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Functional bacterial and archaeal dynamics at slightly acidic conditions revealed by pyrosequencing in a propionate-fed UASB reactor

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ABSTRACT

To reveal the response mechanism of syntrophic propionate degrading communities to slightly acidic environments, the performance and microbial community structure at pH 7.0, 6.0 and 5.0 in a propionate-fed upflow anaerobic sludge blanket (UASB) reactor were investigated. The propionate removal and the specific methane production rate were reduced by 33.9%–69.5% and 10.9%–18.9% independently as pH decreased to 5.0 from 7.0. Pyrosequencing based on 16S rRNA gene showed that the microbial diversity in the UASB reactor was increased as pH decreased. *Syntrophobacter* was the dominant propionate-oxidizing bacteria (POB) at pH 7.0 and its relative abundance reached 23.0%. When the pH decreased from 7.0 to 5.0 in stages, the dominant POB were replaced by *Smithella* and *Pelotomaculum*. The hydrogenotrophic and acetotrophic methanogens were dominated by *Methanospirillum* and *Methanosaeta* at pH 7.0. pH decrease resulted in the relative abundance of acetotrophic methanogens decreased by 88.6% and hydrogenotrophic methanogens became the major methanogens present. The relative abundance of *Methanobacterium* was up to 5.0% at pH 5.0. pH decrease broke the normal metabolic network, and then further exacerbated the instability of process. These results reveal the microbial mechanism of process instability caused by pH reduction and provide guidance for the microbial management of anaerobic digesters.

Keywords: Upflow anaerobic sludge blanket; Slightly acidic conditions; Microbial community; Propionate-oxidizing bacteria; Methanogens

1. Introduction

As a feasible biomass energy conversion technology, anaerobic digestion (AD) has been widely applied in treating various industrial wastewaters, excess sludge, food waste, agricultural wastes, etc. [1–3]. AD process contains four main metabolic reactions: hydrolysis, acidogenesis, acetogenesis and methanogenesis [4]. In a stably operating digester, these steps maintain balance through microbes having different metabolic capabilities. However, operational mismanagement or toxic substances can inhibit anaerobic procedure by reducing pH and metabolic levels of microbes, which is resulted from the accumulation of volatile fatty acids (VFAs) including propionate [5]. Previous studies have shown that the performance of digesters can be limited when propionate levels exceed the tolerance levels of microbes involved in the anaerobic fermentation process, even if pH is maintained near neutral levels [6,7]. Propionate is a common intermediate in the AD of organic matter [8]. Approximately 20%–43% methane production comes from the complete oxidation of propionate and butyrate, mainly relying on the reactor configuration and substrate characteristics [9]. Propionate accumulates readily in anaerobic digesters, due to the fact that propionate oxidation is highly endergonic [10]. Therefore, propionate degradation is considered as a rate-limiting step during the AD [11].

Propionate is converted through the cooperation of propionate-oxidizing bacteria (POB) and hydrogen-consuming microorganisms (e.g., hydrogenotrophic methanogen), which rely on each other for maintaining energy and

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growth [9]. The syntrophic association between POB and methanogens is completed by interspecies electron (H_2 or formate) transfer [10]. Syntrophic propionate-degrading consortia can be found in various anaerobic ecosystems, including bioreactors, freshwater sediments and landfills, etc. [12–14]. Some investigators have described the factors affecting propionate degradation in AD systems treating some organic wastewaters [7,15,16]. Also, some studies about the response of propionate degradation on pH, temperature, and macro-/micro-nutrients in upflow anaerobic sludge bed (UASB) reactors containing a high concentration of propionate have been conducted [17–19]. In order to comprehend the microbial mechanism of propionate degradation, the composition, distribution and quantity of propionate-degrading consortia have been investigated by molecular biology techniques based on 16S rRNA genes [8,20]. For instance, *P. schinkii* was recognized as the dominant propionate degraders in a UASB reactor when the temperature was lower than 20ºC [18]. Ariesyady et al. found high proportions of *Smithella* and low proportions of *Syntrophobacter* in anaerobic sludge, but they did not determine *Pelotomaculum* [20]. Ahlert et al. found that *Syntrophobacter* was replaced by *Pelotomaculum* with decreasing propionate concentration [14]. In recent years, high throughput sequencing technology has been widely used to detect microbial community structure and diversity of AD habitats [3,21,22]. For example, Joyce et al. revealed that the microbial community structure and function during the acidified anaerobic digestion of grass by Illumina [21]. Using Illumina sequencing, Peng et al. clarified correlations between ammonia accumulation, microbial community dynamics and process stability during AD of food waste [3].

Although some research about propionate degrading communities have been done, very few high throughput-based studies have been conducted on microbial communities under pH stress in UASBs containing propionate individually as substrate. As a high-throughput sequencing technique, pyrosequencing provides a relatively unbiased overview of the community structure at a fine scale and coverage. Therefore, this study focused on the succession of microbial community, in conditions of decreasing pH in a UASB reactor containing propionate as the sole carbon source, based on pyrosequencing of 16S rRNA gene. The objectives of this study were to investigate the linkages between pH decrease, microbial dynamics, and process performance, providing guidance for the microbial resource management of anaerobic digesters.

2. Materials and methods

2.1. Reactor operation

A lab-scale UASB reactor with working volume of 4 L was used as bioreactor in present study. The internal diameter and height were 70 mm and 700 mm, respectively.

