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Characterization of extracellular polymeric substances and microbial diversity in anaerobic co-digestion reactor treated sewage sludge with fat, oil, grease

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HIGHLIGHTS

- Two anaerobic co-digesters of sewage sludge and fat, oil, grease operated 180d.
- Fat, oil, grease addition enhanced anaerobic co-digestion performance significantly.
- Sequencing technology comprehensively revealed the dynamic change of microbial communities.
- Co-digestion simulated the degradation of extracellular polymeric substances.

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ABSTRACT

Performance of co-digesters, treated of sewage sludge (SS) with fat, oil and grease (FOG), were conducted semi-continuously in two mesophilic reactors over 180 days. Compared with SS mono-digestion, biogas production and TS removal efficiency of co-digestion were significantly enhanced up to 35% and 26% by adding upper limit FOG (60% on VS). Enhancement in co-digestion performance was also stimulated by the release of extracellular polymeric substances (EPS), which was increased 40% in both loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS) than that of mono-digester. Based on high-throughput sequencing (HTS), analysis of microbial 16S rRNA gene comprehensively revealed the dynamic change of microbial community. Results showed that both bacterial and archaeal undergone an apparent succession with FOG addition, and large amount of consortium like *Methanosaeta* and *N09* were involved in the process. Redundancy analysis showed the acetoclastic genera *Methanosaeta* distinctly related with biogas production and EPS degradation.

1. Introduction

Sustainable management and efficient disposal of sewage sludge (SS) have become an issue of particular concern for resent decades due to the rapid increasing amounts and variable operation issues. Anaerobic digestion (AD) of organic components convert to biogas is acknowledged as the most cost-effective way for waste sludge treatment and green-energy production. SS, the inevitable by-product of municipal waste water treatment plants (WWTPs), was widely used for AD as the main substrate (Mata-Alvarez et al., 2014). However, the efficiency of SS mono-digestion is largely limited by the low carbon-to-nitrogen (C/N) ratio (6–9) and relatively slow hydrolysis process (Toreci et al., 2009). Consequently, applying some high organic wastes as possible substrates for anaerobic co-digestion (ACoD) has attracted great interest due to the advantage of adjusting the C/N ratio, increasing the methane yields, diluting harmful substances, and also mediating the hydrolysis process (Kawai et al., 2014). Therefore, it seems clear that co-digestion of high organic wastes would be a promising method and this opportunity that requires further research.

Fat, oil and grease (FOG) collected from the food service industry has been cited as a desirable co-digestion substrate due to its high organic contents and excellent biodegradability (Kabouris et al., 2009). On the premise of the system stability, higher FOG means higher waste treatment capacity and methane yield.

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Nevertheless, ACoD with excessive FOG has been regarded as an adverse condition for the application. Excessive FOG presents important challenges related to the accumulation of volatile fatty acids (VFAs), which result in the acidification of systems (Kawai et al., 2014). Therefore, it is significant to investigate the relationship of FOG addition and digester performance, and conduct a suitable ratio of FOG to balance the digestion capacity, methane production and sustainable operation.

As a syntrophic biological degradation process, AD was related to various microbe according to its origin, substrate, operational condition and environment parameters (Hagen et al., 2014). At least three functional groups of microorganisms mainly regulated the mutual metabolic interactions under anaerobic conditions. The first community hydrolyzes complex polymeric substances (e.g. lipids, cellulose and protein) to fundamental structural building blocks such as glucose and amino acids. The second community subsequently ferments these products to fatty acids, acetate and hydrogen. This acidogenesis process was reported to be the most important step among decomposition processes of anaerobic digestion. The third community converts acetate and hydrogen to methane and carbon dioxide (methanogenesis). Accordingly, stable operation and effective conversion of organic matter to biogas relies on all of these species living together in dynamic equilibrium. It is well known that, a deeper investigation of the syntrophic cooperation and microbial community evolution, as well as the optimization of FOG addition, would provide valuable insight into the viable utilization of anaerobic digestion for sludge treatment. Studies of digestion microbial communities focused on reactors with stable performance have been extensively reported in literature (Pope et al., 2013). However, to our knowledge, fewer efforts have been made to evaluate the shifts in microbial communities linked to the instability and variation of anaerobic co-digesters treating SS and FOG (Ziganshin et al., 2013). Nowadays, comprehensive understanding depth of the microbial community is impeded by the low sequencing depth. Many traditionally molecule technology approaches, such as denaturing gradient gel electrophoresis (DGGE) and (terminal restriction fragment length polymorphism) T-RFLP, are limited to reveal higher microbial diversity in the environment. Based on the analysis 16S rRNA gene, high-throughput sequencing (HTS) technologies such as illumina Hiseq 2500 have been applied to fully explore the microbial composition and diversity in the environment, which can provide less biased, more robust and higher coverage information. This method could support many unrevealed details about the mechanism of microbial response to the FOG enhancement.

Beside, no previous work comprehensively described microbial metabolism and functional community structures in the anaerobic co-digester of SS with FOG yet.

Extracellular polymeric substances (EPS) of sludge aggregates are a general metabolism of microbial consortia. Yu et al.(2012) pointed out that EPS was partly result from the microbial metabolism, which is affected by the microbial community composition and its activity. The production and composition of EPS mainly come from bacterial active secretion, cell surface material shedding, cell lysis and desorption from the surface of an external matrix (Sheng et al., 2010). Different growth conditions with different quantity of EPS expressed, which in turn to exert impacts on the anaerobic digestibility and biogas yield. Contributions of different microorganism to EPS secretion are not yet clear. Consequently, a comparative study on the substrates degradation pathways and the by-products of EPS subfractions could provide additional information of long-term effects of microbial activity on anaerobic co-digester.

In order to understand these facts, two group (control and test system) continuously stirred tank reactors (CSTR) were conducted to test the co-digestion performance of SS and FOG over 180 days. Phylogenetic differentiation of bacterial and archaeal communities in two digesters was analyzed by HTS. Furthermore, the relationship between metabolism EPS subfractions and digestion process was revealed by redundancy analysis (RDA). This gave a unique opportunity to study the microbial community dynamics and the metabolism degradation in the FOG enhanced ACoD reactors.

2. Materials and methods

2.1. Inoculum and digestion substrates collection

Waste activated sludge (WAS) and dewatered sludge (DS) were collected from a WWTP in Changsha, China. FOG was collected over 5 consecutive working days from a typical Chinese local restaurant in Changsha. The top layer of FOG was separated from the settable solids and water layers. The collected samples were transported to laboratory within 1 h and stored at 4 °C for no more than 3 days. Pre-treatment should be practiced before pumping WAS and DS into reactors, as described by Xu et al. (2015). All of them were brought to room temperature before utilization. The main characteristics of inoculum and digestion substrates are summarized in Table 1.

2.2. Reactor set-up and running mode

Anaerobic co-digestion of SS and FOG was conducted in two mesophilic CSTRs with working volume of 1.0 L each. Corresponding operation strategies are shown in Table 2. Control reactor (C) lasted for a total progress of 180 days without FOG addition, receiving mono-sludge at a "safe" organic loading rate (OLR) of 3.0 g VS L^{-1} d^{-1} (g volatile solids per reactor volume per day). The operational conditions of test reactor (T) were the same as those in the control test for the first 45 days. Next, the co-digestion stage started on day 46 with 1–3 g VS FOG were introduced in the following 3 phases (45 days per phase). As a consequence of the addition, OLR slightly increased to 4, 5, 6 g VS L^{-1} d^{-1}, respectively. Both two reactors were identically operated at 35 ± 1 °C water bath with a 15 days hydraulic retention time (HRT). Each reactor was initially inoculated 70% seed sludge and 30% co-substrates (Xu et al., 2015). From the next day, the co-substrates were prepared daily and fed to the reactors. 66 mL of digested materials was extracted with a vacuum pump once a day, always following the same volume of substrate was added. The pH values were initially adjusted to approximately 7.0 by adding 1M NaOH/HCl solution.

