Shannon index of HRT 6 h (6.21) was larger than 6.08 in HRT 4 h. Pyrosequencing revealed new microbial species continued to appear even after 5,000 reads sampling, although propionate as sole carbon source in this UASB reactor (Fig. 1). However, the Good's sampling coverage was reached 98.8%–99.5%, indicating the sequencing depth has captured a majority of microbial community (Table 2).

3.3. Comparative analysis of microbial communities

In order to comprehend in detail the succession of microbial community structure, the top 20 abundance genera were selected from each sample for hierarchical cluster analysis as suggested previously (Fig. 2) [29]. The result showed that each group was clearly different from the other two groups, even though these three microbial communities contained some same microorganisms. PCA analysis based on OTUs further confirmed that each group was separated from the other two groups, suggesting there is a significant divergence among them (Fig. 3). Principal components 1 and 2 explained 84.7% and 15.3% of the total community variations, respectively. These results showed that the microbial community structure was dramatically influenced by HRT in the range of 10–4 h.

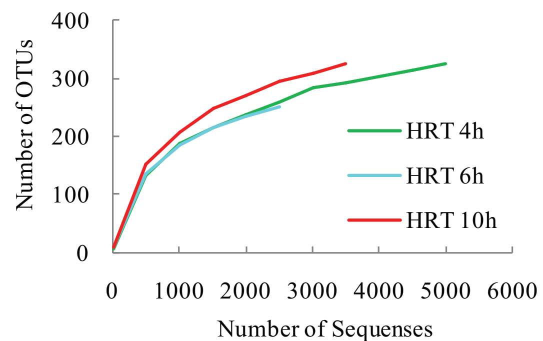

Fig. 1. Rarefaction curves base on pyrosequencing of all samples. The operational taxonomic units (OTUs) were defined by 3% distances.

Table 1
Operational performance of the UASB under different HRT conditions

Stage	Acetate content (mg/L)	Propionate removal (%)	Biomass (gMLVSS/L)	Specific COD removal rate of anaerobic sludge (kg COD/kg VSS·d)	Specific methane production rate (LCH_4/kg $COD_{removed}$/d)
HRT 10 h	66.3 ± 4.9	96.9 ± 2.5	6.1 ± 0.2	1.1 ± 0.04	200.1 ± 31.7
HRT 6 h	66.7 ± 3.9	94.7 ± 3.6	7.7 ± 0.5	1.5 ± 0.05	338.9 ± 17.6
HRT 4 h	58.2 ± 7.5	94.1 ± 1.6	10.5 ± 0.4	1.7 ± 0.06	302.2 ± 36.5

Table 2
Sequence reads, diversity/richness indices, coverage and operational taxonomic units (OTUs) at 97% sequence identity

Sample	Sequence reads		OTUs	Diversity/richness indices			Good's sampling coverage (%)
	Raw reads number	Effective reads number		Shannon diversity index	Simpson diversity index	Chao1 estimator	
HRT 10 h	9,503	5,318	226	6.80	0.98	387.32	99.2
HRT 6 h	6,772	3,957	198	6.21	0.96	296.12	98.6
HRT 4 h	13,618	7,273	198	6.08	0.96	397.73	99.5

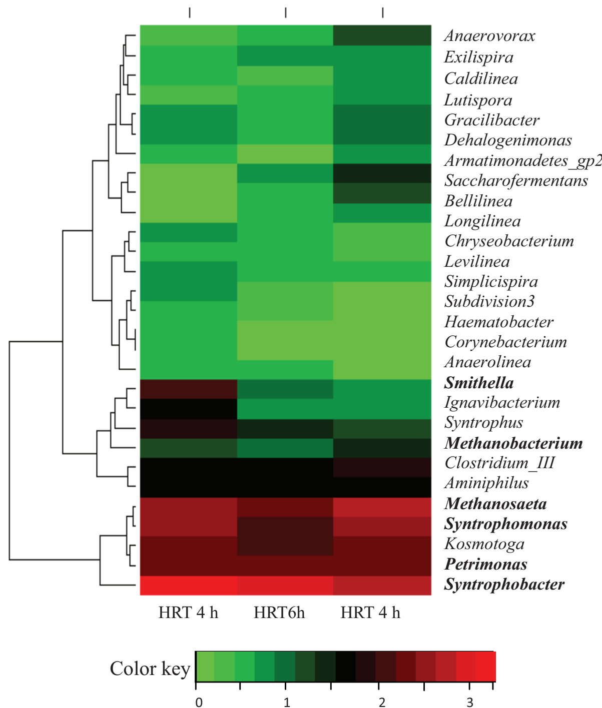

Fig. 2. Heat map of top 20 genera in each sample. The top 20 abundant genera in each sample were selected and compared with their abundances in other samples. The color intensity (log10 transformed) in each panel shows the number of a genus in each sample. Those in bold font are the core genera in different samples.

