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Bioproduction of Short Chain Fatty Acids in Primary and Excess Sludge Fermentation: Performance and Microbial Community Structures Comparison

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ABSTRACT

The fermentation characteristics of activated biomass from two types of sludge, namely primary sludge (PS) and excess sludge (ES) were compared under three pH conditions (uncontrolled, 4, 10) over a period of 8 days. Results show the highest production of short-chain fatty acids (SCFAs) in PS and ES of 352 ± 7 and 324 ± 13 mg COD/g VSS, respectively at pH 10, on the 2nd day in PS fermentation, and the 5th day in ES. Consequently, MiSeq high-throughput sequencing was applied to analyze the succession of microbial community structures in an alkaline condition, since SCFAs production was higher at the pH 10 than the other two pH conditions. For bacteria at the genus level, *Bacillus* and *Anaerobacillus* were only detected in the fermented sludge. The relative abundance of *Bacillus and Anaerobacillus* showed a big difference in the two types of sludge fermentation systems. *Bacillus* accounted for 62.4% and 33.1%, in PS and ES, respectively, while *Anaerobacillus was* 18.5% and 40.3%. More acidogenesis bacteria were found in PS than in ES fermentation. Thus, PS under alkaline fermentation could provide a promising carbon source for WWTPs.

Keywords: Primary sludge fermentation; excess sludge fermentation; hydrolysis and acidification; short-chain fatty acids (SCFAs); Microbial community

1. INTRODUCTION

Activated sludge process is a widely used technique for wastewater treatment (Frigon et al., 2006; Nyholm et al., 1996; Wang et al., 2016). However, if there is limited availability of readily biodegradable COD (chemical oxygen demand) in the raw wastewater, the nitrate and phosphorus cannot be completely removed (Liu et al., 2012; Pijuan et al., 2004). It is reported that supply of sufficient short-chain fatty acids (SCFAs) could improve the performance of phosphorus accumulation organisms. Presently, there are two main approaches to achieve the SCFAs augmentation. The first is to add pure SCFAs, e.g., acetate in the anaerobic reactor as a substrate for phosphorus removal (Guerrero et al., 2012; Puig et al., 2008; Tayà et al., 2015; Vargas et al., 2009). The second alternative is to produce SCFAs through fermentation of primary sludge (PS) in a side stream reactor, which is then added to the mainstream anaerobic tank (Park et al., 2011; Wang et al., 2016; Yuan et al., 2006; Zhang et al., 2010). Nonetheless, the addition of pure SCFAs represents relatively large costs, and also, increases carbon

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footprints. Hydrolysis and acidification of activated sludge are thus, regarded as an economical way to augment the SCFAs in wastewater treatment plants, while helping to achieve resource recovery and sludge reduction targets (Zhu and Chen, 2005).

To improve the degradative efficiency of activated biomass in the sludge, the methods for strengthening hydrolytic fermentation process has become a hotspot recently. Many previous research reports indicate that pH, temperature, sludge retention time, and dissolved oxygen concentration were the main factors affecting the degradation of sludge and SCFAs production (Bouzas et al., 2007; Chen et al., 2007; Cokgor et al., 2009; Wu et al., 2009; Yadav et al., 2014). As we know, anaerobic digestion includes three steps: hydrolysis, acidification, and methanogenesis. Since methanogenesis converts SCFAs to methane, hydrolysis is the rate limiting step. In order to improve the production of SCFAs, it means hydrolysis should be enhanced and methanogenesis should be inhibited. pH, SRT_{aci} are the main factors that influence both produced rate and products. Two types of sludge from wastewater treatment plant can be utilized for fermentation, namely PS and ES. PS is a settling material produced from the primary sedimentation tank of WWTPs, which consists of a high portion of organic matter such as feces, vegetables, fruits, textiles and paper, a significant organic carbon fraction for the subsequent nutrient removal processes (Cokgor et al., 2009). Unlike PS, ES originates from the secondary sedimentation tank and contains small amounts of non hydrolyzable particulate materials and biomass, which also can be a potential carbon source for WWTPs (Arnaiz et al., 2006). At present, most researchers are focused on one type of sludge fermentation. There are few discussions on the comparison of the fermentative characteristics of the two types of sludge. Furthermore, studies on the comparison of the different types of sludge only focus on SCFAs. Insight studies on the microbial community succession is still rare (Ucisik and Henze, 2008).

Therefore, the objective of this study was to investigate (i) the degradative efficiency of activated biomass from PS and ES, (ii) the optimal operating conditions of sludge fermentation, (iii) the succession of microbial community structure in PS and ES.

