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RESEARCH ARTICLE

Microbial community dynamics at high organic loading rates revealed by pyrosequencing during sugar refinery wastewater treatment in a UASB reactor

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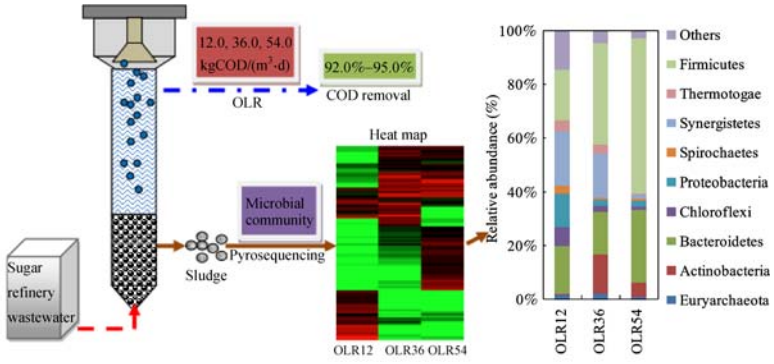
HIGHLIGHTS

- High strength sugar refinery wastewater was treated in a mesophilic UASB.
- Pyrosequencing reveals microbial community succession with OLR increase.
- Diversity of microbial communities in OLR12 is much higher than those in OLR36 and OLR54.0 kgCOD/(kg VSS·d).
- Fermentative bacteria could deal with increasing OLR through the increase of microbial diversity and quantity.
- Hydrogen-producing acotogens and methanogens mainly coped with high OLR shocks by increasing the quantity of community

GRAPHIC ABSTRACT



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ABSTRACT

The performance and microbial community structure in an upflow anaerobic sludge blanket reactor (UASB) treating sugar refinery wastewater were investigated. The chemical oxygen demand (COD) removal reached above 92.0% at organic loading rates (OLRs) of 12.0–54.0 kgCOD/(m^3·d). The volatile fatty acids (VFAs) in effluent were increased to 451.1 mg/L from 147.9 mg/L and the specific methane production rate improved by 1.2–2.2-fold as the OLR increased. The evolution of microbial communities in anaerobic sludge at three different OLRs was investigated using pyrosequencing. Operational taxonomic units (OTUs) at a 3% distance were 353, 337 and 233 for OLR12, OLR36 and OLR54, respectively. When the OLR was increased to 54.0 kgCOD /(m^3·d) from 12.0 kgCOD/(m^3·d) by stepwise, the microbial community structure were changed significantly. Five genera (*Bacteroides*, *Trichococcus*, *Chryseobacterium*, *Longilinea* and *Aerococcus*) were the dominant fermentative bacteria at the OLR 12.0 kgCOD/(m^3·d). However, the sample of OLR36 was dominated by *Lactococcus*, *Trichococcus*, *Anaeroarcus* and *Veillonella*. At the last stage (OLR = 54.0 kgCOD/(m^3·d)), the diversity and percentage of fermentative bacteria were markedly increased. Apart from fermentative bacteria, an obvious shift was observed in hydrogen-producing acetogens and non-acetotrophic methanogens as OLR increased. *Syntrophobacter*, *Geobacter* and *Methanomethylovorans* were the dominant hydrogen-producing acetogens and methylotrophic methanogens in the samples of OLR12 and OLR36. When the OLR was increased to 54.0 kgCOD/(m^3·d), the main hydrogen-producing acetogens and hydrogenotrophic methanogens were substituted with *Desulfovibrio* and *Methanospirillum*. However, the composition of acetotrophic methanogens (*Methanosaeta*) was relatively stable during the whole operation period of the UASB reactor.

1 Introduction

Anaerobic digestion technology is widely used for treating all kinds of organic wastes including municipal sewage, organic wastewaters, animal waste and agricultural wastes,

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and simultaneously generates methane as an energy source (Astals et al., 2012; Yang et al., 2013; Gaur et al., 2017). This process involves four steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis. The performance of an anaerobic digester depends on the metabolic balance between fermentative bacteria, hydrogen-producing acetogens, homoacetogens and methanogens (Ban et al., 2013a). When the system is shocked by temperature fluctuations, toxic substances or high organic load rate (OLRs), the aforementioned four steps become unbalanced, which in turn leads to the instability and failure of anaerobic digesters (Moertelmaier et al., 2014; Amani et al., 2012). Therefore, the performance of an anaerobic digestion system is strongly correlated with the microbial community structure and diversity.

As an efficient anaerobic reactor, the upflow anaerobic sludge blanket (UASB) reactor has been successfully applied in the treatment of various organic wastewaters in recent years (Delforno et al., 2014; Zhang et al., 2015a; Antwi et al., 2017). A comprehensive knowledge of microbial community structure is not only paramount to understanding the mechanism of biological treatment processes, but could also be used for warning inhibitory action (Hesham et al., 2011; Niu et al., 2016). Therefore, some studies have investigated anaerobic consortia in UASB reactors treating several organic wastewaters using molecular techniques based on 16S rRNA genes. For instance, Shi and coworkers investigated the microbial community by DGGE and found that the predominant microorganisms belonged to the phyla *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, and *Synergistetes* and *Euryarchaeota* in a UASB reactor treating vitamin C biosynthesis wastewater (Shi et al., 2012). Some researchers discussed the quantity of anaerobic microorganisms (such as propionate-oxidizing bacteria, anaerobic ammonium-oxidizing bacteria, methanogens) in UASB reactors by FISH or qPCR (Ke et al., 2008; Ban et al., 2013b; Li, 2014). In recent years, high throughput sequencing technology has been widely used to detect microbial community structure and diversity (Zhang et al., 2012; Zhang et al., 2015a; Antwi et al., 2017). Using Illumina Miseq sequencing, Zhang et al. investigated the shift of microbial community structure as hydrolic retention time (HRT) deceased in a UASB treating trichloroethylene wastewater (Zhang et al., 2015a). Illumina Miseq sequencing also revealed that a UASB treating potato starch processing wastewater was dominated by *Firmicutes*, *Chloroflexi*, *Proteobacteria*, *Cloacimonetes*, *Bacteroidetes*, *Synergistetes* and *Euryarchaeota* at the phylum level (Antwi et al., 2017).