The produced biogas was gathered from top of UASB and entered a wet gas meter. This bioreactor was used to treat propionate synthetic wastewater for three months. Propionate at a concentration of 2000±50 mg/L was used as the sole carbon source for synthetic wastewater. The basic media was prepared as described previously [23]. The initial inoculation sludge was gathered from a lab-scale anaerobic baffled reactor (ABR) treating molasses wastewater [24]. The concentration of anaerobic sludge in the UASB was 7.9 g MLVSS /L (mixed liquid volatile suspended solid). The synthetic propionate wastewater was fed into the reactor through a peristaltic pump. The operational temperature and hydraulic retention time (HRT) were maintained at 35±1ºC and 6 h, respectively. pH of the feed was adjusted to 7.0±0.5 with $NaHCO_3$. The reactor was continuously operated for at least two weeks when the performance achieved a steady state or inhibited steady state (the changing magnitudes of propionate removal, biogas production, VFAs content were less than 5%), then the pH in sludge bed decreased to 6.8, 6.5, 6.0, 5.5, 5.0, 4.5 and 4.0 by HCl [3].

2.2. Analysis

Biogas production, pH, biogas composition and VFAs were measured every day, MLVSS was determined at the end of each stage. Biogas production was measured by a wet gas meter (LML-1; Changchun Filter Co., Ltd., Changchun, China). VFAs was analyzed by a gas chromatograph (SP6890, Shandong Lunan Instrument Factory, Zaozhuang, China) equipped with a flame ionization detector and a 2 m stainless (5 mm inside diameter) column packed with Porapak GDX-103 (60/80 mesh). The operational temperatures of the injection port, the column and the detector were 220, 190 and 220°C, respectively. Nitrogen was used as carrier gas at a flow rate of 50 ml/min. Biogas composition was measured methane content by another gas chromatograph (SP-6800A, Shandong Lunan Instrument Factory, Zaozhuang, China) equipped with a thermal conductivity detector (TCD). A 2-m stainless iron column was packed with Porapak TDS-01 (60/80 mesh). Nitrogen was used as the carrier gas at a flow rate of 70 ml/min. The operating temperatures of the injection port, the oven, and the detector were 80°C. The pH and MLVSS were measured as described in the standard methods [25].

2.3. Nucleic acid extraction and pyrosequencing analysis

The sludge samples were taken from the bottom of the UASB in the end of a certain pH stage. Three digestate samples were collected on day 45, 147, 205 with pH 7.0, 6.0, 5.0, respectively. The sludge samples were successively washed once with distilled water and phosphate buffer (pH 7.0), and then the total DNA was extracted using a Powersoil DNA Isolation Kit (MO Bio Laboratories, Carlsbad, CA, USA). The DNA quality was verified using agarose gel (1.0%) electrophoresis, and DNA concentrations were determined using Quant-iTTM dsDNA BR Assay Kit. 454 pyrosequencing based on 16S rRNA gene was conducted for microbial community analysis. The V3-V5 region of the 16S rRNA gene was amplified by the primers forward (5′-CCTACGGGRBGCAGCAG-3′) and reverse (5′-GGACTACMV GGGTATCTA-3′) [26]. A 10-nucleotide barcode was inserted into the 5′ end of forward primer. After being purified and quantified, amplicons were finally pyrosequenced on Roche 454-GS-FLX platform at BGI Co. Ltd (Shenzhen, China). Raw data from pyrosequencing was deposited to the NCBI (National Center for Biotechnology Information) sequence read archive with accession no. SRR3471436, SRR3471437 and SRR3471438. 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 1000 nucleotides. After the low-quality sequences was filtered, 7273 (pH 7.0), 11501 (pH 6.0), 12881 (pH 5.0) effective sequences of 16S rRNA gene were produced.

2.4. Sequence analysis, phylogenetic classification and statistical analysis

All qualified sequences were assigned to operational taxonomic units (OTUs) at a 3% distance by the MOTHUR program (version 1.25.0) (http://www.mothur.org). These sequences were designated to taxonomic classifications by a Ribosomal Database Project (RDP) classifier. The relative abundance of dominant microbes was investigated at phylum, class and genus levels. Shannon/Simpson diversity indices, Chao1 richness index, Rarefaction curves and Venn diagram were generated in QIIME (Quantitative insights into microbial ecology) [26]. Hierarchical cluster analysis was performed using gplots package of R in Linux. Detrended correspondence analysis (DCA) revealed the gradient size was 1.735 in this study. Therefore, redundancy analysis (RDA) was performed to investigate the relationship between microbial communities, environmental variables (pH, VFAs), and reactor performance (propionate removal, methane yield). Both DCA and RDA were conducted using CANOCO 4.5 software (Microcomputer Power Co., USA).

3. Results and discussion

3.1. Performance of the UASB in slightly acidic conditions

The UASB was started up under an influent propionate concentration of 2000 mg/L at 35ºC conditions, and performed for 205 d staged by pH decrease. The process performance, biomass and chemical oxygen demand (COD) balance in the steady state are illustrated in Table 1. Table 1 shows that the total propionate removal reached 93.6% at pH 7.0 and most of the propionate (86%) was degraded by the sludge blanket (Table 1). pH decrease resulted in the reduction of propionate removal in the sludge blanket to 56.6% (pH 6.0), and 26.2% (pH 5.0). Combined with the role of outside of the sludge blanket, the total propionate removals at pH 6.0 and 5.0 were 86.1% and 79.3%, respectively. Feasible methanogenesis could be observed in the neutral conditions (Table 1). At acidic conditions (pH 5.0 and pH 6.0), methanogenesis was inhibited with a specific methane production rate of 257.6 and 283.0 L CH_4 /($kgCOD_{removed}$·d). No hydrogen was detected during the whole operation period or the hydrogen content remained below the detection limit, demonstrating that the hydrogen partial pressure is not a limiting factor for propionate degradation at pH 7.0–5.0. Table 1 also shows that the acetate concentration in the effluent remained low. The COD balance of steady-state periods was above 80%, indicating that the data is reliable. These results indicate that the metabolic ability and quantity of the syntrophic propionate degradation communities might be compromised by the reduction in pH.