2. MATERIALS AND METHODS

2.1 Sources of sludge

Sludge in this study was taken from Xi'an No. 5 Sewage Treatment Plant, China. The sludge was concentrated by settling at 4˚C for 12 h and adjusted to the required concentration, the main characteristics of which are shown in Table 1.

2.2 Batch fermentation experiments

The suspended solids of PS and ES were diluted to 13462.5 mg/L and 11040 mg/L, respectively with the influent of the aerated grit chamber. Following this dilution, the SCFAS concentrations decreased to 94.9 mg COD/g VSS and 17.2 mg COD/g VSS, respectively. In order to investigate the anaerobic fermentation efficiency of PS and ES, six glass reactors (1 L) with continuous low-speed stirring were kept at 35 ± 1˚C in a water bath kettle and closed by plastic wrap. For each type of sludge, there were three pH conditions: uncontrolled, 4 and 10. To maintain the exact pH value, 1 mol/L of NaOH and 1 mol/L of HCl were added to adjust the pH every 3 h.

2.3 Analytical methods

The analysis of COD, SCOD, TSS, VSS, ammonia and phosphate were conducted following standard methods (APHA, 1998). SCOD was measured for samples filtrated with

Whatman GF/C glass microfiber (0.45 μm). SCFAs compositions were analyzed by means of gas chromatography (SHIMADZU GC-2014) with flame ionization detector and DB-FFAP-123-3233 column (30 m × 0.5 μm × 0.32 mm). The pH of samples was adjusted to 4 with phosphoric acid. The injection port and the detector were maintained at 200 and 230˚C, respectively. The oven of GC was programmed to begin at 100˚C and to remain there for 2 mins, then to increase at a rate of 10˚C/min to 120˚C and maintain 120˚C for 2 mins, and to increase for the second time at a rate of 6˚C/min to 200˚C/min for 2 mins. The sample injection volume was 1.0 μL. SCFAs concentrations were converted to COD by using appropriate conversion factors as 1.07 for acetic acid, 1.51 for propionic acid, 1.82 for n-butyric and iso-butyric acids, and 2.04 for n-valeric and iso-valeric acids. Proteins and carbohydrates, however, were converted to COD with the conversion factors of 1.5 and 1.07, respectively (Tong and Chen, 2007).

2.4 DNA extraction and PCR amplification

For the analysis of bacteria in fermentation reactors, sludge samples were collected from PS and ES on the 2nd and 5th day, respectively. The samples were freeze-dried by LAB-CONCO (Model 195, England). DNA was subsequently extracted from the dried sludge using the Fast DNA Kit (BIO 101, Vista, CA). Amplicon libraries were constructed by using the V3 and V4 region primers of bacterial 16S rRNA genes, and high throughput sequencing was carried out on the MiSeq platform.

Primers 338F 5'-ACTCCTACGGGAGGC AGCA-3' and 806R 5'-GGACTACHVGGG TWTCTAAT-3', where the barcode is an eight-base sequence unique to each sample (Dennis et al., 2013). The PCR reactions were performed in triplicate using a 20 μL mixture containing 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 m MdNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase, and 10 ng of template DNA.

2.5 Processing of MiSeq sequencing data

The four DNA samples taken were analyzed by Illuminamiseq sequencing (IlluminaMiseq PE300 platform). Taxonomic classification was carried out to determine the optimized sequences into operational taxonomic units (OTUs). The OTUs were clustered with a 97% similarity cutoff using UPARSE, and the chimeric sequences were identified and removed using UCHIME. A Venn diagram was generated using the Mothur utility (Fouts et al., 2012) based on OTUs.

Table 1 Characteristics of the primary and excess sludge

Parameters	Primary sludge	Excess sludge
pH	7.4 ± 0.2	7.0 ± 0.1
TSS (total suspended solid) mg/L	33778.5 ± 751.6	22710.6 ± 475.1
VSS (volatile suspended solid) mg/L	18473.2 ± 630.2	13355.7 ± 500.5
TCOD (total chemical oxygen demand) mg COD/g VSS	1548.0 ± 63.5	1949.3 ± 46.1
SCOD (soluble chemical oxygen demand) mg COD/g VSS	246.3 ± 12.1	33.3 ± 4.3
SCFAs (mg COD/g VSS)	130.5 ± 1.4	20.9 ± 2.2
NH_4^+-N (mg/g VSS)	14.2 ± 2.7	1.8 ± 0.7
PO_4^{3-}-P (mg/g VSS)	1.6 ± 0.5	3.6 ± 0.3