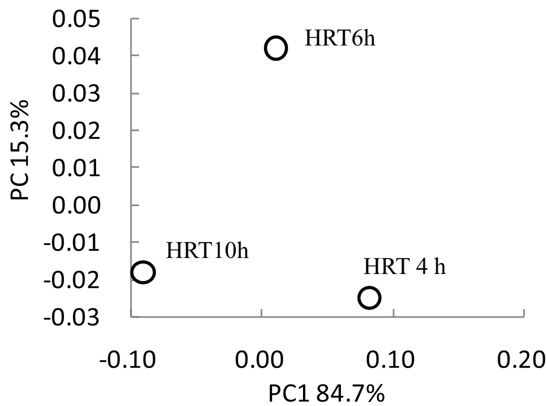

Fig. 3. Principal component analysis (PCA) of microbial communities from HRT 10 h, HRT 6 h and HRT 4 h based on pyrosequencing of 16S rRNA gene.

The total number of clustered OTUs in all three 16S rRNA gene libraries was 290, 125 OTUs (accounting for 43.1% in all detected OTUs) were shared by them (Fig. 4). Most of the shared OTUs (50.4%) were the unclassified bacteria (28.5%) and the family *Syntrophobacteraceae* (21.9%). The family *Syntrophobacteraceae* contains some identified propionate-oxidizing bacteria (the genus *Syntrophobacter*), indicating carbon source strongly shapes the microbial community [30]. HRT 10 h and HRT 6 h shared more OTUs (167) than any of them with HRT 4 h (HRT 10 h/HRT 4 h, 146; HRT 6 h/HRT 4 h, 144). HRT 10 h, HRT 6 h and HRT 4 h contained the number of unique OTUs were 38, 12 and 33, respectively. The sum of these unique OTUs in three communities accounted for 28.6% of total OTUs.

3.4. Microbial taxonomy identification

To evaluate the phylogenetic diversity of these three microbial communities (HRT 10 h, HRT 6 h and HRT 4 h), qualified reads were analysis at phylum, class and genus levels (Fig. 5). There were 10 identified bacterial or archeal phyla were detected in three communities (Fig. 5(a)). Unclassified reads in HRT 10 h, HRT 6 h and HRT 4 h separately accounted for 17.7%, 18.8% and 14.3% of the total reads at the phylum level. Two phyla (*Bacteroidetes* and *Proteobacteria*) reflected clearest difference in distribution between three groups. These two phyla accounted for 34.3% (HRT 10 h), 47.2% (HRT 6 h) and 51.3% (HRT 4 h) of the total reads. So far, all known propionate-oxidizing bacteria were mainly confined to the phyla *Proteobacteria* and *Firmicutes* [11]. In this study, the relative abundance of *Proteobacteria* (15.5%) and *Firmicutes* (18.4%) in HRT 10 h was similar. However, *Proteobacteria* represented the dominant bacterial community in HRT 6 h (30.9%) and HRT 4 h (40.2%). *Synergistetes* is major in wastewater treatment plants. It includes some species that have been identified as sludge degraders in anaerobic digesters [31]. The relative abundance of *Synergistetes* in HRT 10 h was 7.8%, but relatively low in HRT 6 h (4.9%) and HRT 4 h (4.6%). *Thermotogae* is a class of fermentative bacteria and they can degrade complex-carbohydrates for producing hydrogen gas [32]. Their distribution (3.2%–3.6%) was similar in three communities. As a class of universal filamentous bacteria in wastewater treatment processes, *Chloroflexi* are abundant in HRT 10 h [33]. The relative abundance of *Spirochaetes* and *Chlorobi* was less than 1% in each sample. Some *Spirochaetes* populations are involved in syntrophic acetate oxidation in anaerobic digesters [34]. *Euryarchaeota* is the sole archeal phylum in this study. Most identified methanogens belong to the phylum *Euryarchaeota* [35]. *Euryarchaeota* was highest in HRT 10 h with a relative abundance of 7.5%, lower in HRT 6 h (4.2%) and HRT 4 h (4.7%).