3.2. Overall phylogeny and diversity of microbial phylotypes

Three 16S rRNA gene libraries, L1, L2 and L3, were respectively generated from the UASB under pH 7.0, pH 6.0 and pH 5.0 conditions using pyrosequencing. After filtering the low quality reads, the effective reads in samples L1, L2 and L3 were 7273, 11501 and 12881, respectively (Table 2). The length of all effective reads was within 203~574 bp. The effective reads were clustered into 198 (L1), 256 (L2) and 330 (L3) OTUs by setting a 97% similarity. The decreasing pH led to increased amounts of OTUs, indicating an increase in species diversity (Table 2). To further understand the micro-

Table 1
The operational performance during stable phases under different pH conditions

Stage	Propionate removal in sludge blanket (%)	Total propionate removal (%)	Acetate content in effluent (mg/L)	Biomass (gVSS/L)	Specific methane production rate (L CH_4 /kg COD·d)	COD mass balance (%)
pH 7.0	86.0±1.6	95.1±2.3	69.4±10.5	9.5±0.5	317.7±15.6	108.9±2.5
pH 6.0	56.6±8.1	86.1±2.5	58.3±11.5	9.6±0.4	283.0±16.8	83.9±4.6
pH 5.0	26.2±2.3	79.3±2.7	43.7±9.1	6.5±0.8	257.6±19.6	80.1±6.8

All process parameters were expressed as average ± standard deviation.

Table 2
Sequence reads, diversity/richness indices, coverage and 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	
L1 (pH 7.0)	13618	7273	198	6.10	0.95	357.91	99.5%
L2 (pH 6.0)	20756	11501	256	6.30	0.95	463.95	99.6%
L3 (pH 5.0)	24320	12881	330	7.14	0.99	539.41	99.7%

bial community diversity, the Shannon/Simpson diversity indices and Chao1 were estimated by QIIME. Shannon index and Chao1 showed an increasing tendency towards diversity as pH decreased. It was deduced from the Good's sampling coverage (99.5%–99.7%) that the sampling depth of the selected samples was adequate for UASB system (Table 2). In this study, the rarefaction curve showed similar patterns for all samples (Fig. 1).

Similarity among samples was investigated using Heat map (Fig. 2a) and Venn diagram (Fig. 2b). As shown in Fig. 2a, the heat map indicated that the community structure of each sample was markedly different from the other two samples. Venn diagram revealed the shared and distinct OTUs among the different samples (Fig. 2b). The unique OTUs in samples from L1, L2 and L3 were 36, 26 and 89, respectively. The 109 OTUs were shared in three samples. These shared OTUs were clustered to 15 families and 1 unclassified bacterial family. They were related to propionate degraders, syntrophic fatty-acid oxidizing bacteria, methanogens and fermentative bacteria [10,27,28].

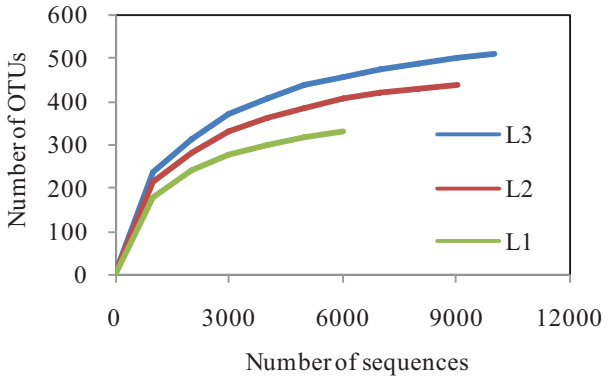

Fig. 1. Rare faction curves base on pyrosequencing of all samples. The OTUs were defined by 3% distances. L1, L2 and L3 represent the sludge samples at pH 7.0, 6.0 and 5.0, respectively.

3.3. Microbial taxonomic identification and relative abundance

All these bacterial and archaeal groups formed a relative stable community structure at each pH condition after long-term cultivation on propionate as the sole carbon source. To evaluate the microbial succession with decreases in pH during the propionate degradation process, qualified reads were analyzed at phylum, class and genus three levels (Fig. 3). Pyrosequencing based 16S rRNA gene revealed that the relative abundances of bacteria were 95.3%, 95.9% and 93.8% for L1, L2 and L3, respectively. The percentage of archaea was only 4.1%–6.2% in three samples. Unclassified reads in samples L1, L2 and L3 separately accounted for 14.1%, 13.4% and 11.7% at the phylum level (Fig. 3a). The unclassified reads were increased to 20.6%–28.9% at the class level (Fig. 3b) and 59.9%–76.0% (Fig. 3c) at the genus level.