3. RESULTS AND DISCUSSION

3.1 Effect of pH on acidification performance

3.1.1 SCFAs accumulation

pH was an essential factor in SCFAs accumulation from sludge fermentation and also affected the degradation efficiency both in PS and ES (Cokgor et al., 2009; Wu et al., 2009; Yuan et al., 2006). The concentration of SCOD and SCFAs at different pH and bio-production time are shown in Figure 1. SCOD in the two types of sludge showed similar tendencies of increasing to a peak, and then gradually decreasing thereafter. Three pH values (uncontrolled, 4, 10) were studied for each type of sludge. SCOD concentration in both fermenters was as high as 395 ± 11 and 516 ± 16 mg COD/g VSS, respectively, in the PS and ES at pH 10. When pH is 10, both the production of SCOD and SCFAs is higher than the other two pH. Similar findings have been reported whereby pH 10 led to higher SCFAs accumulation than pH 4 and uncontrolled pH (Yue et al., 2015). This can be explained by the fact that SCFAs could be consumed by methanogenic archaea to produce methane at pH 4 and uncontrolled pH, which lowered SCFA accumulation. Besides, SCOD concentration in ES was higher than that in PS (Figure 1a).

The biodegradable organic matter can be divided into two fractions: readily biodegradable and slowly biodegradable. The quantity of readily biodegradable COD is much more important for enhanced nutrient removal in wastewater treatment (Henze et al., 2000; Wentzel et al., 2001). Therefore, the production of SCFAs was measured in this study (Figure 1b). The concentration of SCFAs in PS and ES measured were as high as 352 ± 7 and 324 ± 13 mg COD/g VSS, respectively, which accounted for 89% and 63% of the SCOD on the 2nd day and the 5th day, respectively. Thus, SCFAs was the main component of SCOD. The ratio of SCFAs to SCOD in PS on the 2nd day was significantly higher than that in ES on the 5th day. These results are consistent with that of Ucisik and Henze (Ucisik and Henze, 2008) that fermentation of PS produced higher amounts of SCFAs compared to ES in their experiments performed with both batch and semi-continuous reactors. This fact might be due to the different percentages of organic constituents in the sludge. PS was reported to include high amounts of protein, carbohydrate and lipid (Ucisik and Henze, 2008). Besides, the differences in the inorganic matter fraction among the activated sludge also influence SCFAs production. Another explanation for the variation of SCFAs production is the nature of the sludge. The majority of organic matter is separate and more available in PS, while organic matter is polymer form in ES.

As for the sludge retention time in the reactor (SRT_{aci}), neither too long nor too short are good for sludge acidification. It was reported that methane production is more likely to occur at longer retention time, which could reduce the yield of VFAs (Luo et al., 2014). Thus, the sludge retention time needs to be carefully controlled. It was reported that at SRT_{aci} of over 8 days, methane would be generated in the fermentation process (Moser-Engeler et al., 1998). According to Jiang et al. (2007), the optimum fermentation time for SCFAs production is 6 days. Thus, in this study, a SRT_{aci} of 8 days was chosen for the batch reactors. The best SRT_{aci} to achieve a high SCFAs production was 2 days in PS and 5 days in ES.