Fig. 5(b) shows that the identification of three communities (HRT 10 h, HRT 6 h and HRT 4 h) in class level. The sum of 12 classes was obtained from three communities by pyrosequencing, including nine bacterial classes and three archeal classes. Most of sequences were distributed in nine classes. HRT 10 h is primarily consisted of δ-*Proteobacteria*, *Clostridia*, *Thermotogae*, *Synergistia*, *Anaerolineae*, *Bacteroidia* and *Methanosarcinales*. HRT 6 h and HRT 4 h had a similar microbial community composition and highly enriched in classes of α, δ-*Proteobacteria*, *Clostridia*, *Bacteroidia*, *Thermotogae*, *Synergistia*, *Anaerolineae* and *Methanosarcinales*. The unclassified microbes at the class level increased to 34.4% (HRT 10 h), 31.5% (HRT 6 h) and 24.7% (HRT 4 h) of the total reads.

Depending on the genus enables us to further speculate the microbial functions (Fig. 5(c)). The present study revealed that *Syntrophobacter* was the dominant propionate-oxidizing bacteria during the whole operational period. *Syntrophobacter* spp. degrade propionate by methylmalonyl-coenzyme A pathway in the presence of methanogens [11,36,37].

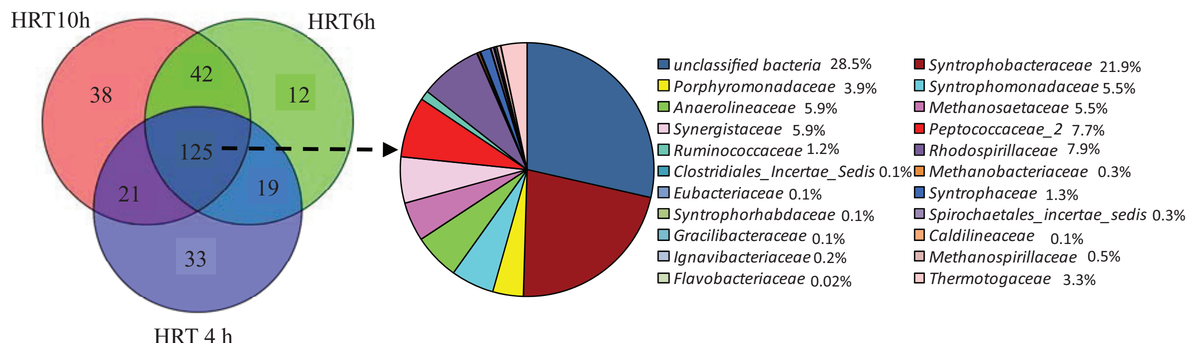

Fig. 4. Overlap of three microbial communities from HRT 10 h, HRT 6 h and HRT 4 h based on OTUs (3% distance), and the taxonomic identities of the shared OTUs at family level. The value in each of the circles represents the corresponding number of OTUs in that community.

The relative abundance of *Syntrophobacter* was 9.2% in HRT 10 h. When the HRT decreased to 6 and 4 h, its relative abundance was significantly raised to 18.5% and 23.0%, respectively. It is related to UASB reactor conditions, which have a highly selective for *Syntrophobacter* spp. [38]. Similarly, some previous studies also indicated that the genus *Syntrophobacter* was the dominant propionate-oxidizing bacteria in the UASB reactors treating sucrose, butyrate–propionate–acetate and propionate process under OLR of 5.0–11.8 kg COD/(m^3 d) conditions [13,17]. However, Ariesyady et al. [20] found that the primary propionate degraders in anaerobic digesters were from the genus *Smithella*. It was found that high dilution rates were favorable to *Pelotomaculum* spp. whereas low dilution rates stimulate *Syntrophobacter* spp. in chemostat experiments containing propionate as the sole carbon source [38].

Simultaneously, we found low abundance of *Smithella* (propionate-oxidizing bacterium) existed in this system. Its proportion was increased to 1.2% (HRT 4 h) from 0.1% (HRT 10 h). *Smithella* contains only an identified species *S. propionica*, which oxidizes propionate to acetate and butyrate via an integration of two molecules of propionate, followed by syntrophic β-oxidation of butyrate to acetate [39]. Butyrate degradation is thermodynamically easier than propionate so that product inhibition is easily eliminated, leading to the promotion of propionate oxidation by *S. propionica* [40]. Indeed, some syntrophic fatty acid-oxidizing bacteria (*Syntrophomonas* and *Syntrophus*) can be observed in this system. They can use C_4–C_8 compounds in co-culture with methanogens or *Desulfovibrio* spp. [40–42]. The dominant syntrophic fatty acid-oxidizing bacteria in all HRTs were *Syntrophomonas* spp. with relative abundance of 3.1%–6.8%. The richness of *Syntrophus* was increased to 1% in HRT 4 h from 0.2% in HRT 10 h as increasing available substrate butyrate, which was produced by *Smithella*.