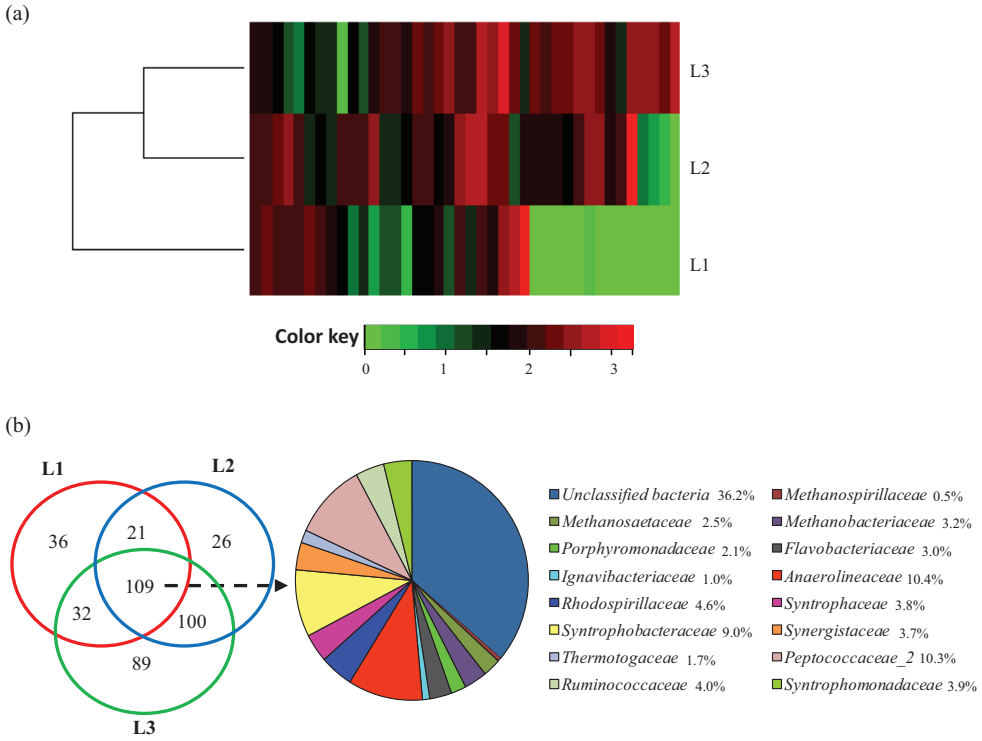

Fig. 2. Heat map (a) and Venn diagram (b) based on pyrosequencing of all samples. L1, L2 and L3 represent the sludge samples at pH 7.0, 6.0 and 5.0, respectively. a. The y-axis is the clustering of the 40 most abundant OTUs in reads. The color intensity (log10 transformed) in each panel shows the number of an OTU in each sample. b. The number in Venn diagram represents the total number of OTUs in that community. The taxonomic identities of the shared OTUs were conducted at family level. Unclassified bacteria represent the total sum of unclassified bacteria in family level.