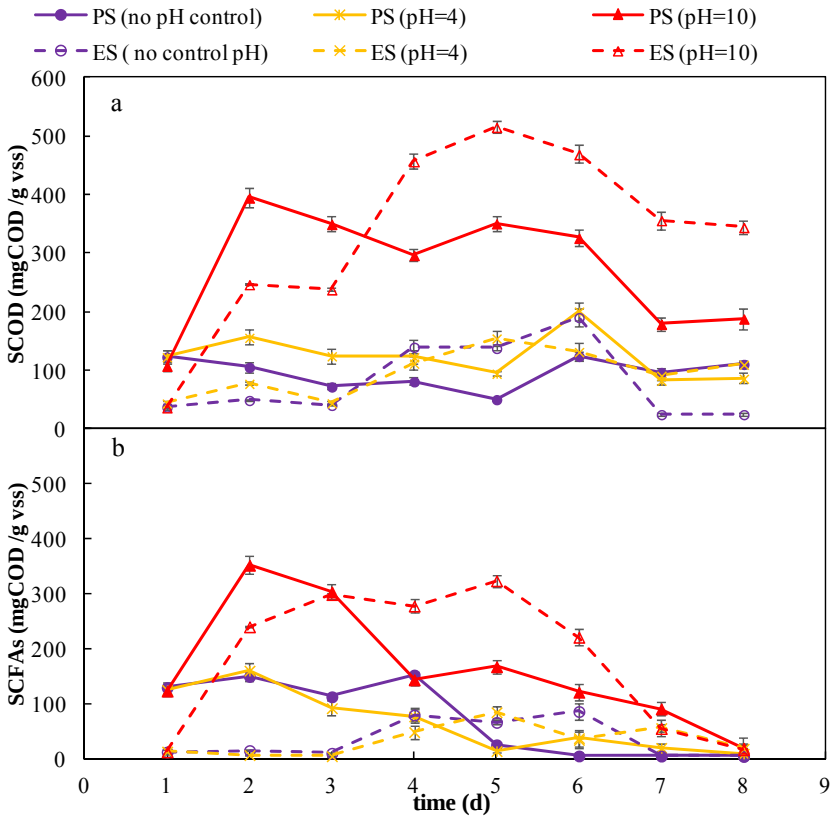

Figure 1 Effects of pH on production of SCOD and SCFAs

3.1.2 Release of NH_4^+-N and PO_4^{3-}-P

The phosphorus and ammonia in the bacterial cells were released as NH_4^+-N and PO_4^{3-}-P during sludge fermentation. The release of NH_4^+-N was from the biodegradation of nitrogenous matters and the PO_4^{3-}-P was released from the decomposition of lipid bilayer and polyphosphate particles in microbial cells. The concentrations of ammonia and phosphate were found to increase slowly with fermentation time (Figure 2). The concentration of NH_4^+-N was the lowest at pH 10, since NH_4^+ could react with OH-. For phosphorus, the release was the lowest at the uncontrolled pH. Overall, ammonia was released in PS while more phosphorus was released in ES. A similar finding was reported in an earlier study that the amount of NH_4^+-N released was higher than the PO_4^{3-}-P released in an ES fermentation system (Chen et al., 2007). The mechanism of phosphorus and nitrogen removal is totally different. Previous studies have reported that phosphorus can be precipitated as struvite to achieve phosphorus recovery, and that no SCFAs are consumed in the chemical precipitation. Nevertheless, NH_4^+-N needs to be removed before applying SCFAs-rich hydrolysate to denitrification and biological phosphorus removal processes; otherwise, ammonia will consume SCFAs. Besides, it was reported that free ammonia inhibited the hydrolytic acidification of protein, changed the intracellular pH of hydrolytic acidification of protein, increased the energy requirement, and inhibited enzyme reaction in pure cultures (Zhang et al., 2010). The noncompetitive inhibition of ammonia on biomass was negligible, since the ammonia concentration was below 0.03 g/L in this study, which was far less

than the concentrations of ammonia reported to inhibit the processes mentioned above (1.7-14 g/L (Chen et al., 2008).

Considering that the fermentation liquor is a good carbon resource for WWTPs, increasing the concentration of ammonia and phosphorus would also contribute nutrient load as well. The ratio of PO_4^{3-}-P to SCFAs in PS and ES were 0.006 and 0.05 respectively at pH 10. It is reported that when the ratio of PO_4^{3-}-P/SCFAs is higher than 0.05, biological phosphorus removal is inefficient.

3.2 SCFAs components under alkaline condition

Following the discussion on the gross amount of SCFAs in 3.1, it is also important to know the composition of the SCFAs. It is reported that in the enhanced biological nutrient removal (EBNR) process, acetic acid and propionic acid are the preferred substrates (Yuan et al., 2006). In this study, six components of SCFAs were measured in the sludge over 8 days. Considering the finding that the SCFAs production was higher at the pH 10 than the other two pH conditions (Figure 1b), the components of SCFAs were examined at this pH (Figure 3). The composition of SCFAs produced from the two types of sludge showed significant differences. On the 2nd day in PS, acetic acid, at 63% (223.4 mg COD/g VSS) of the total SCFAs concentration, was the major SCFAs component, followed by iso-valericat at 14.7% (51.9 mg COD/g VSS), n-butyric at 13.0% and iso-butyricaleric at 8.8%. For ES, the highest SCFAs production was recorded on the 5th day. Unlike PS, the majority of SCFAs in ES was n-butyric (236.2 mg COD/g VSS), which accounted for 76% on the 5th day. On the other hand, the percentage of acetic acids was only 20.1% (62.9 mg COD/g VSS). The amount of acetic acid was found to decrease with fermentation time. The experiments performed by PS and ES of approximately the same SS concentration showed that the PS produced the largest percentage of acetate with butyric. Thus, the hydrolysate obtained from fermentation of PS has greater potential for the application as carbon source to nutrient removal processes compared to the ES. There are several possible reasons for the different distribution of VFAs, such as the sludge quality, sludge components, pre-treatment methods, and operating conditions. In general, two mechanisms can be stated here to explain. The first is that the bacteria are using different metabolic pathways to ferment hydrolysis products in the sludge with different VSS concentrations. The other mechanism is that the operating conditions affect the types of particulates being hydrolyzed, and these, in turn, affect the distribution of the VFAs (Eastman and Ferguson, 1981). In this study, since the experiments were carried out at the same operational conditions, the differences in the bacterial metabolism pathways might be the reason for the observed changes in the distribution of SCFAs. Therefore, it is necessary to discuss the microbial communities in detail (see section 3.3).