There are other bacterial microbes that could be detected in all samples (Fig. 5(c)). The fermentative acidogenic bacteria in three samples were from genera *Petrimonas* (1.9%–3.9%), *Kosmotoga* (3.0%–3.4%) and *Aminiphilus* (0.6%–0.9%). Their richness was not significantly changed with HRT decrease. These fermentative acidogenic bacteria can utilize some organic compounds (such as carbohydrates, pyruvate, fumarate and malate) as substrates and acetate was the major end product [43–45]. Pyruvate, fumarate and malate are the intermediate products of propionate degradation by methylmalonyl-coenzyme A pathway, which is possessed by the dominant propionate degrader *Syntrophobacter* spp. in this study [7]. The cellulose/cellobiose-digesting bacteria *Clostridium*_III accounted for 0.7%–1.5% [46]. The relative abundance of *Ignavibacterium* (anaerobic photoautotrophic green sulfur bacteria) was low (0.1%–0.5%) in three samples [47].

Methanogenesis is important for propionate anaerobic oxidation in methanogenic environments [11,48]. The important role of methanogens is to eliminate acetate and H_2/CO_2 from propionate degradation and then promote the reaction process. Methanogens from four genera (*Methanosaeta*, *Methanoculleus*, *Methanospirillum* and *Methanobacterium*) were observed in three detected samples. *Methanosaeta* was the major acetotrophic methanogens in all samples and its relative abundance was 3.5%–7.1%. *Methanosaeta* spp. is a specialist in utilizing acetate [35]. *Methanosaeta* species were previously identified as the dominant acetotrophic methanogens in various anaerobic reactors with low concentration of acetate [49]. They can often promote sludge granulation, which in turn lead to a stable performance [50,51]. The granulation of sludge is favorable for achieving a high rate of methanogenesis with propionate. Because granular provides a close spatial microbial proximity compared with suspended cultures [5,11]. *Methanoculleus*, *Methanospirillum* and *Methanobacterium* are considered as hydrogenotrophic methanogens and they can use H_2/CO_2 and formate as substrate for growth [35]. The activity of hydrogenotrophic methanogens is essential to maintain low hydrogen partial pressure in methane fermentation systems [52]. The syntrophic degradation of propionate requires the critical hydrogen partial pressure (1×10^{-4} atm) [53,54]. *Methanoculleus* spp. was unique in HRT 10 h and the relative abundance was 0.1%. *Methanobacterium* spp. was dominant hydrogenotrophic methanogens in HRT 10 h with the relative abundance of 0.4%. The dominant hydrogenotrophic methanogens was shifted to *Methanospirillum* spp. (1.0%) with HRT increased to 4 h, indicating *Methanospirillum* spp. has a higher specific growth rate. These methanogens allowed the acetate concentration to always be less than 70 mg/L in effluent and the hydrogen content be lower than detection limit of gas chromatograph during the whole operational period. The relative abundance of acetotrophic methanogens in each sample was much higher than hydrogenotrophic methanogens by 1.9–13.2 times, indicating that methane was mainly