2.3. Reactor performance and EPS analysis

The daily biogas production was quantified by water-replace method. Shimadzu gas chromatograph (GC 2010) was used to measure the methane (CH_4) content. The GC was used with a thermal

Table 1
Main characteristics of inoculum and digestion substrates.

Item	Inoculum	Substrates	
	WAS	DS	FOG
Density (g mL^{-1})	0.91	1.16	0.92
pH	7.72 ± 0.1	7.53 ± 0.1	4.16 ± 0.1
sCOD (g L^{-1})	0.6 ± 0.2	18.3 ± 0.2	137.4 ± 0.2
TS (g L^{-1} substrate)	46.2 ± 0.5	314.5 ± 0.5	856.1 ± 0.5
VS (g L^{-1} substrate)	15.6 ± 0.5	141.6 ± 0.5	827.8 ± 0.5
VS/TS (%)	33.8 ± 0.5	45.0 ± 0.5	96.7 ± 0.5
Volatile fatty acid (mg L^{-1})	673.5 ± 17	567.8 ± 14	672.5 ± 21
Alkalinity (mg L^{-1} as $CaCO_3$)	1718.9 ± 20	817.4 ± 10	1344.5 ± 20
C/N (w/w)	6.6 ± 1.3	6.9 ± 0.7	19.3 ± 2.4

WAS = waste activated sludge; DS = dewatered sludge; FOG = fat, oil, grease.

Table 2
Operation mode and general performance of 180 days anaerobic digestion.

Item	Control				Test			
Phase	I (0–45d)	II (46–90d)	III (91–135d)	IV (136–180d)	I (0–45d)	II (46–90d)	III (91–135d)	IV (136–180d)
Feeding	DS	DS	DS	DS	DS	DS + FOG	DS + FOG	DS + FOG
OLR (g VS L^{-1} d^{-1})	3	3	3	3	3	4	5	6
pH*	7.51 ± 0.1	7.43 ± 0.1	7.45 ± 0.1	7.39 ± 0.1	7.48 ± 0.1	6.91 ± 0.1	7.05 ± 0.1	7.23 ± 0.1
VFA/Alk*	0.35 ± 0.2	0.27 ± 0.2	0.27 ± 0.2	0.28 ± 0.2	0.37 ± 0.2	0.54 ± 0.2	0.39 ± 0.2	0.30 ± 0.2
sCOD*	440.6 ± 45	553.1 ± 56	576.2 ± 60	542.0 ± 73	451.7 ± 54	1148.5 ± 90	1055.4 ± 70	678.7 ± 73
TS (g L^{-1})*	42.2 ± 0.7	38.8 ± 1.1	32.9 ± 0.7	26.5 ± 0.1	43.1 ± 0.8	43.3 ± 1.0	37.1 ± 0.2	31.3 ± 0.5
VS (g L^{-1})*	13.8 ± 0.3	16.0 ± 0.8	17.2 ± 0.5	14.7 ± 0.9	13.6 ± 0.5	22.1 ± 0.8	21.4 ± 0.9	17.3 ± 0.2
CH_4 (%)*	56.4 ± 2.8	64.4 ± 7.3	62.4 ± 7.5	65.4 ± 1.9	54.4 ± 2.7	85.1 ± 6.1	83.7 ± 9.0	75.5 ± 3.4
PN/PS in LB-EPS*	1.00	0.98	1.36	0.53	1.00	1.64	1.89	1.45
PN/PS in TB-EPS*	0.68	0.88	1.18	1.24	0.68	0.96	1.20	0.96

DS = dewatered sludge; FOG = fat, oil, grease; VFA = volatile fatty acid; Alk = alkalinity; PN = protein; PS = polysaccharides; LB-EPS = loosely bound EPS; TB-EPS = tightly bound EPS.
* Shown effluent data was mean value of each phase.

conductivity detector (TCD) equipped with a 2 m × 3 mm stainless-steel column packed (Porapak Q, 80/100 mesh). The digested sludge samples were collected every 15 days. Effluent samples were centrifuged at 10,000*g* for 20 min, then filtered through disposable Millipore filter units (0.45 μm pore size) for the reactor parameters analysis, including pH, volatile fatty acid (VFA), alkalinity (Alk), soluble chemical oxygen demands (sCOD) and total nitrogen (TN). pH was determined using pH meter (Mettler Toledo FE 20). VFAs was measured by a gas chromatograph (Agilent 7890A, USA) with a flame ionization detector (FID) and a DB-FFAP (Agilent, USA) column (30 m × 0.32 mm × 0.50 μm). Alk, sCOD, TN, total solids (TS) and volatile solids (VS) were quantified according to the Standard Methods (APHA, 2005).

Centrifuged pellets was used for EPS extraction by modified two-step extraction method, as described by Li and Yang (2007) Polysaccharides (PS) and proteins (PN) in EPS were measured by the anthrone method and Coomassie brilliant blue assay. All the above analyses were conducted in triplicate. The results were expressed as mean value ± standard deviation.

2.4. DNA extraction

Total genomic DNA was extracted from approximately 0.5 g settled biomass using Fast DNA® Spin kit for soil (Biomedical, USA) according to the protocol described by the manufacturer. Micro-Ultraviolet spectrophotometer (NanoDrop Inc., USA) was used to determine the quality of the genomic DNA.

2.5. 16S RNA amplification and illumina Hiseq sequencing

The variable V3–V4 region of 16S rRNA gene was amplified using barcoded 341F (5′-adaptor + barcode + CCTAYGGGRBGC ASCAG-3′) and 806R (5′-adaptor + GGACTACNNGGGTATCTAAT-3′) . PCR amplification was performed at a final volume of 20 μL following previously reported study (Yu et al., 2005). The PCR cycles started with a 98 °C for 1 min, 30 cycles of denaturation at 98 °C for 10 s, 50 °C annealing for 30 s and 72 °C for 30 s extension. A final extension at 72 °C for 5 min was included before holding at 4 °C. All the reactions were done in triplicates. PCR raw products were gel-purified using the GENECLEAN Turbo Kit (MP bio, USA) and the pure PCR production was detected by 1.5% (w/w) agarose gel. An equal volume of each amplicon was mixed to prepare amplicon pools, and then sent to Illumina sequencing (Zheng et al., 2014a).

2.6. Sequencing data analysis

Analysis of 16S rRNA sequencing data was conducted using the microbial ecology community software program Mothur. To reflect the diversity and structure of microbial community, Chao1 index and phylogenetic diversity index were calculated from each sample. The UniFrac matrix were performed with principal coordinate analysis (PCoA) and cluster analysis in R (v.2.15.0) (Zheng et al., 2014b). A phylogenetic tree of 16S rRNA genes was constructed in MEGA 5.0 using neighbor-joining method with the representative sequences at 0.03 genetic distance. Nearest relatives were retrieved from the NCBI database. The correlation between the species composition and process performance and environmental parameters were analyzed by RDA using the Canoco (v.4.5 for Windows).

3. Results and discussion

3.1. Digestion general performance

Two reactors (control and test) were carried out with different FOG contents and the corresponding experimental results are presented in Fig. 1. Operational conditions and mean values of several process parameters are also summarized in Table 2. After the adaption of 45 days, the control and test digester reached similar condition during the phase 1, in terms of TS removal efficiency, daily biogas production and methane production. In the following 3 phases, control reactor received the same substrates as initial feeding and the corresponding digestion performance were kept in a stable range. However, after the startup phase, different FOG (1–3 g VS) was introduced into test reactor as a comparison. The corresponding relative OLR increased 30%, 60% and 100% (Fig. 1a). It could be observed that TS removal efficiency increased significantly with more FOG addition, leading to increased biogas yield and volumetric methane content. TS removal efficiency reached 50% in test reactor, compared with 30% in control reactor.