Considering that acetate was the dominant product, a visible decrease of acetic acids was found both in PS and ES fermentation. Acetic acid might be degraded directly by methanogens, as reported in a previous study (Shin et al., 2010). However, strong alkaline pH (such as 10.0) can inhibit the growth of methanogens and produce more VFAs (Chen et al., 2007; Yuan et al., 2006). So, the main reason might be that the acetate was consumed for bacteria growth.

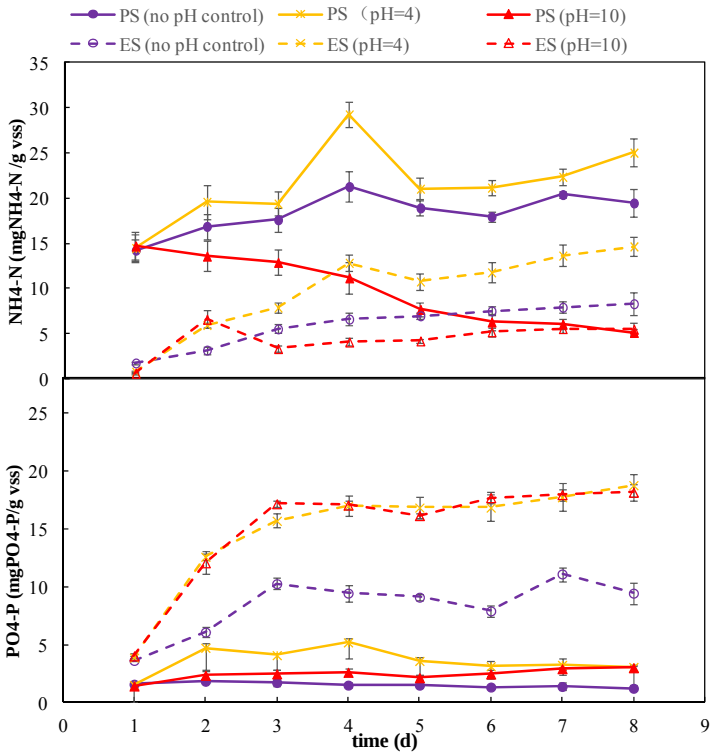

Figure 2 NH_4^+-N and PO_4^{3-}-P release in fermentation

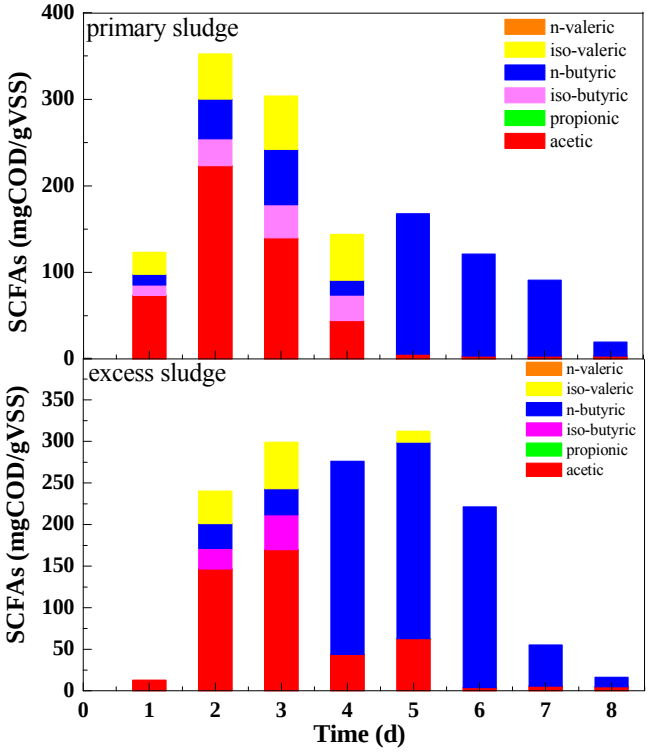

Figure 3 SCFAs components in sludge fermentation

3.3 Microbial community structure analysis

3.3.1 Community structure and dominant functional bacterial species

It is reported that the yield of SCFAs and SCFAs components from PS and ES fermentation were closely associated with microbial communities. Bacterial communities in fermented (pH 10) and unfermented sludge were analyzed using MiSeq sequencing, in which 39532 (PS) and 23372 (ES) for fermentation, and 4233 (PS) and 31658 (ES) for unfermented sludge trimmed sequences were obtained.

The relative abundance (> 0.1%) of microbial communities in the two types of sludge was characterized at phylum level (Figure 4). 