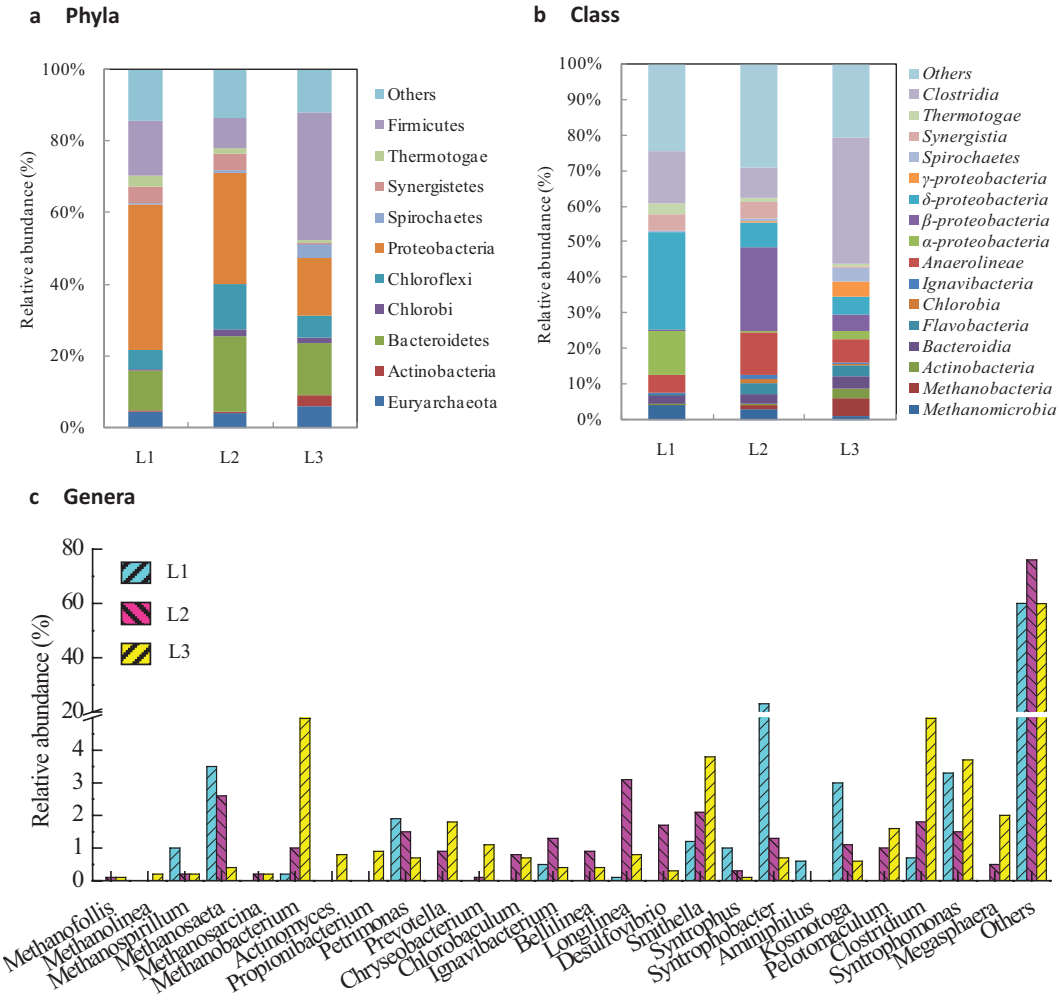

Fig. 3. The relative abundance of microbial communities at the (a) phylum, (b) class, and (c) genus levels in all samples (L1, L2 and L3). L1, L2 and L3 represent the sludge samples at pH 7.0, 6.0 and 5.0, respectively. Taxa represented occurred at > 0.5% abundance for bacteria or > 0.1% abundance for 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".

There were ten phyla identified existed in the UASB system. Among them, *Firmicutes*, *Proteobacteria* and *Euryarchaeota* frequently were recognized as dominant phyla in propionate anaerobic degradation [8,11]. In this study, *Firmicutes* (8.6%–35.7%), *Proteobacteria* (16.3%–40.2%), *Chloroflexi* (5.6%–12.2%) and *Bacteroidetes* (11.1%–21.0%) were the dominant phyla. *Proteobacteria* was highly enriched in sample L1 (accounting for 40.2%) and it exhibited a decreasing trend with decreases in pH. The percentage of *Proteobacteria* in L3 (pH 5.0) was only 16.2%. The proportion of *Firmicutes* (8.6%) was lowest in L2 (pH 6.0), and then increased to 35.7% in L3. *Chloroflexi* and *Bacteroidetes* showed a tendency of increasing first and then decreasing with pH decrease. *Chloroflexi* and *Bacteroidetes* include some fermentation bacteria, which utilized the products of microbial distegration as substrates in the UASB system containing propionate as the sole carbon source [28]. Although the relative abundance (4.1%–6.2%) of *Euryarchaeota* in three analyzed samples was low, it plays an important role in propionate degradation, which requires the syntrophic cooperation between POB and methanogens [10].

Sixteen classes were identified in the total microbial community, including 14 bacterial classes and 2 archeal classes (Fig. 3b). The domain classes were *Clostridia*, α-*proteobacteria*, β-*proteobacteria*, δ-*proteobacteria* and *Anaerolineae*. *Clostridia* was relatively abundant in all samples, and reached a maximum (35.5%) at pH 5.0. The relative abundance of α, δ-*proteobacteria* was sharply reduced as pH decreased from 7.0 to 5.0. Their proportions were respectively 2.3% and 5.0% in sample L3. The relative abundance of *Anaerolineae* and β-*Proteobacteria* exhibited a trend of increasing first and then decreasing with pH decrease. Two classes were related to archaea. *Methanomicrobia* displayed a decreasing trend as pH decreased from 7.0 to 5.0. In contrast, pH decreases led to a significant increase in the proportion of *Methanobacteria* to 5.0% from 0.2%.

A total of 25 genera were classified among the tested samples (Fig. 3c). Nineteen bacterial genera could be observed in three samples. *Syntrophobacter*, *Smithella* and *Pelotomaculum* are associated with propionate degradation in the presence of methanogens [10]. *Syntrophobacter* (23.0%) was noted as the dominant genera in sample L1 and showed a

decreasing trend as pH decreased. *Smithella* and *Pelotomaculum* exhibited an increasing tendency with pH decreased to 5.0 from 7.0. *Syntrophus* and *Syntrophomonas* were identified as syntrophic fatty acid (C_4-C_8)-oxidizing bacteria and their relative abundances were 1.0%–0.1% and 1.5%–3.7%, respectively [29,30]. *Petrimonas* (1.9%–0.7%), *Ignavibacterium* (0.4%–1.3%) and *Kosmotoga* (3.0%–0.6%) were the fermentative bacteria, which can utilize some organic compounds (such as carbohydrates, pyruvate, fumarate, malate) as substrates and acetate was the major end products [27,28,31]. The relative abundance of *Clostridium* showed an increasing trend with pH decrease. Although most of the species identified in *Clostridia* are hydrolytic fermentative bacteria, most of the syntrophic acetate-oxidizing bacteria (SAOB) known so far belong to the *Clostridia* class [3]. The percentages of *Longilinea* (0.1%–3.1%) increased first and then decreased. *Aminiphilus* (0.6%), a genus of anaerobic amino-acid-degrading bacteria, was present only in neutral environments (pH 7.0) [32]. In contrast, the low abundance (0.3%–2.0%) of *Actinomyces*, *Propionibacterium*, *Prevotella*, *Chryseobacterium*, *Chlorobaculum*, *Bellilinea*, *Desulfovibrio* and *Megasphaera* only appeared under acidic conditions (pH 6.0 and pH 5.0). The high diversity and succession of bacterial taxa in acidic conditions could be related to emerging acid-tolerant fermenting bacteria, which utilized the microbial biomass of dead cells resulting from the decrease in pH. Furthermore, both the number of OTUs and diversity indices also showed that the microbial diversity at pH 5.0 were higher than in other samples (Table 1). Emerging species might contain some unknown acid-tolerant propionate degraders, which favor propionate oxidation in low pH conditions.