Although the microbial community structure during the degradation of several organic wastewaters in UASB reactors has been investigated, knowledge about the shift of microbial community with OLR increase in UASB reactors treating sugar refinery wastewater is still incomplete. High throughput sequencing can provide an insight about 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 microbial consortia as OLR increases in a fed-sugar refinery wastewater UASB reactor by 454 pyrosequencing.

2 Materials and methods

2.1 Bioreactor operation

The study was performed in a laboratory-scale UASB reactor made of transparent plexiglass. The effective volume was 11 L. As shown in Fig. 1, four evenly distributed sampling ports and a solid outlet were installed over the height of the column. The feed was pumped to the reactor by a peristaltic pump. The reactor was wrapped by electro-thermal wire and was maintained at 35°C by a temperature controller. The evolved biogas was collected by the gas-solid-liquid separator, entered a water lock and was measured daily using a wet gas meter (Changchun Filter Company, China). The water lock and wet gas meter were filled with water of pH 3.0 to prevent dissolution of the biogas.

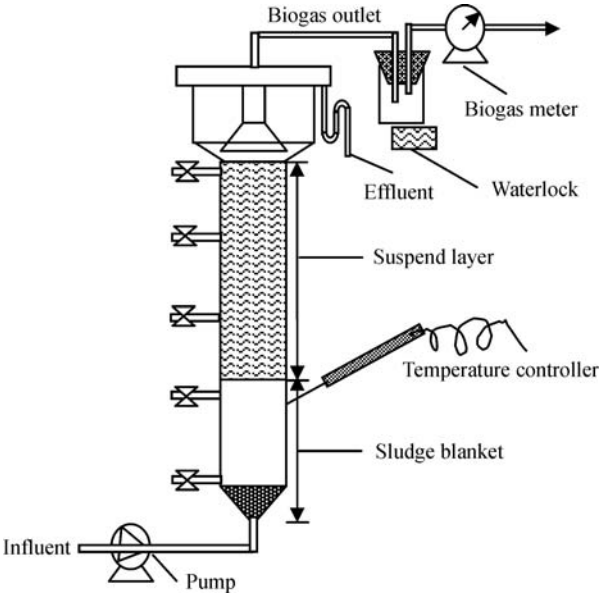

Fig. 1 Schematic diagram of the UASB in this study

The inoculated sludge was originally collected from a second settling tank in a local brewing wastewater treatment plant. A diluted sugar refinery wastewater was used as substrate from startup. The characteristics of sugar refinery wastewater have been described in a previous study (Li et al., 2007). The UASB was started up at a HRT of 36 h and influent COD of 1000 mg/L. The pH of the reactor was remained at around 7.0 by $NaHCO_3$. After a stable performance was achieved, the HRT was shorted to

8h and influent COD was increased to 2000 mg/L by stepwise. Then the OLR was increased from 6.0 to 12.0, 36.0 and then 54.0 kgCOD/(m^3·d), by enhancing the influent COD concentration from 2000 to 4000, 12000, and 18000 mg/L.

2.2 Analytical methods

The liquid samples from influent and effluent were centrifuged at 10, 000 rpm for 5 min, followed by 6 M HCl acidification for volatile fatty acids (VFAs) measure. VFAs content 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. The 0.5mL of biogas was sampled from the headspace of the UASB reactor to measure 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 70ml/min. The operating temperatures of the injection port, the oven, and the detector were 80°C. The pH and MLVSS (mixed liquor volatile suspended solid) were measured as described in the standard methods (APHA, 1995).

2.3 Nucleic acid extraction

Microbial samples were obtained on 38d, 79d, 110d with OLR of 12.0, 36.0 and 54.0 kgCOD/(m·d), respectively. Anaerobic sludge was collected from the sludge blanket of UASB reactor. The sludge samples for microbial analysis were immediately stored at −20°C until DNA extraction was conducted. 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 Pyrosequencing 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 (Wang and Qian, 2009). 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 number SRP127155. To minimize the impact caused by random sequencing errors, 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 were deleted. Pyrosequencing produced 10463 (OLR12), 10456 (OLR36) and 10210 (OLR54) 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 (Lu et al., 2012). The effective sequences were designated to taxonomic classifications by an RDP (ribosomal database project) database (http://www.mothur.org/wiki/Silva_reference_files) (Crawford et al., 2009). 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 in per sample. Rarefaction curves, Shannon diversity index, Simpson diversity indices and species richness estimator of Chao1 were generated in QIIME (Quantitative insights into microbial ecology) for each sample. Hierarchical cluster analysis was performed using gplots package of R in Linux. Venn diagram and PCA (Principal component analysis) were conducted by QIIME (Caporaso et al., 2010).