70%~90% bacteria belonged to five phyla (*Proteobacteria*, *Bacteroidetes*, *Chloroflexi*, *Actinobacteria* and *Firmicutes*). Previous studies found that *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Chloroflexi* were the key phyla in conventional anaerobic digesters (Kwon et al., 2010; Nelson, 2011). *Proteobacteria*, *Bacteroidetes* and *Chloroflexi* were abundant in raw sludge at 23.1%, 22.5%, 13.4%, respectively in PS and 32.4%, 32.9% and 8.2% in ES. Compared with raw sludge, fermentation reduced the relative abundance of *Proteobacteria* and *Chloroflexi*, and enhanced *Firmicutes*. *Firmicutes* were the major microbes at the phylum level in the fermentation sludge system, accounting for 93.5% and 92.7% in PS and ES, respectively. *Firmicutes* were found abundant in this study, and also other studies. Yue et al. (2015) found that *Firmicutes* were the typical microbial community in sludge fermentation in alkaline condition. Most bacteria in the fermentation reactors were affiliated to *Proteobacteria* and *Firmicutes* (Zheng et al., 2013). It was reported that *Firmicutes* could produce ectoenzymes to metabolize protein, fat and carbohydrates (Zeng et al., 2010). Furthermore, *Bacteroidetes* could convert proteins and carbohydrates to propionate and acetate in anaerobic sludge fermentation (Abe et al., 2012; Kwon et al., 2010).

Considering that there is no obvious difference between PS and ES at the phylum level, the relative abundance at genus level was explored.

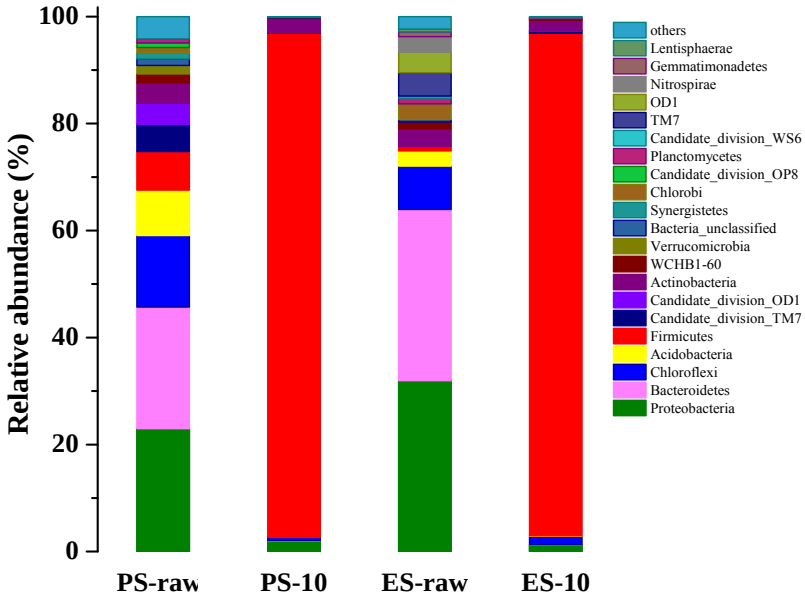

Figure 4 Bacterial community of PS and ES at phylum level (PS-10, on the 2nd day fermentation; ES-10, on the 5th day fermentation)