In addition to bacteria, six genera were identified taxonomically as methanogens, including *Methanofollis*, *Methanolinea*, *Methanospirillum*, *Methanosaeta*, *Methanosarcina* and *Methanobacterium*. *Methanosaeta* accounted for the largest percentage of methanogens in the sample L1 (up to 3.5%), and displayed a reducing tendency as pH decreases. In contrast, the abundance of *Methanobacterium* was least at pH 7.0 (only 0.2%), pH decreases resulted in *Methanobacterium* was increased, reaching a maximum (5.0%) at pH 5.0. Notably, *Methanofollis*, *Methanolinea* and *Methanosarcina* can be found at acidic conditions.

3.4. Succession of dominant microorganisms involved in the propionate degradation

Propionate is a common intermediate during the anaerobic degradation process of organic matter. Under methanogenic conditions, propionate is oxidized to acetate and H_2/CO_2, which are subsequently converted to methane by methanogens. In this study, POB were mainly involved in aforementioned three genera. Under favorable conditions, *Syntrophobacter* is a major species of POB in UASB reactors [8,34,35]. Similarly, the present study also found that *Syntrophobacter* was the dominant POB with a relative abundance of 23.0% in the propionate-fed UASB reactor (Fig. 4). The members of *Syntrophobacter* can degrade propionate through methylmalonyl-coenzyme A (MMC) pathway, indicating that MMC might be the pathway used under neutral conditions (pH 7.0) (Fig. 5). The major POB was gradually substituted by *Smithella* and *Pelotomaculum* with pH decreased to 5.0 (Fig. 4). Some previous studies indicated that *Smithella* and *Pelotomaculum* can compete more successfully with *Syntrophobacter* for the substrate propionate in anoxic habitats, high dilution conditions and the absence of molybdenum, tungsten and selenium [8,36]. The current study confirmed that *Smithella* and *Pelotomaculum* competed more successfully than *Syntrophobacter* in propionate consumption in slightly acidic conditions. It can be known from Fig. 5 that propionate was decomposed through two pathways at pH 6.0. They were the MMC pathway and a unique metabolic pathway, which is proposed for *Smithella propionica*, the only isolated species in genus *Smithella* [37]. The latter became the master mode for propionate degradation at pH 5.0. In this pathway, two molecules of propionate were disproportionated to one molecule of acetate and one molecule of butyrate, which was further oxidized by β-oxidation to acetate and H_2/CO_2 [37].

Meanwhile, *Methanospirillum* and *Methanosaeta* were the dominant methanogens at pH 7.0. *Methanospirillum* can use H_2/CO_2 and formate as substrate for growth [38]. *Methanospirillum* spp. are the ideal syntrophic partners for *Syntrophobacter* spp. and all species from the genus *Syntrophobacter* have been isolated in syntrophy with *Methanospirillum hungatei* up to now [10]. *Methanosaeta* are generally considered to be the dominant acetotrophic methanogens in anaerobic reactors with low concentrations of acetate [39]. *Methanosaeta* species can often promote sludge granulation, which in turn lead to stable performance [40]. Previous studies showed that acetate cleavage is the main methanogenic acetate degradation pathway in UASB systems [24,41,42]. It can be concluded that the high propionate removal (86.0%) and specific methane production rate (317.7 L CH4 /kg COD removed·d) were achieved in the UASB by the MMC coupled with the acetoclastic pathway for propionate degradation at pH 7.0 (Table 1 and Fig. 5). When pH was decreased to 6.0, *Methanosaeta* was still the dominant acetotrophic methanogens while *Methanobacterium* became the dominant hydrogenotrophic methanogens in the system. The hydrogenotrophic methanogens (*Methanobacterium*) dominated over acetotrophic methanogens at pH 5.0. The decrease in aceticlastic methanogens and increase in hydrogentrophic methanogens with decreasing pH was caused by a comparatively higher tolerance of *Methanobacterium* to low pH. It has been reported that acetotrophic methanogens were inhibited strongly at pH < 6.2 [38]. However, some hydrogenotrophic methanogens can grow in pH as low as 4.0 [20,43]. Herein, the pyrosequencing result indicated that *Methanobacterium* was moderately acid tolerant. Similarly, Williams and Crawford also reported that an unclassified *Methanobacterium* can tolerate pH 4.0 [44]. The succession of methanogens harboring the different pathways as pH decreased indirectly revealed an alteration in the methane generation pathway. This study showed that the acetate cleavage pathway might be replaced by the syntrophic acetate oxidation coupled with the hydrogenotrophic pathway at pH 5.0. Some studies have also reported that acetotrophic methanogens are more susceptible to stress conditions (such as high-level ammonium, low pH, high hydrogen partial pressure etc.) than hydrogenotrophic ones and the acetoclastic methanogenesis is replaced by H_2-dependent methanogenesis under stress conditions [14,45]. To date, there are a restricted number of syntrophic acetate-oxidizing bacteria (SAOB) have been identified, *Clostridium ultunense*, *Syntro-*