3 Results and discussion

3.1 Bioreactor performance

A UASB reactor treating sugar refinery wastewater was operated at different OLRs (12.0, 36.0 and 54.0 kgCOD/(m^3·d)) in order to understand the influence of increases in OLR on performance. The performance of the UASB during the steady-state is illustrated in Table 1. The results showed that the concentration of VFAs in effluent was increased from 147.9 mg/L to 451.1 mg/L as the influent COD was increased from 4000 mg/L (OLR = 12.0 kgCOD /m^3·d) to 18000 mg/L (OLR = 54.0 kgCOD/(m^3·d)). Acetate and propionate together accounted for 89.1%–95.3% in VFAs. In addition to acetate and propionate, a small amount of butyrate was also observed in the liquid samples. The UASB system achieved a high COD removal (92.0%–95.0%) at OLRs in the 12.0–54.0 kg COD /(m^3·d) range. The specific methane production rate improved by 1.2–2.2-fold with a maximum of 827 L CH_4/(kg VSS ·d) at OLR 54.0 kgCOD/(m^3·d). Therefore, the degradation of sugar refinery wastewater was not affected by the OLR increases in the range of 12.0–54.0 kgCOD/(m^3·d) in the UASB reactor.

Table 1 Operational performance of the UASB under different OLR conditions

OLR(kgCOD/(m^3·d))		12.0	36.0	54.0
VFAs (mg/L)	Acetate	52.9±8.3	65.0±3.9	140.8±9.6
	Propionate	88.0±1.9	106.0±4.6	281.7±21.2
	Butyrate	7.0±1.4	21.0±4.7	28.6±9.4
	Total VFAs	147.9±10.2	192.0±18.3	451.1±36.9
COD (mg/L)	Influent	4000±51	12000±186	18000±201
	Effluent	238±11	540±41	1446±95
COD removal (mg/L)		94.2±2.0	95.0±3.3	92.0±4.1
Biomass (gMLVSS/L)		18.7±1.3	23.9±0.8	28.5±2.7
Specific methane production rate (LCH_4(kg VSS/d))		258±13	560±21	827±16

3.2 Richness and diversity of microbial phylotypes

High-rate methanogenesis is essential for transforming organic pollutants to methane. Monitoring microbial community dynamics is very helpful for predicting and explaining functional changes in treating sugar refinery wastewater under different OLR conditions. In the present study, three 16S rRNA gene libraries (named OLR12, OLR36 and OLR54) were generated using 454 pyrosequencing. OLR12, OLR36 and OLR54 communities contained 10463, 10456 and 10210 high-quality V3–V5 tags, respectively (Table 2). The average read length of all effective reads was 298 bp. Bacteria were the dominant microbial group in these three samples, accounting for 97.8%–98.8%. The relative abundance of archaea in the anaerobic sludge was 1.8%, 2.2% and 1.2% for OLR12, OLR36 and OLR54, respectively. These effective reads were clustered into 353 (OLR12), 337 (OLR36) and 233 (OLR54) OTUs by setting a 97% similarity (Lu et al., 2012). It is seemingly suggested from the numbers of OTUs that the diversity of microbial communities in OLR12 is much higher than those in OLR36 and OLR54.

As shown in Table 2, Shannon and Simpson diversity indices exihibited a similar trend in observed OTUs. The Shannon diversity index not only represents species richness, but also reveals the abundance of each species in the community (Lu et al., 2012). OLR12 had the highest diversity (Shannon = 6.61, Simpson = 0.99) in these communities. The Shannon index (5.98) and Simpson indices (0.96) of OLR36 was larger than 5.55 (Shannon index) and 0.95 (Simpson indices) in OLR54. Herein, Shannon index showed a range of 6.61–5.98, which was higher than some UASBs treating potato starch processing wastewater, poultry slaughterhouse wastewater, 2,4-dinitrochlorobenzene (Delforno et al., 2014; Jiang et al., 2016; Antwi et al., 2017). Again, a similar trend in community richness was observed using the Chao1 estimator. Pyrosequencing revealed that new microbial species continued to appear even after 5 000 reads sampling (Fig. 2). However, the Good's sampling coverage value was reached 99.5%–99.7%, indicating that the sequencing depth captured a majority of microbial community (Table 2).

3.3 Comparative analysis of microbial communities

To comprehend the evolution of microbial community structure in detail, the top 60 abundance genera were selected from each sample for hierarchical cluster analysis as suggested previously (Fig. 3(a)) (Zhang et al., 2015b). The result showed that each group differed markedly from the other two groups, even though these three microbial communities contained some of the 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(b)). The cumulative percentage variance of species explained by the first axis was 74.1%. The first and second axes explained 100% variance of species. These results showed that the microbial community structure was dramatically

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	
OLR12	18418	10463	353	6.61	0.99	643.67	99.6%
OLR36	17209	10456	337	5.98	0.96	524.41	99.5%
OLR54	16707	10210	233	5.55	0.95	375.28	99.7%

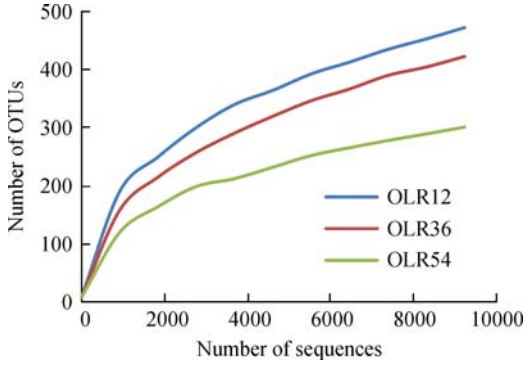

Fig. 2 Rarefaction curves base on pyrosequencing of all samples. The Operational taxonomic units (OTUs) were defined by 3% distances