Besides, mean value for biogas generation in test reactor throughout phase 3 reached 4032 mL until a large reduction was observed in phase 4 (Fig. 1c). At this point, FOG addition (represented 100% of the VS) restricted methane yield by 23.4%. A reduction of methane contents was also observed with FOG addition exceed over 2 g L^{-1} d^{-1}. It can assumed that co-digestion with more than 2 g L^{-1} d^{-1} FOG resulted in negative methane accumulation. A similar result was obtained in previous studies (Kabouris et al., 2009). The cause of this promotion/reduction in biogas generation was investigated by reviewing substrates conversion. Degradation of lipids is conducted in a specific anaerobic chain reaction and a metabolic route. Firstly, the neutral fats are hydrolyzed into free long-chain fatty acids (LCFAs) and glycerol catalyzed by extracellular lipases. Secondly, the free LCFAs are converted into acetate and H_2 by acetogenic bacteria through a β-oxidation process. Finally, methane is generated by consuming acetate and H_2 with methanogenic bacteria (Masse et al., 2002).

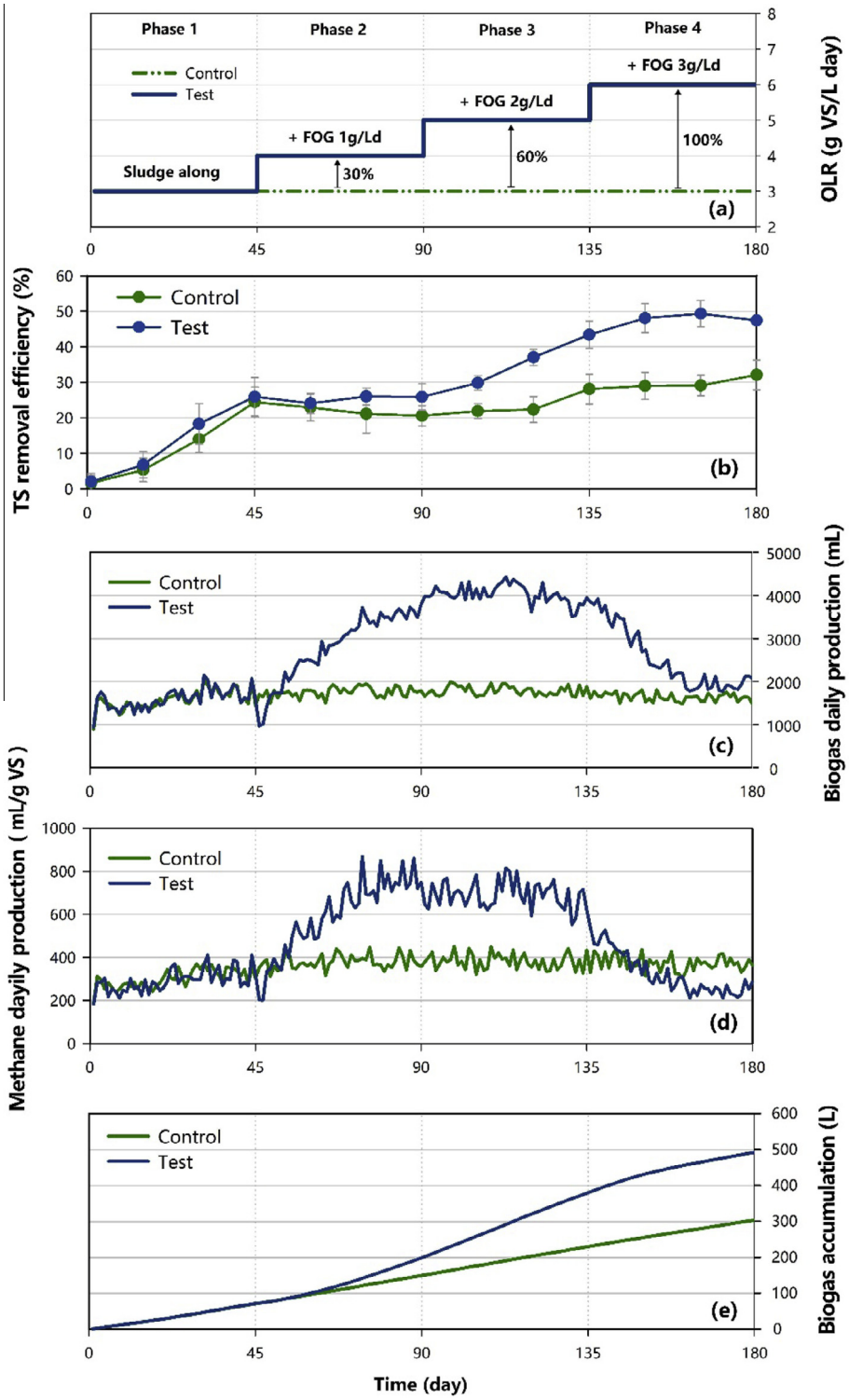

Fig. 1. Anaerobic digestion mode and general performance. (a) OLR (g VS L^{-1} d^{-1}), (b) TS removal efficiency (%), (c) biogas daily production (mL), (d) methane daily production (mL g^{-1} VS) and (e) accumulative biogas (L) in two reactors during 4 phases operation.

As a lipid-rich substrate, FOG is a readily degradable substrate for anaerobic digestion (Kabouris et al., 2009) while sewage sludge is quite refractory to hydrolysis and showed lower biodegradability. However, when the excessive dosage of FOG are introduced into the system, accumulation of LCFAs, propionate and acetate can result in a pH reduction and process restrict due to limited substrate and product transport, damaged cells, and reduced activity of stressed microbial communities (Chen et al., 2008).

Moreover, 304 L and 493 L biogas were obtained throughout the whole study, respectively (Fig. 1e). The promoted ratio of accumulative biogas (60%) was similar with the increased relative OLR (63%), which demonstrated that biogas production was closely related with the OLR of feeding substrates. Furthermore, the evolution of pH, VFA, Alk and the VFA/Alk ratios were associated with performance of the reactors throughout the experiment. During 180 days digestion, pH and VFA/Alk in the both reactors remained

a range of 7.0 ± 0.5 and 0.4 ± 0.2 without any artificial intervene (Table 2), which is generally considered as an optimal condition for ACoD degradation process. The stable situation indicates that the application of OLR (3–6 g VS L^{-1} d^{-1}) in this research is acceptable and higher treatment potential could be explored.

3.2. Variations of different EPS subfractions during ACoD

The structure of microbial EPS is generally subdivided into a two layer model. The internal layer (tightly bound EPS, TB-EPS) is composed of polymer with a certain shape and tightly bound with the cell surface. The external layer (loosely bound EPS, LB-EPS), which is consist of a loose and dispersible slime layer without an obvious edge. Published studies on the composition of EPS have been inconsistent (Ras et al., 2011; Liu et al., 2004). The ingredients and quantities of EPS are strongly dependent on the source of sample, the extraction process and the items of analysis conducted. Fig. 2 compares the overall differences of subfractions in LB-EPS and TB-EPS. Both LB-EPS and TB-EPS obtained from the two reactors showed an "*n*" shape along with digestion time. Further, EPS in test reactor varied more significant with progressive addition of FOG. As pointed out in previous work, the degradation of substrates and release from inherent microbial consortia are considered as the two important resources in this research (Nielsen et al., 1997). Based on the operation strategy of CSTR, EPS fluctuation attributed to the substrates degradation aspect was controlled by receiving the equivalent amounts of feeding identically. In this word, EPS fluctuation observed in current experiment can be largely attributed to the response of microbial activity to environment parameters. Co-digestion of sludge with FOG simulated the microbial activity of some particular consortia. After experiencing an adaption process, microbes could utilize FOG for self-growth and metabolisms while in turn to stimulate the excretion of EPS. Thus, the performance of ACoD maintained stable, and methane production was promoted greatly. As a result, more EPS were obtained in the enhanced phase 3 (90–135 days). Also, the essential role played by EPS in the toxicity response of microbial aggregates to LCFAs has drawn much attention. The addition of FOG contributed to a stepwise rise in EPS might be associated with the acute toxicity of LCFAs, which resulted in the lysis of some planktonic cells in bulk liquor or absorbed on the surface of sludge flocs and, subsequent release of biopolymers (Lu et al., 2014). However, a further FOG addition (phase 4) did not result in greater methane productivity (Fig. 1d). This can be explained by the direct inhibition of LCFAs, or by the fact that the biomass could not degrade the extra organic loading (6 g VS L^{-1} d^{-1}). The restriction of microbial activity resulted in the reduction of EPS accumulation in phase 4.

