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Comparative Analysis of Performance and Microbial Characteristics Between High-Solid and Low-Solid Anaerobic Digestion of Sewage Sludge Under Mesophilic Conditions S

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High-solid anaerobic digestion of sewage sludge achieves highly efficient volatile solid reduction, and production of volatile fatty acid (VFA) and methane compared with conventional low-solid anaerobic digestion. In this study, the potential mechanisms of the better performance in high-solid anaerobic digestion of sewage sludge were investigated by using 454 high-throughput pyrosequencing and real-time PCR to analyze the microbial characteristics in sewage sludge fermentation reactors. The results obtained by 454 high-throughput pyrosequencing revealed that the phyla Chloroflexi, Bacteroidetes, and Firmicutes were the dominant functional microorganisms in high-solid and low-solid anaerobic systems. Meanwhile, the real-time PCR assays showed that high-solid anaerobic digestion significantly increased the number of total bacteria, which enhanced the hydrolysis and acidification of sewage sludge. Further study indicated that the number of total archaea (dominated by *Methanosarcina*) in a high-solid anaerobic fermentation reactor was also higher than that in a low-solid reactor, resulting in higher VFA consumption and methane production. Hence, the increased key bacteria and methanogenic archaea involved in sewage sludge hydrolysis, acidification, and methanogenesis resulted in the better performance of high-solid anaerobic sewage sludge fermentation.

Keywords: Anaerobic digestion, high solid, sewage sludge, microbial characteristic, pyrosequencing

Introduction

With the rapid improvement of urbanization level, large amounts of sewage sludge, a major byproduct of the biological wastewater treatment process, is being produced in China. To transform organic material into an energy-rich biogas (60–70 vol% of methane, CH_4) and yield final products with high nutrient, anaerobic digestion (AD) is considered to be one of the most technically mature and cost-effective processes [21]. This technology has been successfully implemented in the production of methane as a renewable biofuel source from various types of organic wastes, including agricultural wastes, food wastes, and sewage sludge [9].

Compared with the traditional low-solid AD, high-solid AD, with a low total solid content of the feedstocks, typically less than 85% (w/w) [32], is believed to be favorable owing to such factors as smaller reactor volume, less material handing, and lower energy requirement for heating [9, 10]. In China, over 80% of the sewage sludge has already been dewatered before further treatment and disposal, which makes it feasible to solve the disposal problems caused by sewage sludge using high-solid AD technology. However, the research of this new AD technology is still at a starting point and many problems in this field need to be resolved. On the basis of our previous research, it was found that the physical and chemical parameters in a high-solid AD process were obviously different from that in low-solid

AD, resulting from the difference of total solid (TS) content of feeding sewage sludge and that the high-solid AD process could show better performances such as higher volumetric methane production rate.

It is known that anaerobic digestion, including hydrolysis, acidification, and methanogenesis, is actually a microbial process of organic matter degradation and stabilization [18]. Therefore, characterization of the microbial community structure in high-solid anaerobic fermentation reactors is critical to better understand the underlying mechanisms of highly efficient methane production. However, most of the microbial studies on the AD sewage sludge in available literature were conducted for biogas production in low-solid AD, and there have been no reports on the microbial characteristic in the mesophilic high-solid anaerobic condition [1, 16, 18]. Although the effect of organic loading rate on reactor performance and archaeal community structure were evaluated in mesophilic anaerobic digesters treating municipal sewage sludge with 13.3% TS content [12], the results obtained were still very limited. It was reported in the literature that the TS content affects the global anaerobic digestion performance, based on experimental observations and ADM1 simulations [2], suggesting that the microbial characteristic in high-solid anaerobic fermentation reactors will change with the increase of TS content. However, so far, the microbes and their competition or cooperation correlation in sewage sludge hydrolysis, acidification, and methanogenesis in high-solid anaerobic digestion processes under mesophilic conditions have not yet been fully investigated. Thus, information is rather scant.

As an alternative to the traditional culture-dependent techniques, which may lead to underestimating the real microbial diversity [19], various molecular methods have been applied in a large number of scientific research studies to better understand the microbiomes in anaerobic fermentation systems and their influence on the stability and efficiency of anaerobic digestion [30]. High-throughput sequencing, which is a next-generation sequencing technology, has revolutionized the microbial diversity study in recently years. The 454 pyrosequencing platforms can generate a large amount of DNA data through a massively parallel sequencing-by-synthesis approach [33], and identify more than thousands of operational taxonomic units (OTUs) [19, 31]. As of now, 454 high-throughout pyrosequencing has been successfully applied to investigate the microbial community in various environmental samples and been used to elucidate microbial diversity in AD [25].