To further elucidate the microbial community functions, phylogenetic classification of the 16S rRNA gene sequences at the genus level were performed. Figure 5 summarizes the relative abundance (> 0.1%) of acidogenes is microbial communities at the genus level for the four samples. Previous studies found that *Bacillus*, *Anaerobrancillus* and *Anaerolineaceae* in *Firmicutes* were the main bacterial genus in alkaline fermentation systems. *Bacillus* and *Anaerobacillus* can degrade organic matter to acetic acid (Yamada et al., 2005). The results in this study indicate that *Bacillus*, *Anaerobacillus*, *Erysipelothrix* and *Anaerolineaceae* are the typical bacteria in fermented sludge while acidogenesis bacteria are barely detected in raw sludge. *Saprospiraceae*, *Blastocatella*, *Anaerobacillus* (Figure 5) were the main microbes in raw PS with relative abundance of 8.4%, 7.1%, 6.1%, respectively. Similar microbe types were found in the raw ES. The relative abundance of *Saprospiraceae*, *Anaerolineaceae* were 20.1% and 4.5%. It was reported that *Anaerolineaceae* belonged to *Chloroflexi*, which is the dominant bacteria in the mesophilic anaerobic digesters for biogas production, and also consumed SCFAs (Hao and Hui, 2014). In this study, *Anaerolineacae* was only detected in raw sludge since it was inhibited by the alkaline condition. The types of microbes showed significant changes in fermentation. In fermented PS and ES, the proportion of Bacillus, which was the most abundant acidogenesis bacteria and belonged to *Firmicutes*, were 62.4% and 33.1%, respectively of the total microbial communities. As another type of functional bacteria, *Anaerobacillus* accounted for 18.5% and 40.3%, respectively in fermented PS and ES. The dominant functional bacteria in the two types of sludge were the same, while the relative abundance showed significant differences. The total relative abundance of acidogenesis bacteria (*Bacillus*, *Anaerobacillus* and *Erysipelothrix*) in fermented PS was higher than it in ES. Since there are more acidogenesis bacteria in fermented PS, when there are almost similar suspended solids for both PS and ES, it means acidification is more active than in ES. It also strengthen the finding that SCFAs production in PS in higher than in ES.

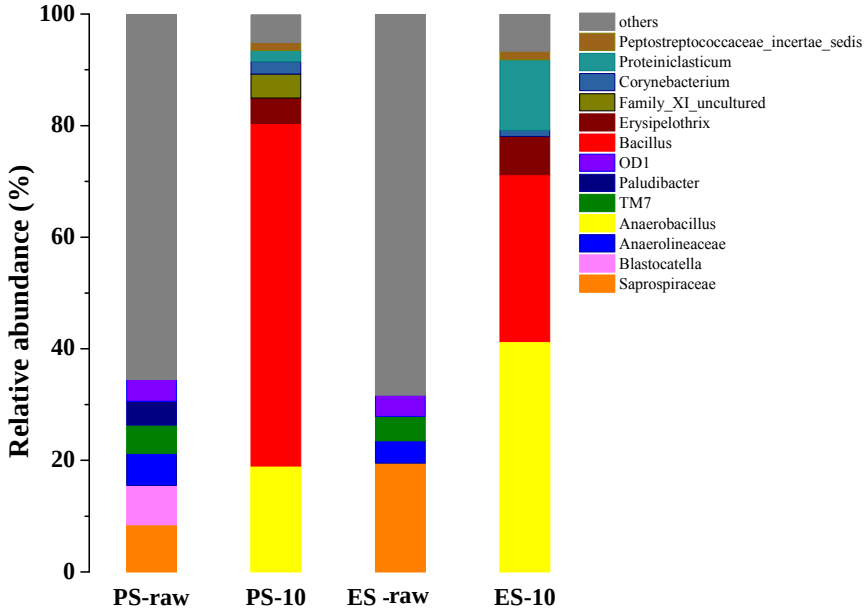

Figure 5 Bacterial community of PS and ES at genus level (PS-10, on the 2nd day fermentation; ES-10, on the 5th day fermentation)

3.3.2 Microbial species diversity and abundance

Principal Component Analysis (PCA) showed significant separation of the microbial community for activated biomass from raw sludge and fermented sludge at pH = 10 (Figure 6). The diversity of raw sludge samples were small and the two points were clustered together (Fer-1 and Fer-3, Figure 6), while biomass from fermented samples seems divergent (Fer-2 and Fer-4, Figure 6). Detailed differentiation of the microbial community can be seen in the Venn diagram (Figure 7).