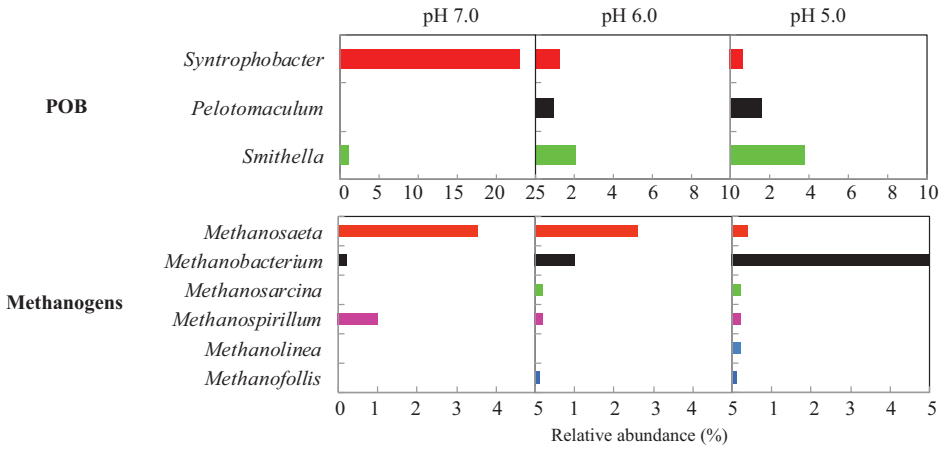

Fig. 4. Succession of dominant microorganisms involved in propionate degradation process. POB: propionate-oxidizing bacteria.

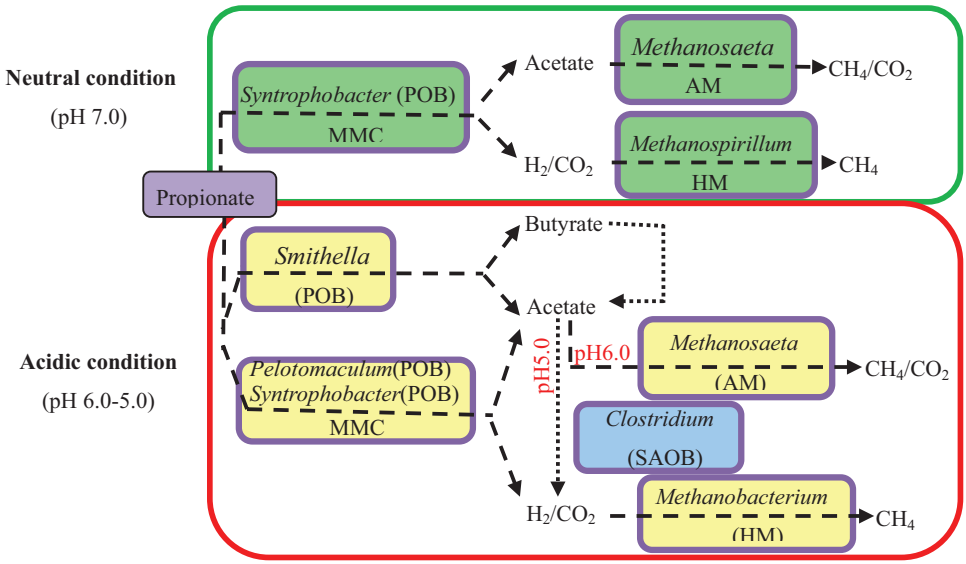

Fig. 5. Presumed metabolic pathway at slightly acidic conditions in propionate degradation process. POB: propionate-oxidizing bacteria; MMC: methylmalonyl-conenzyme A pathway; AM: aceticlastic methanogens; HM: hydrogenotrophic methanogens; SAOB: syntrophic acetate- oxidizing bacteria.

phaceticus schinkii, *Tepidanaerobacter acetatoxydans*, *Thermacetogenium phaeum* and *Thermotoga lettingae* [46]. In the present study, microorganisms from genus *Clostridium* were present in the UASB and it showed an increasing trend as pH decreased. The relative abundance of *Clostridium* achieved a maximum of 13.6% at pH 5.0 (Fig. 3c). In addition, Lee et al. reported the phylum *Spirochaetes* is involved in syntrophic acetate oxidation during anaerobic methane production [47]. The present study also found that the abundance of *Spirochaetes* was significantly increased to 4.1% (pH 5.0) from 0.4% (pH 7.0) at the phylum level (Fig. 3a). The change in metabolic pathways at pH 5.0 caused the propionate oxidation and methanogenesis to be significantly inhibited. Under pH 5.0 condition, propionate removal in the sludge bed was only 26.0% and the specific methane production rate was also reduced by 18.9% compared to what it was at pH 7.0. These results showed that a marked microbial succession occurs in the process of pH reduction, indicating that the propionate degrading community is very sensitive to low pH stress.

3.5. Linkages between operational parameters and dominant microbial groups

To better reveal the relationships between environmental variables (pH, VFAs), reactor performance (Propionate removal, methane yield) and dominant functional microbial genera in the process of pH decrease, RDA was conducted (Fig. 6). As shown in Fig. 6, RDA1 and RDA2 together explained 100% of total variance. Fig. 6 shows that microbial community structure is affected by VFAs and pH levels. The reactor performance is negatively correlated with propionate, but is positively correlated with acetate and pH.