The overarching goal of this study is, therefore, to investigate the underlying mechanisms of the highly efficient methane production by characterizing and comparing the microbial characteristics between high-solid and low-solid anaerobic fermentation reactors treating sewage sludge. The 454 high-throughput pyrosequencing method was employed to analyze the bacterial and archaeal 16S rRNA genes, and to investigate the microbial community in anaerobic digestion systems with different total solid contents. Meanwhile, the quantitative real-time PCR was performed to quantify the total number of bacteria and archaea to explore the correlation between bacteria and methanogenic archaea in the high-solid and low-solid anaerobic fermentation reactors.

Materials and Methods

Substrates and Inoculums

The dewatered sewage sludge used as substrates in this study was obtained from Anting municipal wastewater treatment plant (WWTP) (Shanghai, China), which is in the charge of Shanghai Environmental Protection (Group) Co., Ltd. There is no prohibition from obtaining dewatered sewage sludge from Anting WWTP. The TS content of dewatered sludge ranged from 23% to 25% (w/w) and volatile solid (VS) accounted for 47% to 49% of TS. The sludge was directly stored at 4°C and heated to 35°C before feeding. The anaerobic sludge used as the inoculums was obtained from a full-scale anaerobic digestion reactor with a capacity of 7,000 m^3 at Bailonggang WWTP in Shanghai, China. It had TS of 5.52% (w/w) and VS was 45.33% of TS. The main characteristics of the dewatered sludge and inoculums for the investigation are shown in Table 1.

Reactors and Operation

Two sets of identical anaerobic fermentation reactors with three replicate reactors in each set were equipped with helix-type stirrers, which were set at a rate of 60 rpm with 10 min stirring and 10 min break continuously. Each reactor with a 6.0 L volume

Table 1. Characteristics of the substrates and inoculums.

Parameters	DS[a] 1 (days 1–60)	DS2 (days 61–120)	Inoculums
pH	7.60 ± 0.25	7.43 ± 0.14	7.77 ± 0.21
TS[b] (%, w/w)	23.38 ± 1.32	24.53 ± 1.25	5.52 ± 0.14
VS[c]/TS (%)	49.03 ± 1.80	47.12 ± 1.22	45.33 ± 1.45
TAN[d] (mg/l)	734 ± 8	793 ±6	339 ± 2
FAN[e] (mg/l)	31 ± 1	35 ± 1	36 ± 1

The data are the averages and their standard deviations in duplicate tests.

[a]Dewatered sludge.

[b]Total solids.

[c]Volatile solids.

[d]Total ammonia-nitrogen.

[e]Free ammonia-nitrogen.

liquid working was operated semi-continuously at 35 ± 1°C. After the addition of 6.0 L of anaerobic seed sludge, these anaerobic reactors were continuously flushed with nitrogen gas to remove oxygen and then sealed. According to our previous publication, each reactor was fed with sewage sludge with its designed TS level of 6% and 24% diluted with de-ionized water before feeding [9], respectively. Daily feeding was carried out by pushing dewatered sludge through a feeding piston. As the digestate of dewatered sludge was semi-fluid, draw-off can be easily carried out by directly opening the discharge valve. A photo of the reactor and the simplified drawing were reported and shown in the previous study [10]. All reactors were operated at least five SRTs at 20-day SRT (solid retention time). The biogas production and VS reduction efficiency in each reactor were measured during the anaerobic fermentation period, and were observed to reach a relatively steady state after 60 days of operation. For a full understanding of the microbial characteristics, sewage sludge samples from fermentation reactors were analyzed on day 120 before feeding fresh sewage sludge.

DNA Extraction and Real-Time PCR assay

The Fast DNA Spin Kit (BQIO Gene, Carlsbad, CA, USA) was used to extract total DNA from the sludge sample with three replicates in each set according to the manufacturer's instructions, and then pooled together and stored at −20°C prior to use. All DNAs were extracted in triplicates. Total 16S rRNA genes of bacteria and archaea were estimated by real-time PCR analysis using the domain-level universal primers 341F (5′-CCTACGGGA GGCAGCAG-3′) and 534R (5′-ATTACCGCGGCTGCTGG-3′), and Arch344F (5′-ACGGGGYGCAGCAGGCGCGA-3′) and Arch915R (5′-GTGCTCCCCCGCCAATTCCT-3′) for bacteria and archaea [34], respectively. Real-time PCR analysis was performed using a LightCycler 1.2 instrument (Roche Diagnostics, Mannheim, Germany). The PCR system was 300 nM of both primers, 5 µl of diluted template DNA, 4 µl of LightCycler Faststart DNA Master SYBR-Green I (Roche Applied Science, UK) and 9 µl of sterile Mullipure water. All PCR amplifications were carried out in triplicate and a negative control throughout was performed with sterile Millipure water (no template). The cycling profile for the assay was done as follows: 95°C for 30 sec, then 40 cycles of denaturation at 94°C for 30 sec, and combined annealing and extension at 60°C for 30 sec. A standard curve, generated by using 10-fold serial dilutions of linearized plasmids containing the target fragment of bacteria (or archaea) as a template, was used to calculate the total copy number of bacteria (or archaea) per gram TS.