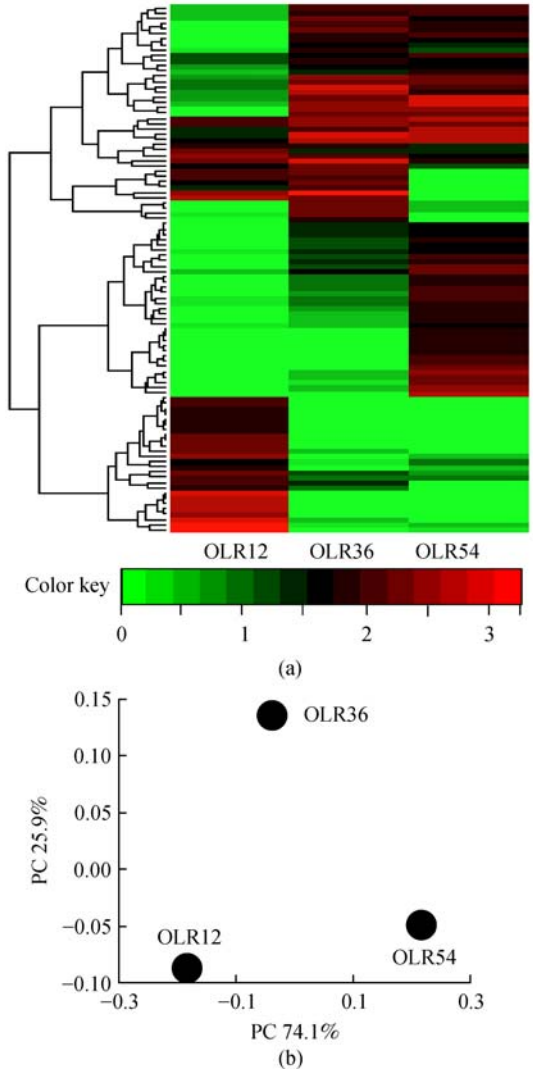

Fig. 3 Heat map (a) and Principal component analysis (PCA) (b) base on pyrosequencing of all samples. (a) The y-axis is the clustering of the 60 most abundant OTUs (3% distance) in reads. The color intensity (log10 transformed) in each panel shows the number of an OTU in each sample

influenced by OLR in the range of 12.0–54.0 kgCOD/($m^3 \cdot d$) even though similar levels of COD removal were observed.

As shown in Fig. 4, a Venn diagram was constructed for evaluating the distribution of OTUs among the different OLR samples. The total number of clustered OTUs in all three 16S rRNA gene libraries was 583 and 60 OTUs (accounting for 10.3% in all detected OTUs) were common in three samples. Most of the shared OTUs were the unclassified bacteria (41.2%–71.7%) at the genus level. The number of common OTUs between OLR12 and OLR36, OLR36 and OLR54, and OLR12 and OLR56 were 218, 106 and 76, respectively. Observably, the number of unique OTUs from OLR12, OLR36, and OLR54 were 119, 73 and 111, respectively. The OTUs unique to each of the three communities accounted for 21.7%–47.6% of the total OTUs.

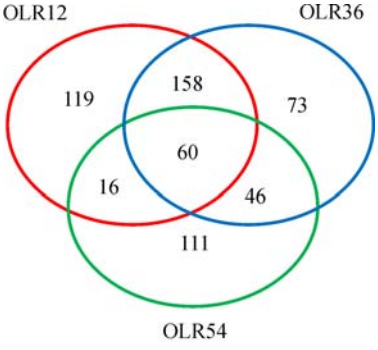

Fig. 4 Venn diagram of OLR12, OLR36 and OLR54 based on OTUs (3% distance), and the taxonomic identities of the shared OTUs at family level. The number in parentheses represents the total number of OTUs in that community

3.4 Microbial taxonomic identified

To reveal the microbial shift in response to increasing OLR during sugar refinery wastewater degradation, qualified reads were analyzed at the phylum, class and genus levels (Fig. 5). In this study, there were 10 different phyla, 14 different classes and 27 different genera were detected in the three communities (Fig. 5). As illustrated in Fig. 5, unclassified sequences were least at the phylum level. Unclassified reads in OLR12, OLR36 and OLR54 separately accounted for 14.2%, 4.5% and 2.5% of the total reads at the phylum level, respectively (Fig. 5(a)). Whereas the unclassified reads was increased to 13.3%–24.6% at the class level (Fig. 5(b)) and 41.2%–68.4% (Fig. 5(c)) at the genus level in three detected samples.

Four (4) mainly phylogenetic groups related to phyla (Fig. 5(a)) emerged in all samples (OLR12, OLR36 and OLR54). These phyla contained *Bacteroidetes*, *Proteobacteria*, *Synergistetes* and *Firmicutes*. These four phyla