Furthermore, TB-EPS obtained from the microbial aggregates were always higher than that of LB-EPS (Fig. 2). Such a discrepancy may be explained by the fact that harsh external force was applied in the extraction for TB-EPS (heating in 60 °C water bath and vortexed twice). Besides, outer layer EPS (LB-EPS) are readily degraded in ACoD process, with a loose and dispersible slime layer, also should be taken into account. EPS are mainly represented by PN and PS. Differences were further highlighted when the PN/PS ratio was calculated on the LB-EPS and TB-EPS (Table 2). Test reactor contains a higher portion of PN than control reactor (PN/PS ratio = 1.00–1.89 in LB-EPS and 0.68–1.20 in TB-EPS). In general, the degree of change in PN appeared to be more significant than that in PS. PS and PN are believed to play an important role in the structure, properties and functions of sludge aggregates. Because of its importance and complexity, the influence of EPS subfractions on the sludge handing has been highlighted. The variation in the PN contents might be attributed to the large amounts of exoenzymes existed in the microaggregates. Sponza (2003) also reported glucose and acetate, the readily degradation and uptake of biodegradable organic substrates lead to a high level of exoenzymes in the EPS matrix.

3.3. Overall analysis of HTS data

Effluent samples for bioinformatics analysis were collected at 45, 90, 135 and 180 days both in control (C1–C4) and test (T1–T4) reactors, represented 4 typical sampling date of each phase. A total of 289,192 and 288,733 high-quality reads (average length of approximately 453 bp) were obtained from two systems, after filtering and normalization. 7470, 7214, 4282, 4157 operational taxonomic units (OTUs) at 3% distance were detected for C1–C4 and 7432, 5467, 5701, 4035 for T1–T4, respectively. The observation of OTUs showed that bacterial community was more diverse than archaeal community (Fig. S1). Also, OTUs obtained from the sequencing contained a large proportion of unassigned representatives at different phylogenetic taxa level. Almost half of bacterial and archaeal genus have not been allocated into genus level. Two α-diversity indexes (Chao1 index and phylogenetic diversity) were calculated to estimate the richness and diversity of the microbial community (Fig. S2). Based on the Chao1 index, FOG treated reactor had higher bacterial/archaeal species richness, the maximum theoretical OTUs were 1,21558 for test reactor and 1,1985 for control reactor, respectively. According to the phylogenetic diversity, the communities developed in the test reactor were more diverse than that of control reactor. Notably, the richness and diversity of microbial community both in two reactors were reduced along with digestion time. Further, PCoA was used to estimate the discrepancy and similarity of microbial community

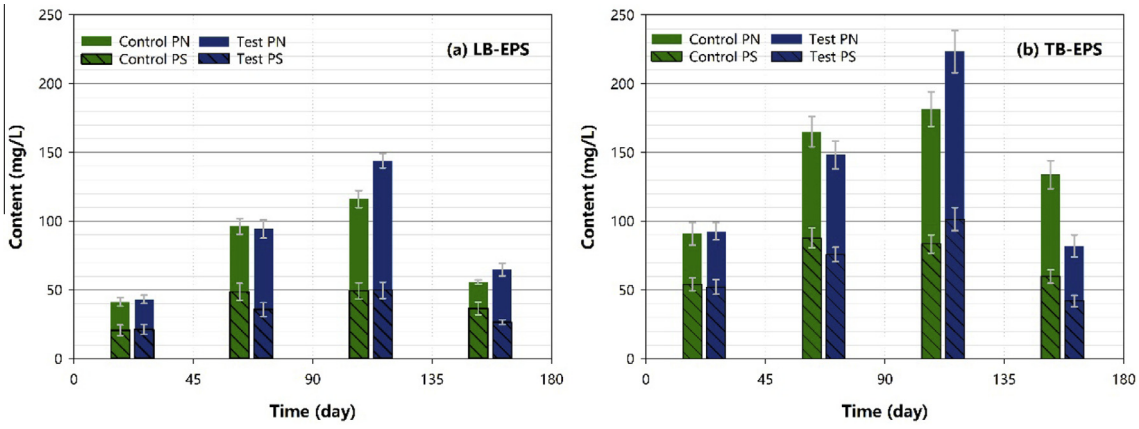

Fig. 2. Different EPS subfractions of (a) loosely bound EPS (LB-EPS) and (b) tightly bound EPS (TB-EPS) in two reactors.

(Fig. S3). Weighed PCoA analysis reflected the community diversity and richness, the principal components 1 and 2 accounted for 48.3% and 16.7% of the total community variations. Based on the distribution distance, co-digestion samples (T2–T4) were well separated from the mono-digestion samples (C2–C4). This result indicated that the microbial community in co-digesters undergoes an apparent ecological succession.

3.4. Microbial phylotypes at different taxonomy level

Phylogenetic analysis of the 16S rRNA gene sequences was assigned to different phylogenetic taxa to explore the dynamic of microbial consortia. Clear changes were observed in Figs. 3 and 4. Generally, the adaptation period (phase 1) did not significantly affect the microbial composition, since the community of C1 and T1 showed high similarity on each taxonomy level. This result also proved that two anaerobic system achieved an identical condition after 45 days adaptation, as discussed in Section 3.1, and the results of following FOG test were reasonable. After that, the microbial community of the test system showed much difference from the control system, which revealed the microbial structure in two reactors were disturbed by FOG significantly.

Actinobacteria, *Firmicutes*, *Bacteroidetes*, *Chloroflexi* and *Proteobacteria* were the most 5 predominant bacterial phyla accounting for 28.4%, 22.9%, 12.5%, 4.0% and 3.5% of total 16S rRNA gene sequences, respectively (Fig. 3). Archaeal representatives were much less diverse than bacterial. The majority of the archaeal OTUs were assigned to phyla *Euryarchaeota* (16.9%), which is a deep branch composed methanogenic organisms. In the start phase, C1 and T1 were identically comprised of 30% *Bacteroidetes*, 28% *Actinobacteria*, 14% *Firmicutes*, 5% *Chloroflexi* and 5% *Proteobacteria*, in addition to 5% *Euryarchaeota*. After that, a great relative intensity of abundance of phyla *Firmicutes* and *Euryarchaeota* could be detected at the co-digestion reactor (T2–T4). While the mono-digestion system were dominated by *Actinobacteria*, which was fade out gradually when adding FOG to the test system from phase 2. Until the phase 4, a dramatic reduction of *Euryarchaeota* and a stepwise increase of *Actinobacteria* were detected. Other three main phyla, *Bacteroidetes*, *Chloroflexi* and *Proteobacteria* were slightly restricted in phase 2 and 3. The predominant phylum composition of *Firmicutes*, *Chloroflexi* and *Proteobacteria* also have been reported as the typical anaerobic digester biomass (Rivière et al., 2009). *Firmicutes*, which are reported to be a critical role in the anaerobic hydrolysis and acidification process (Bertin et al., 2012), were highest in T2 (56.5 %), and detected to be lower in T3 (32.6%) and lowest in T4 (10.4%). Leven et al.(2007) reported *Firmicutes* could produce cellulases, lipases, proteases and other extracellular enzymes that carry out the degradation of substrates (including protein, lipids, lignin, cellulose, sugars and amino acids). Its lack may explain the poorer performance in phase 4. Moreover,