PCR Amplification, Pyrosequencing, and Sequence Analysis

To investigate the microbial community structures in high-solid and low-solid anaerobic fermentation reactors, the primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 533R (5′-TTACCG CGGCTGCTGGCAC-3′) [20] were used for the amplification of the 16S rRNA genes of bacteria, whereas the primers Arch344F and Arch915R were adopted for that of archaea. The 454 adapter for the primers was A′ (5′-CCATCTCATCCCTGCGTGTCTCCG ACGACT-3′) and B (5′-CCTATCCCCTGTGTGCCTTGGCAGTCG ACT-3′). Barcodes (ACACGACGAC and ATGCTACG) that allow sample multiplexing during pyrosequencing were incorporated in the 5′ end of primers 533R and Arch915R, respectively. PCR systems (50 µl) were prepared in duplicates and each contained 1 µl of genome DNA, 5 µl of 10 polymerase buffer, 200 µM of mixed dNTP, 0.2 µM of both primers, and 2.5 U Taq DNA polymerase. The thermocycling steps for bacteria consisted of 98°C for 2 min, then 25 cycles at 98°C for 10 sec, 55°C for 5 sec, 72°C for 15 sec, and a final extension step at 72°C for 5 min. For archaea, the thermocycling was similar to that for bacteria except that the annealing temperature was 57°C.

Before sequencing, the PCR products were purified using an E.Z.N.A. Gel Extraction Kit (Omega Bio-Tek, USA), quantified with a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and then pooled at equal concentrations. The final PCR products were sequenced on the Roche GS FLX 454 pyrosequencing platform in Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). To minimize the effect of random sequencing errors, the Mothur software was used to check and filter all raw sequences before further analyses [26]. The sequence reads with one or more ambiguous base(s), shorter than 200 bp, checked as chimeric artifact were eliminated. Based on the Silva database, the rest of the sequences were trimmed, qualified, and then classified using the Bayesian approach following a previous procedure [23]. Mothur software was also used to assign the optimized sequences to OTUs (97% similarity) and calculate Good's coverage, richness estimators of Chao 1, abundance-based coverage estimator (ACE), Shannon diversity, and Simpson diversity indices according to the literature [30]. For each library, more than 10,000 sequence reads were retrieved from the bacterial community and 5,000 sequences from archaea. The sequences from the pyrosequencing run have been submitted to the NCBI Short Read Archive database under the accession numbers SRR1302083 and SRR1302089 for bacterial sequences, and SRR1302074 and SRR1302078 for archaeal sequences.

Analytical methods

The concentrations of TS, VS, pH, total ammonia-nitrogen (TAN), free ammonia-nitrogen (FAN), and total alkalinity (TA) were measured according to the standard methods [3]. The produced biogas was collected with a gas collector and sampled by a 1 ml syringe to analyze the CH_4 content that was measured by a gas chromatograph (GC) (Agilent Technologies 6890N, CA, USA) [9]. To analyze volatile fatty acids (VFA), the samples from the reactors were centrifuged at 10,000 ×*g* for 15 min. The supernatants were passed through a 0.45 mm microfiber filter paper. The filtrate was collected in a 1.5 ml gas chromatography (GC) vial. Formic acid (5%) was added to adjust the pH to approximately 4.0. The degradation or removal level based on VS (*i.e.*, VS reduction) was calculated with the same equation as described in a previous publication [9].

Table 2. Summary of parameters on system stability and inhibition in reactors.

Reactor	SRT (day)[a]	OLR[b] (kg VS m^{-3} d^{-1})	pH	TA[c] (mg/l)	TAN[d] (mg/l)	FAN[e] (mg/l)
R1 (6% of TS)	20	1.4	7.39 ± 0.07	4,200 ± 105	1,322 ± 48	58 ± 3
R2 (24% of TS)	20	5.6	7.87 ± 0.07	14,090 ± 220	3,954 ±103	392 ± 16

[a]SRT, solid retention time.

[b]OLR, organic loading rate.

[c]TA, total alkalinity.

[d]TAN, total ammonia-nitrogen.