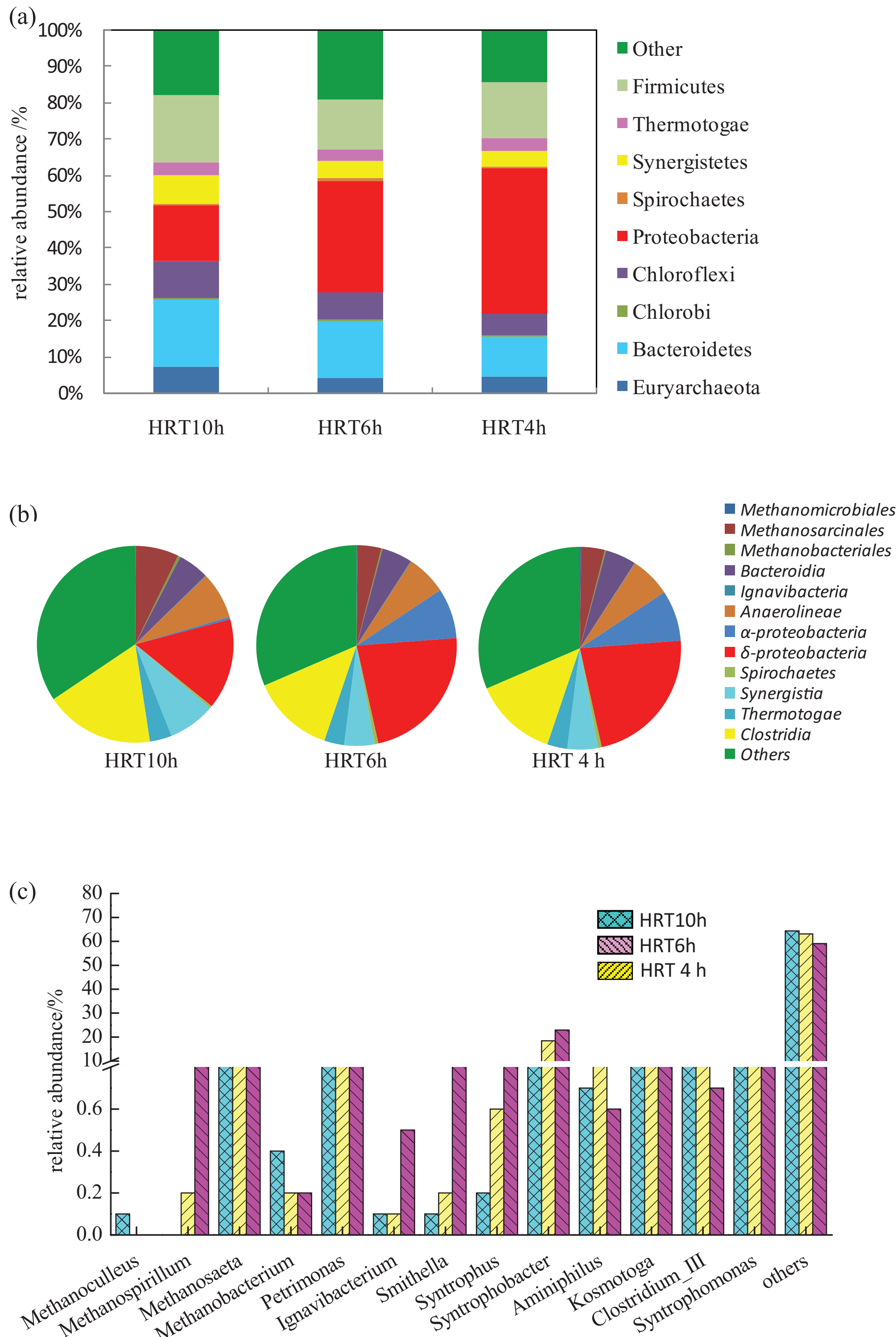

Fig. 5. The relative abundance of microbial communities at the (a) phylum, (b) class, and (c) genus levels in all samples (HRT 10 h, HRT 6 h and HRT 4 h). Taxa represented occurred at >0.5% abundance for bacteria or >0.1% abundance for methanogens in at least one sample. Phyla, classes and genera making up less than 0.5% for bacteria or 0.1% for methanogens of total composition in all three libraries were classified as "other".

produced by acetate cleavage in this UASB reactor. In methanogenic environment, approximately 70%–80% of methane is produced by acetate oxidation [35]. A previous study also showed the amount of acetotrophic methanogens in an UASB reactor was obviously higher than that of hydrogenotrophic methanogens [19].

This study suggested that decreased HRT has not changed the dominant propionate-degrading groups, and high propionate removal at low HRT (6 h and 4 h) conditions is achieved by increasing number of propionate-oxidizing bacteria. Also, the proportion of propionate-oxidizing bacteria was significantly higher than that of methanogens (4.2%–7.6%) during the whole operation. But the hydrogen and acetate were not remarkably accumulated in three stages, suggesting the metabolic potential of propionate-oxidizing bacteria was lower than that of methanogens.

4. Conclusion

In summary, three microbial library (HRT 10 h, HRT 6 h and HRT 4 h) based on 16S rRNA gene were constructed by pyrosequencing. *Syntrophobacter* spp. was considered as the dominant propionate-oxidizing bacteria in all samples. Also, their quantity was significantly increased to 23.0% (HRT 4 h) from 9.2% (HRT 10 h). A small amount of *Smithella* spp. could be detected in this system. Its number was increased to 1.2% (HRT 4 h) from 0.1% (HRT 10 h). The dominant acetotrophic methanogens was *Methanosaeta* spp. with relative abundance of 3.5%–7.1%. The predominant hydrogenotrophic methanogens were *Methanobacterium* (HRT 10 h), *Methanobacterium*/*Methanospirillum* (HRT 6 h) and *Methanospirillum* (HRT 4 h).

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