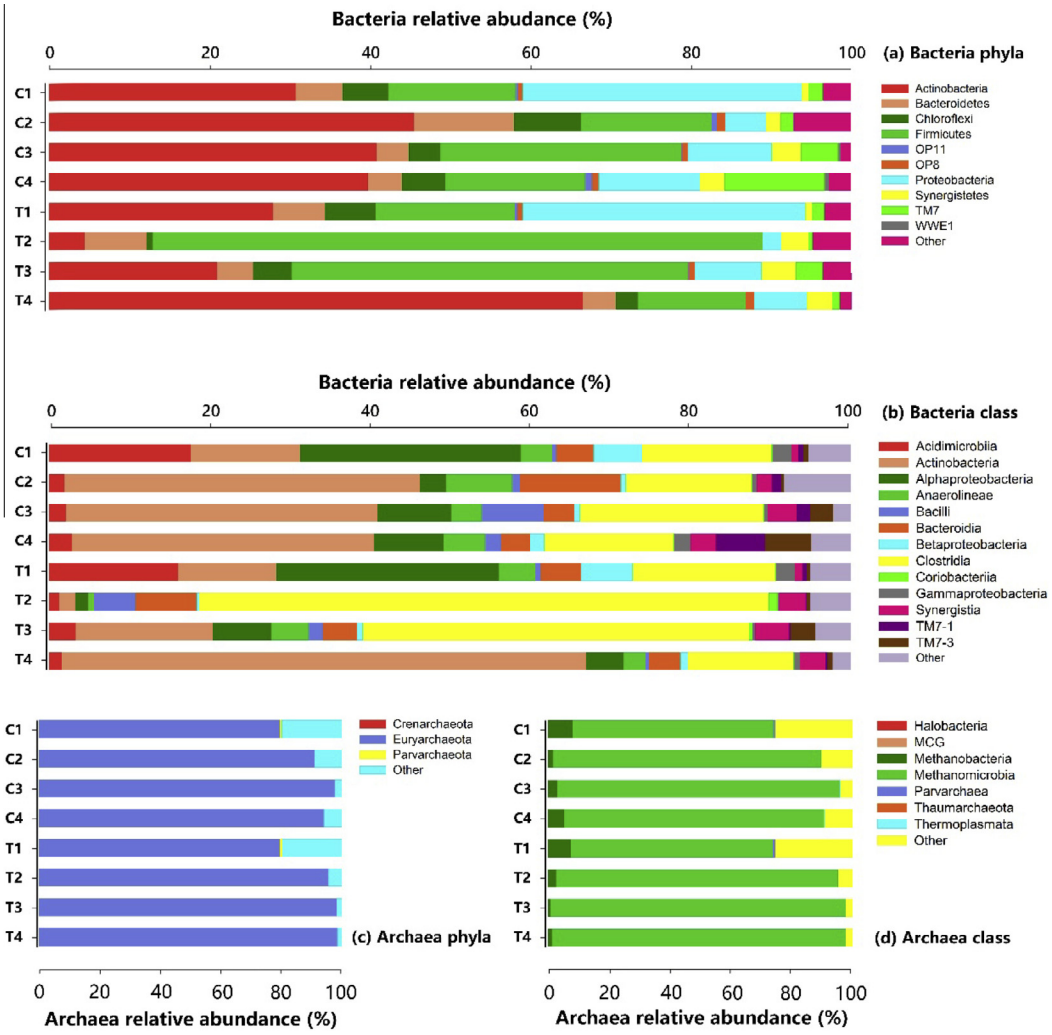

Fig. 3. Dynamic of microbial phylotypes at (a) bacteria phyla level, (b) bacteria class level, (c) archaea phyla level and (d) archaea class level. Fractions are labeled with the relative abundance of top 10 OTUs in each samples.

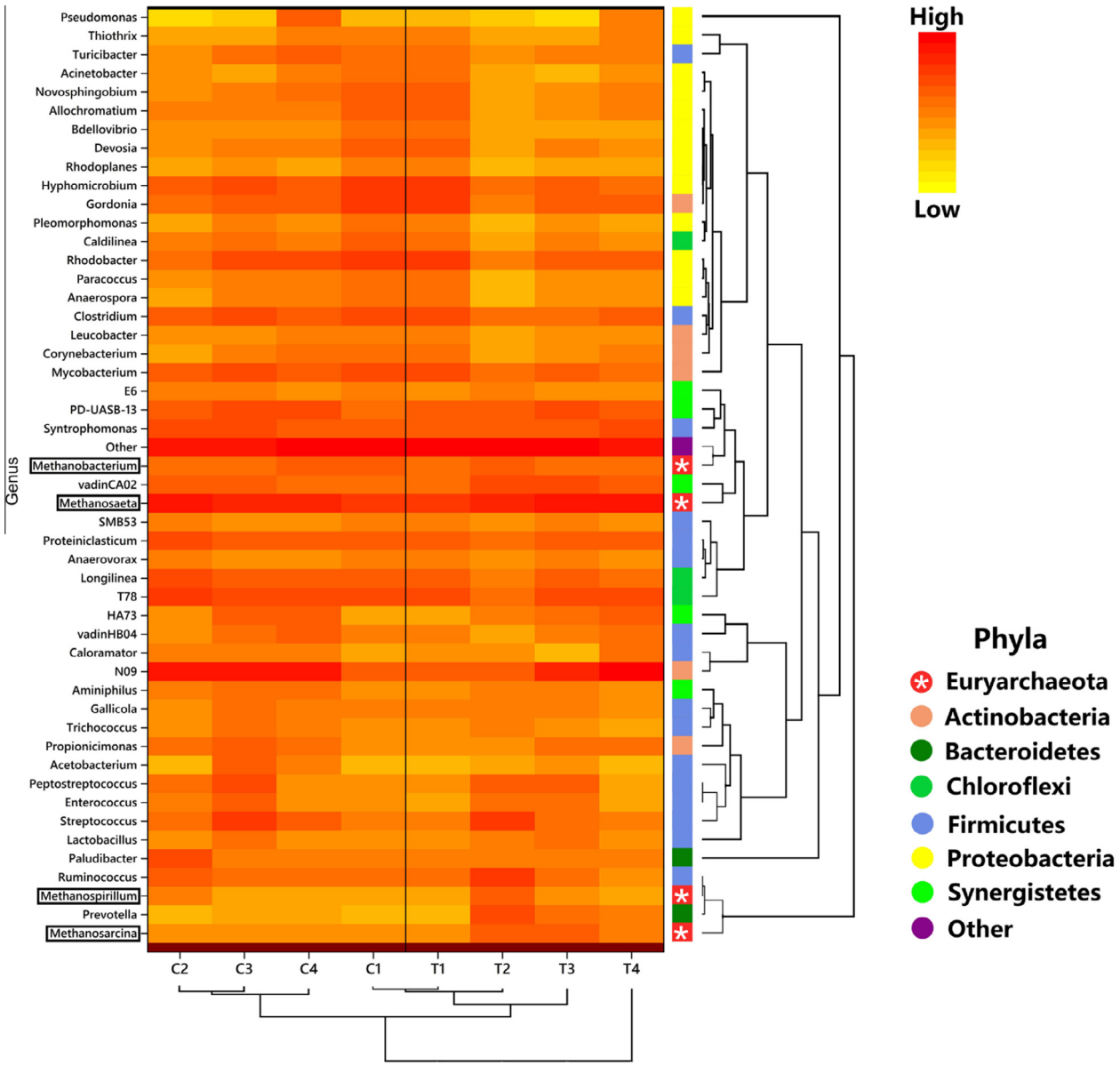

Fig. 4. Hierarchical cluster analysis of microbial communities among the 8 samples. The *Y*-axis is the clustering of the top 50 abundant genus. Different samples were clustered based on complete linkage method. The color intensity of scale indicates relative abundance of each genus. Asterisk represent the genus affiliated to archaea kingdom, others represent the genus affiliated to bacteria kingdom.

the absence of *Firmicutes* could also indicate that there were large amounts of LCFAs in T4 because LCFAs were reported to inhibit such Gram-positive bacteria (Galbraith and Miller, 1973). *Bacteroidetes* are most known to serve several roles in anaerobic degradation, which were reported as sugar fermenters and plant cellulose degraders. In addition, protein degradation and subsequent amino acid fermentation to acetate, propionate and succinate among strains have been documented (Kampmanna et al., 2012). Gao et al.(2010) also reported that *Bacteroidetes* could potentially release more proteinaceous EPS for colonization. Candidate phylum *TM7* was also dynamically changing with different development in the four phases (Fig. 3a). Both these uncultured bacteria divisions have been frequently found in anaerobic processes, however, knowledge regarding their metabolic functions is still poor.