[e]FAN, free ammonia-nitrogen.

Statistical Analysis

All experiments were carried out in the School of Environmental Science and Engineering of Tongji University in China and all assays were performed in triplicates. The performances data of each reactor were expressed as the mean ± standard deviation of the last five samples during the operation period of the sixth SRT. An analysis of variance by SPSS (Statistical Package for the Social Science, ver. 19.0) was used in this study to test the significance of the results, and $p < 0.05$ was considered statistically significant.

Results and Discussion

Anaerobic Digestion Performance of High-Solid and Low-Solid Fermentation Reactors

Table 2 shows the main parameter values of the system at a steady state (determined by constant methane and VS reduction). The data were the average values of the last five collected samples during the period of the sixth SRT. TA and pH, and TAN and FAN indicated the system stability and potential inhibitory chemicals, respectively.

pH is an important parameter greatly affecting the anaerobic fermentation. The anaerobic reactors showed stability and the anaerobic digestion process occurred normally, as a constant pH was obtained for all reactors. The pH value was about 7.87 ± 0.07 and 7.39 ± 0.07 in high-solid AD with 24% TS content and low-solid AD with 6% TS content, respectively. The pH values from 6.5 to 8.5 for anaerobic fermentation were within the permissible range but not the optimal range from 6.8 to 7.4 [22]. It is known that a high VFA concentration results in the decrease of pH value. However, in this study, the pH value in the high-solid reactor was higher than that in the low-solid reactor, which could be explained that a higher buffering capacity was observed in the high-solid reactor with 24% TS content. The total alkalinity value was 14.09 ± 0.2 g $CaCO_3$ l^{-1} in the high-solid anaerobic reactor. It is known that ammonia-nitrogen (especially free ammonia) is an important parameter seriously influencing the stability of anaerobic digestion process. As shown in Table 2, with FAN concentration lower than 400 mg/l and TAN concentration lower than 4,000 mg/l in these reactors, both high-solid and low-solid anaerobic digestions exhibited satisfactory stability according to the inhibition description with difference ranges of TAN and FAN concentration [9].

Fig. 1 shows the variations of the VS reduction efficiencies, VFA, and methane contents in high and low solid anaerobic fermentation reactors during the fermentation period from 60 days to the end of experiment of operation 120 days. As seen in Fig. 1A, the VS reduction of the two sets of anaerobic reactors with different TS contents showed a stable trend with no obvious fluctuation. The average VS reduction efficiency of the high-solid anaerobic fermentation reactor with 24% TS content was 35.4 ± 1.31%, whereas that of the low-solid reactor with 6% TS content was only 27.8 ± 1.27%, which were significantly different ($p < 0.000$). Meanwhile, it can be found that the average concentrations of VFA in the high-solid and low-solid anaerobic fermentation reactors were 1,383 ± 47.69 and 142 ± 4.45 mg/l, respectively (Fig. 1B). Obviously, the accumulation of VFA was highly improved for the anaerobic fermentation reactors with higher total solid content, which could be explained that more organic matter was degraded and transformed to VFA in the reactor with higher OLRs. It was obvious that the acidogenic activity was not influenced significantly based on the high VFA contents and almost steady VS reduction. On the other hand, the volumetric and specific methane production rates based on the added VS in the high-solid reactor of 24% TS content were observed to be 1,144 ± 32 and 196 ± 6 ml CH_4 $l^{-1}d^{-1}$, while that in the low-solid reactor of 6% TS content were around 154 ± 3 and 111 ± 34 ml CH_4 g^{-1} VS^{-1}_{added} d^{-1}, respectively (Fig. 1B and 1C). Obviously, high-solid AD of sewage sludge facilitated the organic matter reduction and VFA production, and reached much higher methane production compared with low-solid AD. Anaerobic digestion is a microbial process that is accomplished jointly by different types of microorganisms in tandem with their