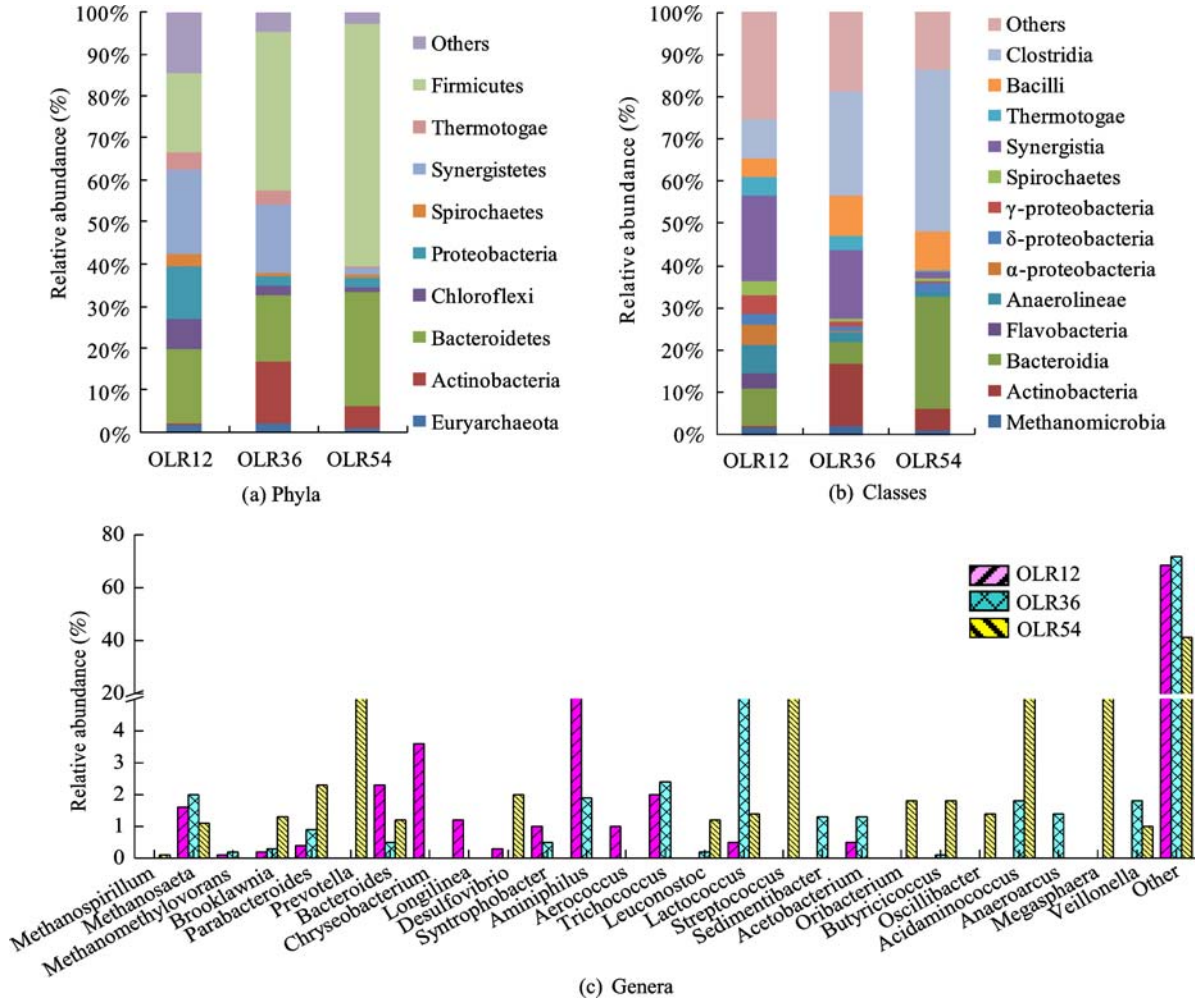

Fig. 5 The Relative abundance of microbial communities at the (a) phylum, (b) class, and (c) genus levels in all samples (OLR12, OLR36 and OLR54). Taxa represented occurred at>0.5% abundance for bacteria or>0.1% abundance for methanogens in at least one sample. Phyla, classes, and genera making up less than 0.5% for bacteria or 0.1% for methanogens of total composition in all three libraries were classified as "other"

accounted for 68.0% (OLR12), 72.1% (OLR36) and 88.5% (OLR54) of the total reads. Previous studies also showed that these phyla were the dominant phyla in anaerobic digesters treating potato starch processing wastewater, food wastewater, trichloroethylene wastewater, etc. (Zhang et al., 2015a; Jiang et al., 2016; Antwi et al., 2017). Some fermentative acidogenic bacteria are included in the phylum *Bacteroidetes* (Grabowski et al., 2005; Yoon et al., 2007; Kitahara et al., 2013). The relative abundance of *Bacteroidetes* was similar in OLR12 (17.6%) and OLR36 (15.9%), and then was increased to 27.1% at an OLR of 54 kgCOD/(m^3·d) condition. Most identified syntrophic acetogens are involved in *Proteobacteria* and low–G + C Gram-positive bacteria *Firmicutes* (Wallrabenstein et al., 1995; Harmsen et al., 1998; Elshahed and McInerney, 2001). In the sample of OLR 12, the percentage of *Proteobacteria* was 12.4%, and it decreased to 2.4% in OLR36 and OLR54. While the percentage of *Firmicutes* was 18.4% in the sample of OLR12, and it showed an increasing trend as OLR increased, becoming the most predominant phylum (57.7%) at the OLR of 54 kgCOD/(m^3·d). *Synergistetes* is abundant in wastewater treatment plants. It includes some species that have been identified as sludge degraders in anaerobic digesters (Riviere et al., 2009). The relative abundance of *Synergistetes* in OLR12 was 19.6%, while it showed a downward trend as OLR increased. *Euryarchaeota* is the sole archeal phylum in this study. Most identified methanogens belong to the phylum *Euryarchaeota* (Liu and Whitman, 2008). The relative abundance of *Euryarchaeota* was highest in OLR36 with a relative abundance of 2.2%, lower in OLR12 (1.8%) and in OLR54 (1.2%).