The taxonomic classification assigned to class level is provided in Fig. 3b and d. *Actinobacteria* (22.8%), *Clostridia* (21.0%), *Methanomicrobia* (16.4%), *Alphaproteobacteria* (9.3%) and *Acidimicrobiia* (4.9%) presented a high relative abundance of OTUs across 8 samples. In control reactor, *Actinobacteria* was the first dominant class with the averaged percentage of 27.5%, followed by *Clostridia* with the averaged percentage of 14.6%. While in test reactor, *Clostridia* became the first dominant class with the averaged percentage of 27.4%, followed by *Methanomicrobia* and *Actinobacteria*. *Clostridia* are a highly versatile class of anaerobic bacteria, which were observed in several previous studies (Ziganshin et al., 2013). Class *Clostridia* present a major group of hydrolyzing and fermentative bacteria, including acetogens and syntrophic acetate oxidizing bacterial. *Clostridia* are reported to produce VFAs by using soluble organics (Kato et al., 2004), which were greatly enhanced in the phase 2 and 3.

At order level, the archaeal population shows a clear shifts to dominance of *Methanosarcinales* (11.7%) (Fig. S4). *Methanosarcinales* have been observed to capable of performing acetate degradation to form CH_4 (Hagen et al., 2014), their high presence was likely a result of increased concentration of acetic acid, indicating

that acetoclastic methanogenesis greatly occurred in the phase 2. Decrease in methane and biogas productions were found out to be closely related with the disappearance of the acetoclastic methanogens (represented by the *Methanosarcinales* order) in the test reactor. Maintenance of *Methanosarcinales* (acetoclastic methanogens) in anaerobic process is critical for stability of performance. Previous studies reported the importance of *Methanomicrobiales* (hydrogenotrophic methanogens), which was the most resistant member in *Methanogens* to toxic substances (Aydin et al., 2015).

Hierarchically clustered heatmap analysis (Fig. 4) provided the microbial community dynamics profiles at genus level. Typically, the beta-diversity and shared OTUs among T1–T4 were presented in Figs. S5 and S6.Test samples (T1–T4) were separated from control samples (C1–C4), suggesting clear distinctions of microbial community structure in two reactors despite the fact they shared the same source of microbial consortia. This was also supported by the PCoA discussed in Section 3.3. *N09* for bacterial (affiliated to *Actinobacteria* phyla) and *Methanosaeta* for archaeal (affiliated to *Euryarchaeota* phyla) were dominated significantly in 645 assigned genus, accounting for 16.5% and 13.2%, respectively. The rest genus each contributed to no more than 2.0% of total abundance. In order to further investigate the microbial composition change along with FOG addition, the integrative and informative investigation of microbial community structure in test system were presented in Fig. 5. Interestingly, genus of *N09* (mainly represented by OTU 3) were not detected in the initial FOG system (T2), while dominated in the excessive FOG condition (T4). Although *N09* was identified as the first dominant group involved in the two systems, their functions in anaerobic digester were still unclear. Genus *Clostridium* (mainly represented by OTU 278) are related to the hydrolysis of complex polymers. The released matters could provide the substances for the rapid colonization of such *Firmicutes* bacteria. *Clostridium* was represented as Gram-positive bacteria and responsible for degradation of organic compounds. Co-digestion with high organic waste has the optimum C/N ratio, then improve the nutrient balance and increase the amount of degradable carbon and, consequently, the biogas yield. Comparing

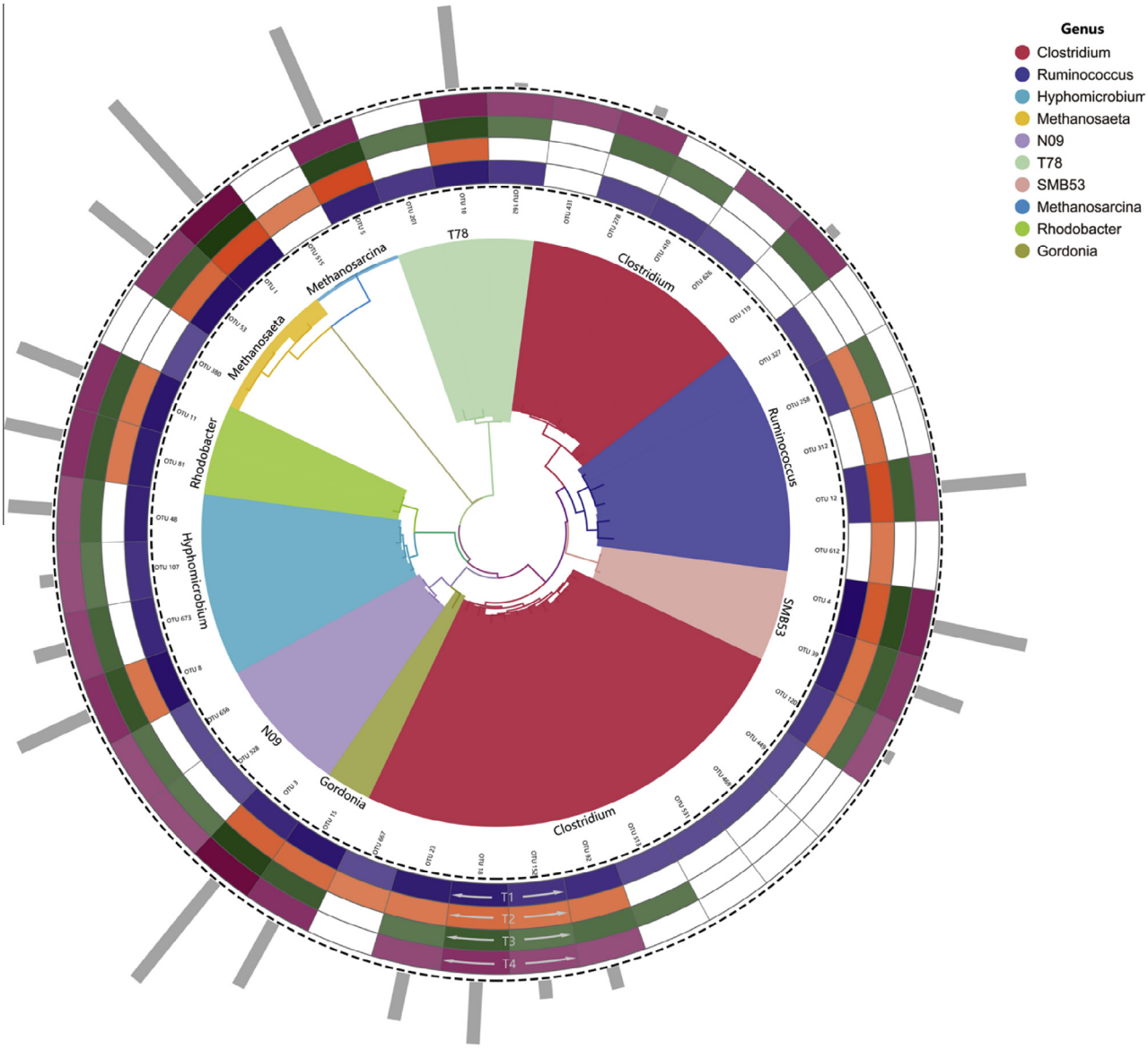

Fig. 5. The phylogenetic tree of relative abundance in four test samples, using circular heatmaps and barplots. Phylogenetic tree of inner circle was constructed by 40 OTUs represented the top 10 genus. Middle circle represented the relative abundance of each OTU, the colors intensity corresponds to species prevalence in each sample. Outer circle represented the total abundance of OTUs in each genus, the bar heights are proportional to total taxa abundance.

to bacteria genus, archaeal community was composed of less diverse groups and only 3 OTUs affiliated to archaeal groups. Acetoclastic methanogenic genus of *Methanosaeta* (mainly represented by OTU 1 and 53) were common to all reactors. *Methanosaeta* has been reported to be dominated in the anaerobic reactor fed with food waste (Williams et al., 2013). In this study, both *Methanosaeta* and *Methanosarcina* were found and only *Methanosaeta* formed the predominant archaeal group in the FOG system. This is probably because only two types of methanogens are known within the trophic chain: acetoclastic (e.g. *Methanosaeta*) and hydrogenotrophic methanogens (e.g. *Methanospirillum*). The acetogenic and methanogenic populations have been described as the populations most sensitive to LCFA inhibition. With regard to methanogenic archaea, acetoclastic populations show a higher inhibition degree than hydrogenotrophic in terms of substrate utilization rate, growth rate and cell yield (Palatsi et al., 2010). Lim et al. (2013) suggested that the predominance of *Methanosaeta* and absence of *Methanosarcina* species indicated test system was at less than optimal OLR. The OLR could be further promoted biogas production.