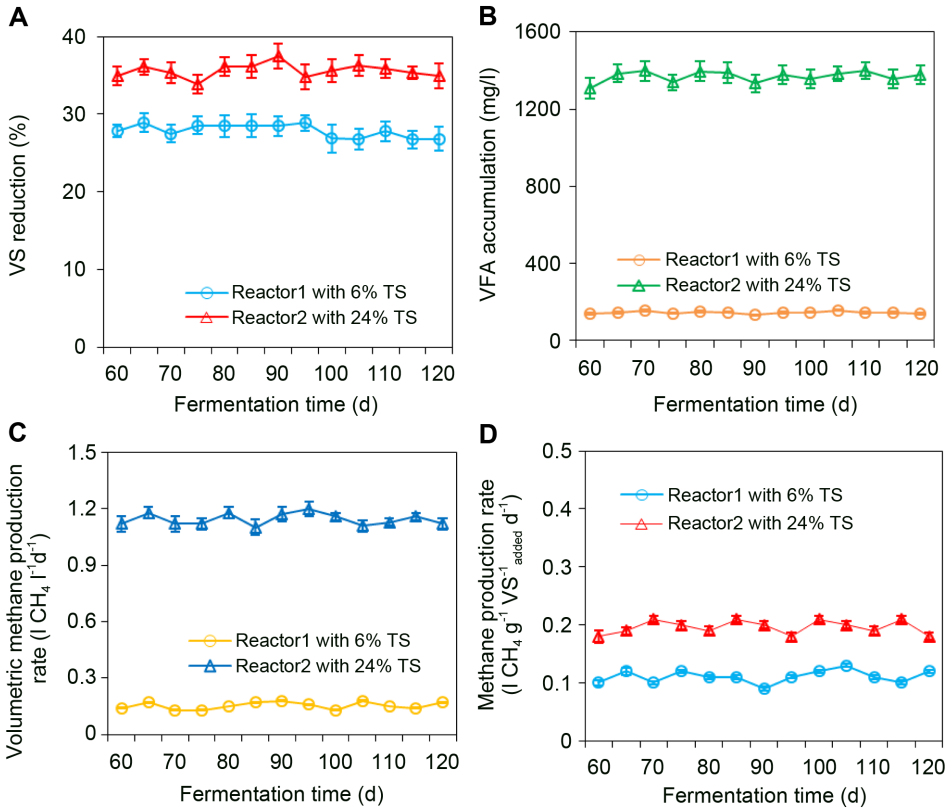

Fig. 1. VS reduction efficiency (**A**), VFA accumulation (**B**), volumetric methane production rate (**C**), and methane production rate based on added VS (**D**) in high-solid and low-solid anaerobic fermentation reactors during the fermentation period of 120 days. The VFA consisted of acetic, propionic, isobutyric, *n*-butyric, isovaleric, and *n*-valeric acids. Error bars represent standard deviations of triplicate measurements.

syntrophic interactions. The higher VS reduction efficiency, VFA concentration, and methane production rate in high-solid AD were closely related to the microbiologic characteristics. Therefore, the underlying mechanisms of the better performance of high-solid AD were investigated by analyzing microbial community structures and total numbers of bacteria and archaea in anaerobic reactors with 6% and 24% TS contents.

Bacterial Characteristics in the Two Fermentation Reactors

The latest developed 454 high-throughput pyrosequencing was employed to investigate the microbial community structures and dynamics. Two bacterial 16S rRNA gene libraries were constructed from the pyrosequencing of bacterial populations in the high-solid and low-solid anaerobic fermentation reactors. The diversity and richness indices of bacterial community structure were calculated at a 3% width, as showed in Table 3. Pyrosequencing of the high-solid and low-solid samples yielded 5,124 and 9,909 high-quality sequence tags, respectively. Compared with other investigations and studies on anaerobic fermentation systems with other molecular methods, such as denaturing

Table 3. Community richness and diversity indices for bacterial and archaeal communities.

Sample name	Bacteria							Archaea						
	Effective[a]	OTU[b]	Ace	Chao	Shannon	Simpson	Coverage	Effective	OTU	Ace	Chao	Shannon	Simpson	Coverage
R1-6%	9,909	344	367	361	3.43	0.12	0.98	6,528	15	15	15	0.44	0.83	0.97
R2-24%	5,124	223	252	242	2.81	0.18	0.97	9,307	18	18	18	0.49	0.80	0.98

[a]Trim reads that passed quality controls.

[b]OTU, operational taxonomic units.

gradient gel electrophoresis (DGGE), fluorescence in situ hybridization (FISH), and clone library, which always have insufficient resolutions to reflect the microbial community characteristic in environmental samples [6], the phylogenetic analysis of 454 high-throughput pyrosequencing demonstrated a more effective method for better revealing the microbial community diversity in various environmental samples [31, 33]. In order to evaluate the bacterial structure communities in anaerobic reactors, sequence reads were clustered to OTUs based on 97% sequence similarity. Table 3 demonstrates that the bacterial sequences from the reactor of 6% TS content were identified to be 344 OTUs, which was different from 1,159 OTUs of conventional anaerobic digester reported in the literature [34]. However, the number of bacterial OTUs at 24% TS content was 223, indicating that increasing the TS content reduced the bacterial diversity. Other species richness estimators such as ACE and Chao indices at 24% TS content were also lower than those at 6% TS content (Table 3).