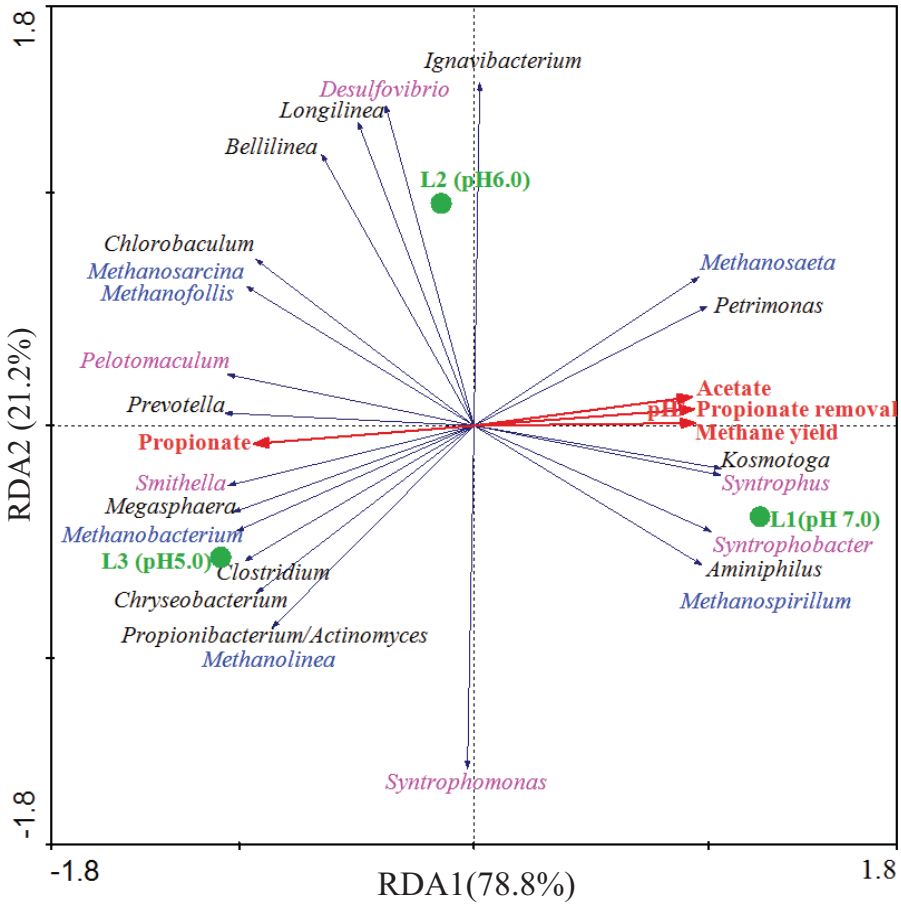

Fig. 6. RDA of ordination diagram (triplot) indicating the relationships between dominant microbial genera, environmental variables, and reactor performance data. The green, red, black, pink, and blue represent samples, environmental variables and reactor performance, hydrolytic fermentative bacteria, syntrophic acetogens, and methanogens, respectively.

The process efficiency was strongly correlated with pH, which is the primary independent variable in this study. As stated in Section 3.1, propionate removal, and specific methane production were significantly reduced as pH decreased. Fig. 6 also reveals the succession of community composition in different samples. As for POB, *Syntrophobacter* is positively correlated with pH levels and methane yield, its relative abundance reached 23.0% at pH 7.0 (Figs. 4 and 6). On the contrary, *Smithella* and *Pelotomaculum* were all negatively correlated with pH levels and methane yield, and they became the dominant POB at pH 5.0 (Fig. 4). Although propionate was used as the sole carbon source in this study, there were some other syntrophic acetogenic bacteria and fermentative bacteria except for POB existed in the bioreactor. *Syntrophus* showed a positive correlation with pH and methane yield, while *Desulfovibri* and *Syntrophomonas* had no significant correlation with pH levels and methane yield. A marked succession in fermentative bacteria is illustrated in Fig. 6. pH and performance were positively correlated with *Petrimonas*, *Kosmotoga* and *Aminiphilus*, and had no significant relationship with *Ignavibacterium*, but were negatively correlated with other fermentative bacteria.

Fig. 6 shows that pH, propionate removal and methane yield were positively correlated with *Methanosaeta* and *Methanospirillum*, but were negatively correlated with *Methanobacterium*, *Methanofollis*, *Methanolinea* and *Methanosarcina.* Theoretically, *Methanosarcina* could become the dominant acetotrophic methanogens or else methane was formed from acetate through the cooperation between SAOB and hydrogenotrophic methanogens when *Methanosaeta* became inhibited [48]. The current study also showed that the abundances of *Methanosarcina* and hydrogenotrophic methanogens reached 0.2% and 6.1% at pH 5.0, and *Methanobacterium* became the dominant hydrogenotrophic methanogens (Fig. 4). *Methanosaeta* and *Methanospirillum* showed a positive relationship with *Syntrophobacter* (POB), while other methanogens were positively correlated with *Pelotomaculum* and *Smithella* (POB). As syntrophic partners, these methanogens prevented the accumulation of both acetate and H_2 during the whole operation period (Table 1).

This study only constructs the interaction network between microorganisms and pH, which is the sole independent variable, but cannot construct the networks of different environmental factors and microbial community. Nevertheless, this study explored the microbial community dynamics at acidic condition in propionate degradation and the correlations between microbial interactions and process performance, which will help to avoid the accumulation of propionate and improve the stability of performance in the engineering application of AD.

4. Conclusion

Propionate removal decreased as pH decreased to 5.0 from 7.0 in a UASB reactor treating propionate synthentic wastewater. Pyrosequencing revealed *Syntrophobacter* was considered as the dominant POB at pH 7.0, whereas *Smithella* and *Pelotomaculum* became the predominant POB as pH decreased to 5.0. *Methanosaeta* was the dominant acetotrophic methanogens at pH 7.0 and pH 6.0 conditions, and its relative abundance was 3.5% and 2.6%, respectively. The hydrogenotrophic methanogens predominated over acetotrophic methanogens at pH 5.0. The dominant hydrogenotrophic methanogens was succeeded as *Methanobacterium* from *Methanospirillum* as pH decreased to 5.0 from 7.0. RDA revealed the correlation between dominant functional microbial groups, pH and reactor performance in the process of pH decreasing.

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