In the Venn diagram (Figure 7), the samples are represented by different colors. The overlapping areas of the circles represent sequences of color-corresponding samples divided into the same OTU. The numbers in the overlapping areas represent the OTU numbers of the microbial communities that are shared between the samples. The Venn diagram with common and unique OTUs on genus level was applied to describe the differences and similarity among PS, ES, fermented PS and fermented ES (Figure 7). In the four samples, the sum of observed OTUs was 1586 OTUs. Raw PS and ES had more shared OTUs (993, 62.6% of total) than fermented sludge (251, 15.8%). The number of OTUs unique to individual communities were 311 (PS-raw), 6 (ES-raw), 133 (PS-10) and 5 (ES-10), accounting for 28.7% of the total number of observed OTUs. Compared with raw sludge, the diversity of bacteria community decreased in fermented sludge. These results correspond with the data for the community diversity at both the phylum and genus levels, and can be explained by the pH effect. The sample was obtained at pH = 10. At this alkaline condition, some bacteria cannot survive. So, the diversity of bacteria community decreased after fermentation. Moreover, adjusting pH seems like an efficient way to select acid-producing bacteria in activated sludge digestion.

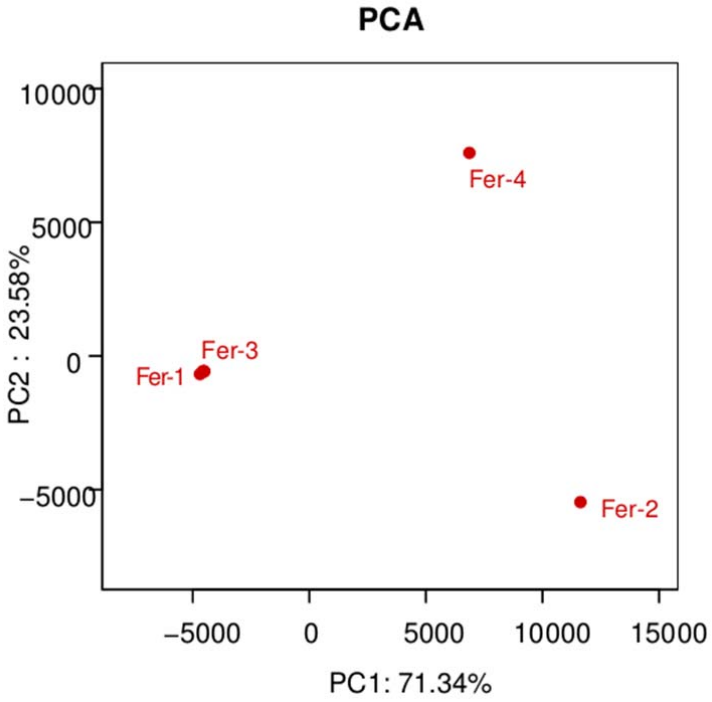

Figure 6 Principal component analysis (PCA) of microbial communities of different sludge samples (Fer-1 raw PS; Fer-2 PS, pH = 10; Fer-3 raw ES; Fer-4 ES, pH = 10)

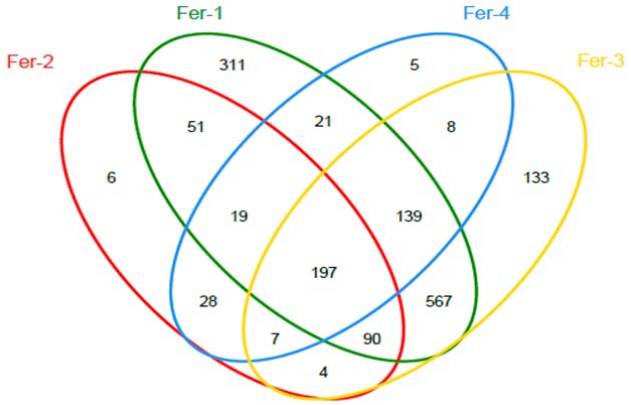

Figure 7 Mothur's Venn diagram showing common and unique OTUs for four sludge samples (Fer-1 raw PS; Fer-2 PS, pH = 10; Fer-3 raw ES; Fer-4 ES, pH = 10)

CONCLUSIONS

PS and ES from a WWTP were fermented in batch tests at three pH conditions (uncontrolled, 4 and 10). Findings show that the fermentation of PS generate significant amounts of SCFAs at short SRT_{aci}, while ES fermentation needs longer SRT_{aci} to reach the same level of SCFAs production. The results of MiSeq high-throughput sequencing analysis indicated that acidogenesis bacteria (*Firmicutes* at phylum level; *Bacillus*, *Anaerobacillus* at genus level) were enriched in the fermentation process. Furthermore, the total relative abundance of acidogenesis communities (genus level) in the PS fermentation system had a higher relative abundance than that in ES. Thus, PS is more suitable for fermentation, which can supply carbon for WWTPs.

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