There were large differences in bacterial diversity in the two sets of anaerobic systems based on the diversity estimators of Chao, ACE, and Shannon and Simpson indices (Table 3). Only 162 OTUs of total 489 OTUs were shared in the two sets of anaerobic fermentation reactors, and the bacterial diversity of the high-solid fermentation reactor of 24% TS content was less than that of the low-solid fermentation reactor of 6% TS content.

Bacterial communities based on the phylum level were analyzed by comparing the bacterial populations between high-solid and low-solid anaerobic reactors with 24% and 6% TS contents (Fig. 2). The phyla of relative abundance lower than 0.5% were grouped into minor groups. It was found

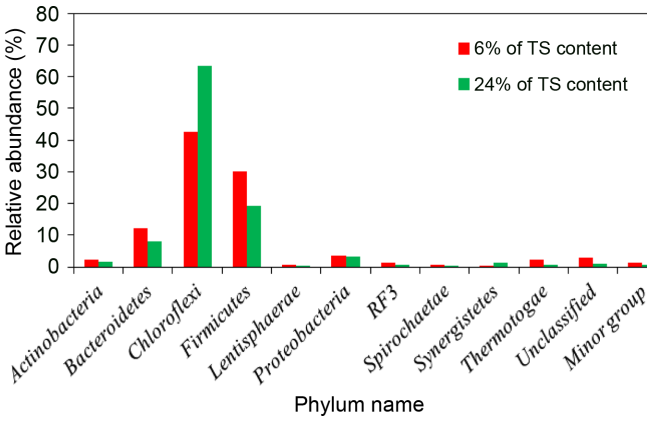

Fig. 2. Taxonomic compositions of bacteria at the phylum level in each sample retrieved from pyrosequencing.
The number in the sample names represents the total solid content.

that the bacterial communities in the two pyrosequencing libraries were nearly identical. The most abundant bacterial populations belonged to Chloroflexi, Bacteroidetes, and Firmicutes, the total relative abundances of which were 84.8% and 90.6%, respectively. The dominance of these three phyla in this study was consistent with previous studies on anaerobic digestion processes investigated by conventional molecular biology methods [25]. The phyla represented by fewer sequences included Actinobacteria, Lentisphaerae, Proteobacteria, RF3, Spirochaetes, Synergistetes, and Thermotogae. Although the majority of bacterial sequences obtained from the two sets of anaerobic fermentation reactors were affiliated to these three phyla, the relative abundance of each group was evidently different. It can be found that Bacteroidetes, Chloroflexi, and Firmicutes in the high-solid anaerobic fermentation reactor with 24% TS content accounted for 12.2%, 42.6%, and 30.0% of total bacterial sequences, respectively, while in the low-solid anaerobic fermentation reactor of 6% TS content were 8.0%, 63.5%, and 19.1%. It was obvious that high-solid AD of sewage sludge increased the relative abundance of Chloroflexi and decreased the relative abundances of Bacteroidetes and Firmicutes.

It was reported that Bacteroidetes, Firmicutes, and Chloroflexi are widely distributed throughout various natural and constructed anaerobic environments, and have the ability to degrade a wide range of complex organic matters [4]. In this study, the identification of these phyla in both high-solid and low-solid anaerobic fermentation reactors by 454 high-throughput pyrosequencing illustrated the superiority of these phyla in the hydrolysis and acidification of complex organic matter in sewage sludge.

The relative abundance of the phylum Chloroflexi in both high-solid and low-solid anaerobic fermentation reactors was the highest, indicating the major impact of this phylum on the conversion of organic matter in sewage sludge. The proliferation of Chloroflexi, a well-known scavenger of biomass-derived organic carbon [25], supports a great influence of difficult-to-biodegrade organic materials in sewage sludge and endogenous decay of the anaerobic biomass. The phylum Chloroflexi currently includes five major classes [14], and over 98.8% of sequences identified in the two systems belonged to the class Anaerolineae (Table S1), which was previous known as "subphylum I" [32]. Previous literature pointed out that this subphylum consists of only a few cultivable organisms, which are all chemoorganotrophs mainly utilizing different carbohydrates and amino acids as substrates for growth [27]. The higher relative abundance of the phylum Chloroflexi per gram TS

in high-solid anaerobic reactors seems to benefit the hydrolysis of carbohydrate and other organic materials in sewage sludge that are difficult to biodegrade.