3.5. Community correlations between dominant genus and environment factors

RDA was used to examine which of the environmental factors explain the variation in microbial composition and demonstrate relationship between environmental factors and major members. In this study, 21 dominant microbial community index, which covered the top 10 abundant genus of 8 samples, and 7 environmental factors were screened for RDA plots calculation (Fig. 6). RDA 1 and RDA 2 explained 96.9% total variation of the microbial abundance and diversity. It was noticed that the 7 environmental factors were divided into 3 groups. TEPS-PS and biogas production were distributed in the same quadrant. While TEPS-PN, LEPS-PN, LEPS-PS and FOG addition clustered together. Additionally, these two groups were separated from factor of pH.

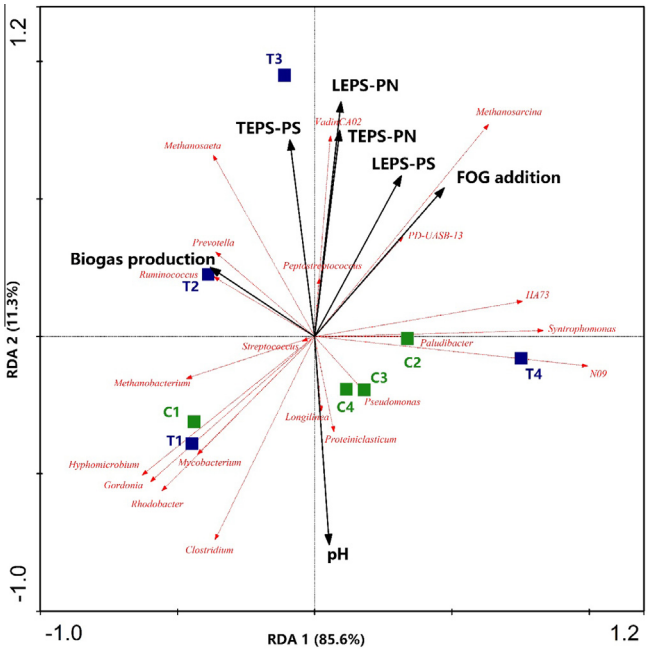

Fig. 6. Redundancy analysis (RDA) triplot showing the correlation between relative abundance of a specific microbial population and environmental factors for different samples. The variables of microorganism structure (at genus level) and environment factors (pH, FOG addition, biogas production, LEPS-PN, LEPS-PS, TEPS-PN and TEPS-PS) are respectively represented by red and black arrows. Color square represented 8 samples taken from two systems. The angles between arrows indicate correlations between two variables. Sequences out of the top 10 in each samples were filtered out. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

From the results, FOG addition and biogas production was not related in a positive way. Since the FOG addition exist a threshold and will not always promoted biogas production. LB-EPS and TB-EPS were in correlation with biogas production. As the indicator of microbial activity to reflex digestion efficiency, different EPS subfractions located closely. Each EPS fraction contains different components and represents rather distinct chemical property. The polysaccharide and protein in TB-EPS are independent of the influent carbon source and C/N ratio. However, the protein content and the carbohydrate content in LB-EPS are related to the influent C/N ratio (Ye et al., 2011). EPS was considered to act as a protective layer for cells against the harsh external conditions, a certain amount of EPS would promote the accumulation of microbes. It is believed that the microbial diversity has a liner relationship with EPS content in a particular range (Yu et al., 2012). Fukuzaki et al. (1995) believed that abundant EPS would be synthesized mainly by carbohydrate degrading bacteria. The extra EPS could help the microbe adhere to the surface and facilitate the development of the colonization.

Anaerobic digestion need a critical mass of microorganisms and nutrition in the substrates to promote methanogenic activity for methane production. The substrate loading with FOG is likely one of the most important parameters in this process, resulted in the differences of microbial composition and methanogenic activity. Higher FOG concentration which exceeds the digestion capacity would increase the risk of system deterioration. Along with digestion process, samples taken from the test system (T2–T4) distributed in a scatter way and well separated from the clustered samples taken from control system (C2–C4), while there was a clear distinction from C1 and T1. The distribution of the samples revealed that the microbial community undergoes unapparent dynamic succession. That means different FOG treatment substantially changed microbial community structures, despite the same original source of microbial consortia was shared in the start phase 1. The microorganisms in a habitat that undergoes major environmental fluctuation would be expected to cause or respond to the environmental variations, such as carbonate balance, VFA balance and ammonia balance. The acclimation of anaerobic sludge to a FOG substrate leads to a new microbial population that can be different from the mother culture or at least result in a new microbial population distribution.

Figs. 4 and 5 showed that a significant number of the sequences classified as *Methanosaeta* with generally increasing abundance throughout the FOG addition, which could reflect a positive response to the biogas production in RDA analysis (Fig. 6). The genus of *Methanosaeta*, *Prevotella* and *Ruminococcus* were mainly located near the arrow of biogas production, which indicated a significant effect on biogas production comparing with that of other species. While the dominant genus of *N09* were neither related to FOG addition or biogas production. Taken the digestion performance into consideration, the proportions of functional microorganisms involved in the hydrolysis and acidification were higher after FOG addition (Fig. 4), which resulted in the higher organic solubilization and hydrolytic enzyme activity. The positive nutrition balance could thus provide a distinct advantage over mono-sludge digestion and promote the implementation of efficiency for sludge treatment. In particularly, *Clostridium* is known for producing VFAs through Stickland reaction and was mainly detected in T1 (startup phase) with 1.5% relative abundance. *Clostridium*, represented as a Gram-positive bacteria, is related to the hydrolysis of complex biopolymers. The released organics would provide the substances for the rapid multiplication of *Clostridium*, and hence the great abundance of these bacteria was observed.

Moreover, T3 sample showed a strong positive correlation between *Methanosaeta* and EPS. Because T3 sample was taken from the phase with the best digestion efficiency, the results demonstrated *Methanosaeta* growth is enhanced and more EPS was obtained by the presence of proper FOG addition.

4. Conclusions

Anaerobic digestion with FOG addition presented a distinct advantage over mono digestion system due to the positive nutrition balance. However, the FOG loading in excess of 2 g L^{-1} d^{-1} were detrimental to biogas production. Using HTS, more than 4000 OTUs were detected and half of them have not been affiliated. RDA indicated that FOG addition was the first important environment factor regulating microbial composition and metabolic activity. Co-digestion system simulated the microbial activity and more EPS were obtained with the progressive addition of FOG. An improved understanding towards optimizing the ACoD need further study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2016.04.046.

References

APHA, 2005. Standard Methods for the Examination of Water and Wastewater, 21st ed. American Public Health Association, Washington, D.C., USA.

Aydin, S., Shahi, A., Ozbayram, E.G., Ince, B., Ince, O., 2015. Use of PCR-DGGE based molecular methods to assessment of microbial diversity during anaerobic treatment of antibiotic combinations. Bioresour. Technol. 192, 735–740.

Bertin, L., Bettini, C., Zanaroli, G., Fraraccio, S., Negroni, A., Fava, F., 2012. Acclimation of an anaerobic consortium capable of effective biomethanization of mechanically-sorted organic fraction of municipal solid waste through a semi-continuous enrichment procedure. J. Chem. Technol. Biotechnol. 87 (9), 1312–1319.

Chen, Y., Cheng, J.J., Creamer, K.S., 2008. Inhibition of anaerobic digestion process: a review. Bioresour. Technol. 99 (10), 4044–4064.