Figure 5(b) shows the identification of members of the three communities at the class level. The 14 classes identified included 13 bacterial classes and 1 archeal class. OLR12 is primarily consisted of δ-*Proteobacteria*, *Clos-*

tridia, *γ-proteobacteria*, *Synergistia*, *Anaerolineae*, *Bacteroidia*, *Thermotogae*, *Bacilli* and *Methanomicrobia*. OLR36 and OLR54 were similar and were highly enriched in the classes of *Clostridia*, *Bacilli*, *Bacteroidia*, *Synergistia*, *Actinobacteria* and *Methanomicrobia*. The percentage of *Clostridia* was high in all samples, and it reached a maximum (37.9%) at the OLR of 54 kgCOD/($m^3 \cdot d$). The relative abundance of *α, γ-proteobacteria*, *Anaerolineae*, *Synergistia* and *Thermotogae* were sharply reduced as OLR increased from 12.0 to 54.0 kgCOD/($m^3 \cdot d$). Their proportions were respectively 0.0%, 0.3%, 1.1%, 1.3% and 0.4% in the sample of OLR54. *Methanomicrobia* exhibited an upward trend at first and then a downward trend with increased OLR.

Depending on the genus enables us to further speculate the microbial functions (Fig. 5(c)). Seventeen classified genera were recognized as fermentative acidogenic bacteria. *Brooklawnia*, *Parabacteroides*, *Bacteroides* and *Lactococcus* were present in the samples of all the OLRs. These species can utilize several carbohydrates to produce organic acids (e.g., lactate, butyrate, propionate, acetate and formate) and ethanol (Yoon et al., 2007; Buschhorn et al., 1989; Strepis et al., 2016; Yamada et al., 2007; Betian et al., 1977). The relative abundance of *Brooklawnia* and *Parabacteroides* showed an increasing trend as OLR increased. *Bacteroides* exhibited a trend of decreasing at first and then increasing with increase in OLR. On the contrary, the number of *Lactococcus* was increased at first and then decreased. Its portion was 2.0% and 2.4% in samples of OLR12 and OLR36, respectively. *Leuconostoc, Butyricicoccus* and *Veillonella* existed in samples of OLR36 and OLR54. The first two genera showed an upward trend whereas the latter dropped as OLR was raised to 54 kgCOD/($m^3 \cdot d$) from 36 kgCOD/($m^3 \cdot d$). *Chryseobacterium*, *Longilinea* and *Aerococcus* occurred in low OLR condition (12 kgCOD/($m^3 \cdot d$) and their relative abundance was 1.0%–3.6%. *Anaeroarcus* with a relative abundance of 1.4% was unique in the sample of OLR36. Five genera (*Prevotella*, *Streptococcus*, *Oribacterium*, *Oscillibacter* and *Megasphaera*) only existed in the OLR of 36 kgCOD/($m^3 \cdot d$). Among them, the relative abundance of *Prevotella* reached 14.8% and became the dominant fermentative acidogenic bacteria in the sample of OLR54.

As shown in Fig. 5(c), two genera (*Desulfovibrio and Syntrophobacter*) were strongly correlated with hydrogen-producing acetogens. In low-sulfate media, some strains from the genus *Desulfovibrio* (such as *D. vulgaris* and *D. desulfuricans*) could degrade ethanol and lactate to produce acetate, H_2, and CO_2 through synergistic association with H_2-utilizing methanogens (Bryant et al., 1977). Under the OLR of 12 kgCOD/($m^3 \cdot d$) condition, the relative abundance of *Desulfovibrio* was only 0.3% and decreased to 0% in OLR36. However, its richness achieved a maximum (2%) in the sample of OLR54. Species from the genus *Syntrophobacter* are mainly identified as propionate-oxidizing bacteria (Wallrabenstein et al., 1995; Harmsen et al., 1998). The largest percentage (1%) of *Syntrophobacter* occurred in the sample of OLR12 and it showed a decreasing trend as the OLR increased. Members of *Acetobacterium* are described as homoacotogens, which can convert glucose into acetate by heterotrophic pathways or reduce CO_2 to acetate using chemolithoautotrophic way (Buschhorn et al., 1989; Müller et al., 2008). The percentage of *Acetobacterium* changed among the different OLR conditions but did not show any clear trend.

In this study, three genera (*Methanospirillum*, *Methanomethylovorans* and *Methanosaeta*) were described as methanogens (Fig. 5c). *Methanospirillum* is considered as hydrogenotrophic methanogens and it can use H_2/CO_2 and formate as substrates for growth (Liu and Whitman, 2008). The activity of hydrogenotrophic methanogens is essential to maintain low hydrogen partial pressure in methane fermentation systems (Demirel and Scherer 2008). It was unique in OLR54 and the relative abundance was 0.1%. Some strains from *Methanomethylovorans* are considered as obligately methylotrophic methanogens (Lomans et al., 1999). The relative abundance of *Methanomethylovorans* was 0.1% in the sample of OLR12 and was increased by 1 time when the OLR was increased to 36.0 kgCOD/($m^3 \cdot d$), but was not detected in OLR54. *Methanosaeta* was the major acetotrophic methanogen in all samples and its relative abundance was 1.1%–2.0%. *Methanosaeta* is a specialist in utilizing acetate and is identified as the dominant acetotrophic methanogen in various anaerobic reactors with low concentration of acetate (Zheng and Raskin, 2000; Liu and Whitman, 2008). *Methanosaeta* is common acetotrophic methanogen in granular sludge reactors (Uyanik, 2003; Keyser et al., 2006; Worm et al., 2009). The granulation of sludge is favorable for achieving a high rate of methanogenesis. This is because granular cultures provides a close spatial microbial proximity compared to suspended cultures (Amani et al., 2012; Stams et al., 2012). The relative abundance of acetotrophic methanogens in each sample was much higher than hydrogenotrophic methanogens by 9-15-fold, indicating that methane was mainly produced by acetate cleavage in this UASB reactor. In methanogenic environment, approximately 70%–80% of methane is produced by acetate oxidation (Liu and Whitman, 2008). A previous study also showed that the amount of acetotrophic methanogens in a UASB reactor was obviously higher than that of hydrogenotrophic methanogens (Ban et al., 2013b).