On the other hand, the Bacteroidetes are proteolytic bacteria degradating protein [25, 35] and are able to ferment amino acids to acetate and NH_3. Meanwhile, the majority of microorganisms of this phylum also participate in metabolizing amino acids to produce VFA [25]. However, their selective enrichment in the low-solid anaerobic fermentation reactor seems to be in contrast with the low protein-input rate and total VFA production (Fig. 2). The phylum Firmicutes, another very abundant group, which are acetogenic and syntrophic bacteria, could degrade various VFA. Within the phylum Firmicutes, over 80% of the bacterial sequences were assigned to the class of Clostridia for each sample (Table S1). The class Clostridia was usually reported to be capable of cellulose hydrolysis and protein catabolism in anaerobic digestion processes [27]. The syntrophic role of Firmicutes competes for H_2 and CO_2 with methanogens [17] and has immediate implications for the composition of the methanogenic community in anaerobic fermentation reactors. Methanogenic acetate degradation is carried out by either an aceticlastic reaction or an anaerobic acetate-oxidizing reaction. The syntrophic acetate-oxidizing bacteria can oxidize acetate to produce hydrogen/CO_2 only when their products are subsequently utilized by the hydrogen-scavenging methanogens. Surprisingly, some of these bacteria can also axenically grow on hydrogen/CO_2 to produce acetate. This means that the bacteria can utilize both substrates and products reversibly.

It was reported in the literature that the hydrolysis of complex organic substances in sewage sludge was the rate-limiting step in anaerobic digestion processes [28], and a part of organic matter could not be converted to VFA and methane, as indicated by the low VS reduction efficiency. The main organic substrates in the sewage sludge were particulate carbohydrate, protein, and lipid. The hydrolysis of the latter two ingredients was more difficult compared with the hydrolysis of carbohydrate. In the present study, the total number of bacteria was evaluated by quantitative real-time PCR with the specific bacterial primers. It was showed that the average numbers of total bacteria in the high-solid anaerobic fermentation reactor of 24% TS content and low-solid anaerobic reactor of 6% TS content were $8.09 \pm 0.13 \times 10^9$ and $1.38 \pm 0.02 \times 10^5$ copies/g TS, respectively, which had significant difference. Considering the predominance of these three phyla and the important roles played in degrading organic matter, it was suggested that the higher abundance of total bacteria in high-solid anaerobic digestion, especially for the key functional microorganisms, was the main reason for the higher VFA concentration and VS reduction efficiency in the high-solid anaerobic fermentation reactor.

To further reveal the characteristic of bacterial community structures in the two sets of anaerobic fermentation reactors, it is preferable to conduct a comparison of the sequencing data at the subdivision level. The relative abundance of the bacteria in the two samples was calculated at the genus level. The sequence distributions in each sample are shown in Table S2. A total of 162 genera were detected, among which 39 genera with a relative abundance higher than 0.5% in at least one sample were well screened as the abundant genera. Other genera were grouped into the minor groups.

Archaeal Characteristics in the Two Fermentation Reactors

In view of the close association of methanogenic microorganisms with bacterial community and the potential difference of the methanogenic pathway between high-solid and low-solid anaerobic fermentation reactors, the archaeal communities were also investigated by pyrosequencing targeting archaeal 16S rRNA gene segments in the present study. It is common knowledge that VFA production in the acidification period will be converted to methane by the methanogenic archaea in the following methanogenesis process. As seen in Fig. 1, the methane production efficiency of the high-solid anaerobic fermentation reactor in both volumetric methane production and methane production rate based on added VS was significantly higher than that of the low-solid anaerobic fermentation reactor. However, by far, the reasons for the higher methane production efficiency of high-solid AD of sewage sludge have not yet been comprehensively investigated from the aspect of microbiology. Although the anaerobic digestion performances were different in many aspects for these reactors, resulting from the difference of feeding TS content (Table 2 and Fig. 1), interestingly, the two fermentation samples showed similar archaeal community structures at the order level. As shown in Fig. S1, the majority of the sequence reads were identified as methanogens (more than 99% in each individual sample). The notable microbial characteristic of the archaeal communities in high-solid and low-solid anaerobic fermentation reactors was the highest abundance of acetoclastic methanogenic order Methanosarcinales, comprising 90.1% and 91.7% of total archaeal sequence reads, respectively. The hydrogen-utilizing methanogenic Methanomicrobiales was the second major group in the