Fukuzaki, S., Nishio, N., Nagai, S., 1995. High-rate performance and characterization of granular methanogenic sludges in upflow anaerobic sludge blanket reactors fed with various defined substrates. J. Ferment. Bioeng. 79 (04), 354–359.

Galbraith, H., Miller, T.B., 1973. Effect of long chain fatty acids on bacterial respiration and amino acid uptake. J. Appl. Bacteriol. 36 (4), 659–675.

Gao, D.W., Zhang, T., Tang, C.Y.Y., Wu, W.M., Wong, C.Y., Lee, Y.H., Yeh, D.H., Criddle, C.S., 2010. Membrane fouling in an anaerobic membrane bioreactor: differences in relative abundance of bacterial species in the membrane foulant layer and in suspension. J. Membr. Sci. 364 (1–2), 331–338.

Hagen, L.H., Vivekanand, V., Linjordet, R., Pope, P.B., Eijsink, V.G.H., Horn, S.J., 2014. Microbial community structure and dynamics during co-digestion of whey permeate and cow manure in continuous stirred tank reactor systems. Bioresour. Technol. 171, 350–359.

Kampmanna, K., Rateringa, S., Kramera, I., Schmidtb, M., Schnella, S., 2012. Unexpected stability of Bacteroidetes and Firmicutes communities in laboratory biogas reactors fed with different defined substrates. Appl. Environ. Microb. 78 (7), 2106–2119.

Kabouris, J.C., Tezel, U., Pavlostathis, S.G., Engelmann, M., Dulaney, J., Gillette, R.A., Todd, A.C., 2009. Methane recovery from the anaerobic codigestion of municipal sludge and FOG. Bioresour. Technol. 100 (15), 3701–3705.

Kato, S., Haruta, S., Cui, Z.J., Ishii, M., Yokota, A., Igarashi, Y., 2004. *Clostridium straminisolvens* sp. nov., a moderately thermophilic, aerotolerant and cellulolytic bacterium isolated from a cellulose-degrading bacterial community. Int. J. Syst. Evol. Microbiol. 54 (6), 2043–2047.

Kawai, M., Nagao, N., Tajima, N., Niwa, C., Matsuyama, T., Toda, T., 2014. The effect of the labile organic fraction in food waste and the substrate/inoculum ratio on anaerobic digestion for a reliable methane yield. Bioresour. Technol. 157, 174–180.

Leven, L., Eriksson, A.R.B., Schnurer, A., 2007. Effect of process temperature on bacterial and archaeal communities in two methanogenic bioreactors treating organic household waste. FEMS Microbiol. Ecol. 59 (3), 683–693.

Li, X.Y., Yang, S.F., 2007. Influence of loosely bound extracellular polymeric substances (EPS) on the flocculation, sedimentation and dewaterability of activated sludge. Water Res. 41 (5), 1022–1030.

Lim, J.W., Chen, C.L., Ho, I.J.R., Wang, J.Y., 2013. Study of microbial community and biodegradation efficiency for single- and two-phase anaerobic co-digestion of brown water and food waste. Bioresour. Technol. 147, 193–201.

Liu, Y., Yang, S.F., Li, Y., Xu, H., Qin, L., Tay, J.H., 2004. The influence of cell and substratum surface hydrophobicities on microbial attachment. J. Biotechnol. 110 (3), 251–256.

Lu, X., Zhen, G., Liu, Y., Hojo, T., Estrada, A.L., Li, Y.Y., 2014. Long-term effect of the antibiotic cefalexin on methane production during waste activated sludge anaerobic digestion. Bioresour. Technol. 169 (5), 644–651.

Masse, L., Mass, D.I., eacute, Kennedy, K.J., Chou, S.P., 2002. Neutral fat hydrolysis and long-chain fatty acid oxidation during anaerobic digestion of slaughterhouse wastewater. Biotechnol. Bioeng. 79 (1), 43–52.

Mata-Alvarez, J., Dosta, J., Romero-Güiza, M.S., Fonoll, X., Peces, M., Astals, S., 2014. A critical review on anaerobic co-digestion achievements between 2010 and 2013. Renewable Sustainable Energy Rev. 36, 412–427.

Nielsen, P.H., Jahn, A., Palmgren, R., 1997. Conceptual model for production and composition of exopolymers in biofilms. Water Sci. Technol. 36 (1), 11–19.

Palatsi, J., Illa, J., Prenafeta-Boldu, F., Laureni, M., Fernandez, B., Angelidaki, I., Flotats, X., 2010. Long-chain fatty acids inhibition and adaptation process in anaerobic thermophilic digestion: batch tests, microbial community structure and mathematical modelling. Bioresour. Technol. 101 (7), 2243–2251.

Pope, P., Vivekanand, V., Eijsink, V.H., Horn, S., 2013. Microbial community structure in a biogas digester utilizing the marine energy crop *Saccharina latissima*. 3 Biotech. 3 (5), 407–414.

Ras, M., Lefebvre, D., Derlon, N., Paul, E., Girbal-Neuhauser, E., 2011. Extracellular polymeric substances diversity of biofilms grown under contrasted environmental conditions. Water Res. 45 (4), 1529–1538.

Rivière, D., Desvignes, V., Pelletier, E., Chaussonnerie, S., Guermazi, S., Weissenbach, J., Li, T., Camacho, P., Sghir, A., 2009. Towards the definition of a core of microorganisms involved in anaerobic digestion of sludge. ISME J. Multidiscip. J. Microbiol. Ecol. 3 (6), 700–714.

Sheng, G.P., Yu, H.Q., Li, X., 2010. Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems: a review. Biotechnol. Adv. 28 (6), 882–894.

Sponza, D.T., 2003. Investigation of extracellular polymer substances (EPS) and physicochemical properties of different activated sludge flocs under steady-state conditions. Enzyme Microb. Technol. 32 (2), 375–385.

Toreci, I., Kennedy, K.J., Droste, R.L., 2009. Evaluation of continuous mesophilic anaerobic sludge digestion after high temperature microwave pretreatment. Water Res. 43 (5), 1273–1284.

Williams, J., Williams, H., Dinsdale, R., Guwy, A., Esteves, S., 2013. Monitoring methanogenic population dynamics in a full-scale anaerobic digester to facilitate operational management. Bioresour. Technol. 140, 234–242.

Xu, R., Yang, Z.H., Chen, T., Zhao, L., Huang, J., Xu, H., Song, P., Li, M., 2015. Anaerobic co-digestion of municipal wastewater sludge with food waste under different fat, oil, grease contents: study on reactor performance and extracellular polymeric substances. RSC Adv. 5, 103547–103556.

Ye, F., Ye, Y., Li, Y., 2011. Effect of C/N ratio on extracellular polymeric substances (EPS) and physicochemical properties of activated sludge flocs. J. Hazard. Mater. 188 (1–3), 37–43.

Yu, Y., Lee, C., Kim, J., Hwang, S., 2005. Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. Biotechnol. Bioeng. 89 (6), 670–679.

Yu, Z., Wen, X., Xu, M., Huang, X., 2012. Characteristics of extracellular polymeric substances and bacterial communities in an anaerobic membrane bioreactor coupled with online ultrasound equipment. Bioresour. Technol. 117, 333–340.

Zheng, Y., Wang, C., Zheng, Z.Y., Che, J., Xiao, Y., Yang, Z.H., Zhao, F., 2014a. Ameliorating acidic soil using bioelectrochemistry systems. RSC Adv. 4 (4), 62544–62549.

Zheng, Y., Xiao, Y., Yang, Z.H., Wu, S., Xu, H.-J., Liang, F.Y., Zhao, F., 2014b. The bacterial communities of bioelectrochemical systems associated with the sulfate removal under different pHs. Process Biochem. 49 (8), 1345–1351.

Ziganshin, A., Liebetrau, J., Pröter, J., Kleinsteuber, S., 2013. Microbial community structure and dynamics during anaerobic digestion of various agricultural waste materials. Appl. Microbiol. Biotechnol. 97 (11), 5161–5174.