Apart from sugar, sugar refinery wastewater also contained a small amount of protein. In methanogentic environments, proteins are hydrolyzed to peptides and amino acids, which are subsequently fermented to VFAs and finally converted to methane and CO_2 (Antwi et al., 2017). In the present study, two genera (*Aminiphilus* and

Sedimentibacter) were recognized as amino acid degrading bacteria (Díaz et al., 2007; Imachi et al., 2016). The largest percentage of *Aminiphilus* was in the sample of OLR12 (up to 5.4%) and showed a decreasing tendency as OLR increased. While *Sedimentibacter* was unique in OLR36 and its portion was 1.3%.

3.5 Shift of dominant bacteria and archaea as OLR increase

The anaerobic digestion of organic matter is completed by four different trophic microorganisms (fermentative acidogenic bacteria, hydrogen-producing acetogens, homoacetogens and methanogens). Table 3 shows the shift of dominant bacteria and archaea with OLR increase. Under the OLR of 12.0 kgCOD/($m^3 \cdot d$) condition, the total percentage of fermentative acidogenic bacteria was 9.2% and the dominant fermentative acidogenic bacteria from five genera (*Bacteroides*, *Trichococcus*, *Chryseobacterium*, *Longilinea* and *Aerococcus*). Antwi et al. reported that *Trichococcus* was one of the dominant fermentative bacteria in a UASB treating potato starch processing wastewater at the OLR of 13.2 kgCOD/($m^3 \cdot d$) (Antwi et al., 2017). However, the *Clostridium* (24.1%) and *Longilinea* (18.0%) were the dominant fermentative bacteria in an expanded granular sludge bed (EGSB) reactor for beet sugar industrial wastewater treatment under the OLR of 3.2 kgCOD/($m^3 \cdot d$) (Ambuschi et al., 2016). When the OLR was increased to 36 kgCOD/($m^3 \cdot d$) and 54 kgCOD/($m^3 \cdot d$), the composition and distribution of fermentative acidogenic bacteria changed significantly. The sample of OLR36 was dominated by *Lactococcus* (6.5%), *Trichococcus* (2.4%), *Anaeroarcus* (1.4%) and *Veillonella* (1.8%). The total number of fermentative bacteria in OLR36 accounted for 14.1% of total detectable microorganisms. Previous studies indicated that the genera *Lactococcus* was one of the dominant fermentative bacteria in a UASB reactor containing glucose/lactate or molasses as substrates under the OLR of 2.9–14.4 kg COD/($m^3 \cdot d$) conditions (Kim et al., 2015; Zhang et al., 2015a). As shown in Table 3, the diversity of fermentative acidogenic bacteria was markedly increased under OLR of 54 kgCOD/($m^3 \cdot d$) condition. Also, the total percentage of

Table 3 Shift of dominant bacteria and archaea as OLR increase

Trophic group	Taxon classification	Relative abundance (%)		
	Genus	OLR12	OLR36	OLR54
Fermentative acidogenic bacteria	*Brooklawnia*	0.2	0.3	1.3
	Parabacteroides	0.4	0.9	2.3
	Bacteroides	2.3	0.5	1.2
	Lactococcus	0.5	6.5	1.4
	Trichococcus	2.0	2.4	—
	Chryseobacterium	3.6	—	—
	Longilinea	1.2	—	—
	Aerococcus	1.0	—	—
	Leuconostoc	—	0.2	1.2
	Butyricicoccus	—	0.1	1.8
	Anaeroarcus	—	1.4	—
	Veillonella	—	1.8	1.0
	Prevotella	—	—	14.8
	Streptococcus	—	—	6.6
	Oribacterium	—	—	1.8
	Oscillibacter	—	—	1.4
	Megasphaera	—	—	6.2
Hydrogen-producing acetogens	*Desulfovibrio*	0.3	—	2.0
	Syntrophobacter	1.0	0.5	—
	Geobacter	0.2	0.7	—
Homoacetogens	*Acetobacterium*	0.5	1.3	—
Methanogens	*Methanosaeta*	1.6	2	1.1
	Methanospirillum	—	—	0.1
	Methanomethylovorans	0.1	0.2	—

fermentative acidogenic bacteria was highest (41.0%) in the sample of OLR54. Twelve genera became the predominant fermentative acidogenic bacteria in OLR54. They were *Brooklawnia* (1.3%) from the phylum *Actinobacteria*, *Parabacteroides* (2.3%), *Bacteroides* (1.2%) and *Prevotella* (14.8) from the phylum *Bacteroidetes*, *Leuconostoc* (1.2%), *Lactococcus* (1.4%), *Streptococcus* (6.6%), *Oribacterium* (1.8%), *Butyricicoccus* (1.8%), *Oscillibacter* (1.4%), *Megasphaera* (6.2%) and *Veillonella* (1.0%) from the phylum *Firmicutes*. The high microbial diversity and percentage of fermentative bacteria made a high metabolic capacity was achieved in the fermentative acidogenic stage under high OLR condition (54.0 kg COD/($m^3 \cdot d$)).