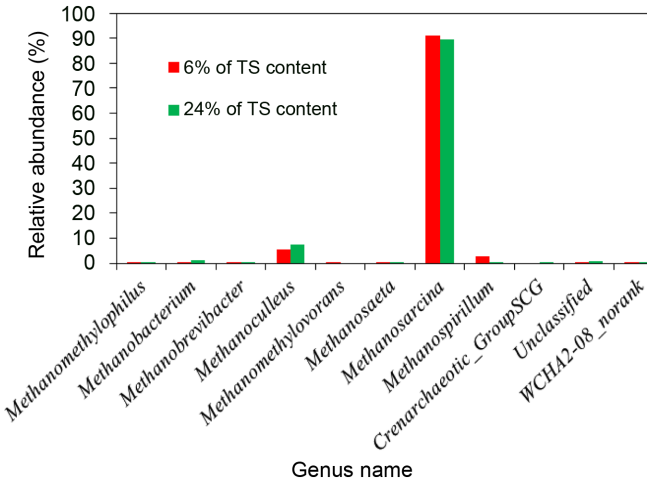

Fig. 3. Taxonomic compositions of methanogens at the genus level in each sample retrieved from pyrosequencing.
The number in the sample names represents the total solid content.

reactors (accounting for 7.7% and 8.1%). However, no Methanococcales-related sequences were identified in our trials, probably due to their requirement of high salt concentration for growth (0.3–9.4% (w/v) NaCl) [5], which was consistent with the results obtained in a previous study [17].

Further comparison of the sequence distributions down to the genus level was conducted in order to reveal more information of the archaeal population between high-solid and low-solid anaerobic fermentation reactors (Fig. 3). As expected, the archaeal diversities were lower than the bacterial diversities, which corresponded with the results obtained from another previous study [15]. From Fig. 3, it was found that there was no large gap in the taxonomic compositions at genus level between high-solid and low-solid samples, which was consistant with the methanogens diversity (Table 3). The main subgroup of the identifiable sequences was related to the genus *Methanosarcina* in both samples, accounting for 89.5% and 91.1%, respectively, which had no significant difference. This is in well agreement with a previous report that showed the marked predominance of *Methanosarcina* in various anaerobic digestion processes [11]. The genus *Methanoculleus* represented the second predominant phylogenetic group in archaeal libraries (7.5% and 5.3%, respectively). Thus, it could be inferred that *Methanosarcina* played a dominant role in anaerobic fermentation of sewage sludge and the major pathway of methane production was based on acetoclastic methanogenesis.

Methanosarcina is a well-known typical and versatile member of acetoclastic methanogens. It was reported that *Methanosarcina* has high growth rates and was more tolerant to sudden pH change from 0.8 to 1.0 units compared with other methanogens that have doubling times of a minimum of 4–6 days and tend to be affected by a pH shock of 0.5 units or even less [7]. In addition, this genus also showed the strong ability of growing in aggregates and forming irregular cell clumps, which can enhance their tolerance to toxic ionic agents [8]. Furthermore, the genus *Methanosarcina* can produce methane from acetate, methanol, monomethylamine, dimethylamine, trimethylamine, H_2/CO_2, and CO; that is, they are able to use both the acetoclastic and the hydrogenotrophic methanogenesis pathways, making them more tolerant to specific inhibitors of the acetoclastic pathway [13]. Therefore, owing to the special physiological characteristics and the flexibility in metabolism, it was suggested that an anaerobic fermentation reactor that is entirely based on the genus *Methanosarcina* could potentially achieve stable methanogenesis [29]. Real-time PCR results showed that the average total numbers of archaea in high-solid and low-solid anaerobic fermentation reactors with 24% and 6% TS contents were $9.54 \pm 2.23 \times 10^7$ and $7.73 \pm 0.14 \times 10^4$ copies/g TS, respectively, suggesting that high-solid AD of sewage sludge significantly increased the number of total methanogenic archaeal microorganisms, which perhaps was the main reason for the high VFA consumption and methane production in high-solid anaerobic digestion. To the best of our knowledge, this is the first creative report on the qualitative and quantitative approaches to look at how bacterial and archaeal characteristics differently evolve in high-solid and low-solid anaerobic fermentation reactors. It can be obviously seen from this study that the high anaerobic digestion efficiency of the high-solid anaerobic fermentation reactor was closely related to its microbial characteristic. This study expands our knowledge on the different microbial responding mechanisms in high-solid anaerobic sewage sludge fermentation to the high TS content compared with low-solid AD, and will help to break the technical bottleneck of low methane yield during anaerobic digestion of sewage sludge.

In conclusion, high-solid AD of sewage sludge increased the number of total bacteria, and the phyla Chloroflexi, Bacteroidetes, and Firmicutes were the key functional microorganisms that benefited the hydrolysis and acidification of organic matter in sewage sludge. Meanwhile, the number of total archaea dominated by *Methanosarcina* in high-solid AD was higher than that in low-solid AD, which was responsible for the high VFA consumption and

methane production. Therefore, the increased abundances of key bacteria involved in sewage sludge hydrolysis/acidification and methanogenic archaea are the main reasons for the higher VS reduction efficiency and methane production.

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