There were three genera were identified as hydrogen-producing acetogens in this study. These hydrogen-producing acetogens showed a clear succession with OLR increase. At low OLRs, the propionate-oxidizing bacteria (*Syntrophobacter*) were the major hydrogen-producing acetogens. The members of *Syntrophobacter* can degrade propionate by methylmalonyl-coenzyme A pathway in the presence of methanogens (Wallrabenstein et al., 1995; Harmsen et al., 1998). Similarly, some previous studies also indicated that the genus *Syntrophobacter* was the dominant propionate-oxidizing bacteria in the UASB reactor treating sucrose, butyrate-propionate-acetate and propionate process at the OLR of 2.9–14.4 kg COD/($m^3 \cdot d$) conditions (Férnandez et al., 2008; Worm et al., 2009; Kovacik Jr et al., 2010; Zhang et al., 2015a). Shigematsu et al. found that high dilution rates favored the growth of *Pelotomaculum* spp. whereas low dilution rates stimulate *Syntrophobacter* spp. in chemostat experiments containing propionate as the sole carbon source (Shigematsu et al., 2006). An increase in OLR to 36.0 kgCOD/($m^3 \cdot d$) from 12.0 kgCOD/($m^3 \cdot d$) resulted in *Syntrophobacter* and *Geobacter* becoming the predominant hydrogen-producing acetogens and their relative abundance was similar. The members of *Geobacter* could degrade ethanol by syntrophic association with methanogens (Summers et al., 2010). When the OLR was further increased to 54.0 kg COD/($m^3 \cdot d$), the main hydrogen-producing acetogens was substituted with *Desulfovibrio*, which can convert ethanol and lactate to acetate, H_2 and CO_2 (Bryant et al., 1977). It can be deduced from the distributed characteristics of hydrogen-producing acetogens that the propionate-type fermentation was formed under the OLR of 12.0 kgCOD/($m^3 \cdot d$) condition and then shifted to mix-acid-type fermentation as OLR increased. These intermediate products (propiontate, ethanol and lactate) were further converted to acetate and H_2/CO_2 by the hydrogen-producing acetogens.

As the last step, methanogenesis is important for maintaining efficient anaerobic digestion. The important role of methanogens is to eliminate acetate and H_2/CO_2 from hydrolysis, acidogenesis, acetogenesis and then promote the reaction process. The diversity of methanogens was low compared with bacteria. In the present study, *Methanosaeta* was the dominant acetotrophic methanogens during the whole operation period of UASB. Its relative abundance was 1.6% (OLR12), 2.0% (OLR36) and 1.1% (OLR54). Although the percentage of acetotrophic methanogens (*Methanosaeta*) in the sample of OLR54 was lower than in other samples, the biomass (28.5 gMLVSS/L) and specific methane production rate (827.0 LCH_4/(kg VSS/d) achieved maximums, leading to the COD removal of 92.0% was reached under the OLR of 54.0 kgCOD/($m^3 \cdot d$) condition. In addition, homoacotogens might reduce CO_2 to acetate by chemolithoautotrophic way under the OLR of 12.0 and 36.0 kgCOD/($m^3 \cdot d$) conditions, resulting in the acetate was increased, which further caused an increase in the abundance of *Methanosaeta*. The methylotrophic methanogen (*Methanomethylovorans*) was observed in the samples of OLR12 and OLR36. Whereas *Methanospirillum* was the hydrogenotrophic methanogens in OLR54. The strains from the genus *Methanomethylovorans* are able to use methylated compounds (such as methanol, dimethylamine, monomethylamine and Trimethylamine), but cannot utilize H_2/CO_2, formate or acetate (Lomans et al., 1999; Jiang et al., 2005; Cha et al., 2013). However, the present study did not find the microorganisms that produced methylated compounds in the samples of OLR12 and OLR36. The mechanism related to this phenomenon warrants further investigation. Under the OLR of 12.0 and 36.0 kg COD/($m^3 \cdot d$) conditions, the H_2/CO_2 from fermentative and hydrogen-producing acetogenic stages might be converted to acetate through a chemolithoautotrophic way by homoacotogen (*Acetobacterium*), resulting in the absence of hydrogentrophic methanogens in the samples of OLR12 and OLR36. Indeed, *Acetobacterium* was observed in OLR12 and OLR36, but did not be detected in OLR54. These results indicated that the acetate cleavage was the main methanogenic acetate degradation pathway in this UASB system during the whole operation period. In addition, the methylotrophic methanogenic pathway was replaced by the hydrogenotrophic methanogenic pathway when the OLR was increased to 54.0 kgCOD/($m^3 \cdot d$) from 12.0 kgCOD/($m^3 \cdot d$).

4 Conclusions

At an OLR of 12.0–54.0 kgCOD/($m^3 \cdot d$), the COD removal reached 92.0%–95.0% with a specific methane production rate of 258–827 LCH_4/(kgVSS/d) in a UASB reactor treating sugar refinery wastewater. Pyrosequencing revealed that the relative abundance of fermentative bacteria was significantly increased to 41.0% from 9.2% when the OLR was increased to 54.0 from 12.0 kgCOD/($m^3 \cdot d$) by stepwise. The diversity of fermentative bacteria at the genus level achieved a maximum in the sample of

OLR54. *Syntrophobacter* was considered as the dominant hydrogen-producing acetogens under the OLR of 12.0 kgCOD/($m^3 \cdot d$) condition. As the OLR increased from 12.0 to 54.0 kgCOD/($m^3 \cdot d$), the dominant hydrogen-producing acetogens was substituted with *Desulfovibrio*. *Methanosaeta* was the only detected acetotrophic methanogens and its relative abundance was 1.1%–2.0%. The *Methanomethylovorans* was observed in the samples of OLR12 and OLR36, while *Methanospirillum* was unique in OLR54. The information will assist in improving the operation and control of UASB system under high OLR conditions.

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