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Determination of the archaeal and bacterial communities in two-phase and single-stage anaerobic systems by 454 pyrosequencing

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ABSTRACT

2-Phase anaerobic digestion (AD), where the acidogenic phase was operated at 2 day hydraulic retention time (HRT) and the methanogenic phase at 10 days HRT, had been evaluated to determine if it could provide higher organic reduction and methane production than the conventional single-stage AD (also operated at 12 days HRT). 454 pyrosequencing was performed to determine and compare the microbial communities. The acidogenic reactor of the 2-phase system yielded a unique bacterial community of the lowest richness and diversity, while bacterial profiles of the methanogenic reactor closely followed the single-stage reactor. All reactors were predominated by hydrogenotrophic methanogens, mainly *Methanolinea*. Unusually, the acidogenic reactor contributed up to 24% of total methane production in the 2-phase system. This could be explained by the presence of *Methanosarcina* and *Methanobrevibacter*, and their activities could also help regulate reactor alkalinity during high loading conditions through carbon dioxide production. The enrichment of hydrolytic and acidogenic *Porphyromonadaceae*, *Prevotellaceae*, *Ruminococcaceae* and unclassified *Bacteroidetes* in the acidogenic reactor would have contributed to the improved sludge volatile solids degradation, and ultimately the overall 2-phase system's performance. Syntrophic acetogenic microorganisms were absent in the acidogenic reactor but present in the downstream methanogenic reactor, indicating the retention of various metabolic pathways also found in a single-stage system. The determination of key microorganisms further expands our understanding of the complex biological functions in AD process.

Introduction

Anaerobic digestion (AD) has been widely applied for sludge treatment at many municipal and industrial wastewater treatment plants. The AD process involves biological hydrolysis, acidogenesis, acetogenesis and methanogenesis (Appels et al., 2008). Fundamentally, these reactions are performed by different microbial groups possessing various metabolic capabilities. Most chemoheterotrophic *Bacteria* are involved in the hydrolysis and acidogenesis reactions of proteins, carbohydrates and lipids (Nelson et al., 2011; Regueiro et al., 2012). Acetogenic microorganisms consist of acetate-producing syntrophic *Bacteria* or

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homoacetogenic *Bacteria* which carry out reversible reduction of carbon dioxide to acetate by utilising hydrogen. The cultivation of syntrophic microorganism requires the presence of its syntrophic partner (*e.g.*, hydrogenotrophic methanogen) to keep the hydrogen partial pressure low (Stams et al., 2012). Lastly, methanogenesis is performed by the methanogenic consortia of *Archaea* which generally utilised either acetate, formate, or hydrogen as electron donor (Liu and Whitman, 2008).

Accumulation of volatile fatty acids (VFAs), caused by an imbalance of the above four steps, is common in single-stage anaerobic systems operated at high organic loadings. VFA accumulation could reduce the reactor pH, inhibit methanogenic activity and subsequently cause system failure (Appels et al., 2008). The hydrolytic and acidogenic bacteria have growth rates which are magnitudes faster than methanogenic microorganisms (Zhang and Noike, 1991). 2-Phase AD configuration separates the two microbial groups through these different growth rates by manipulating solids retention time (SRT) in two connected but separate reactors. Operation of the acidogenic phase at lower SRTs (1–5 days) would maintain the optimal cultivation of hydrolytic/acidogenic bacteria, while in the next reactor, the methanogenic microorganisms and other slower-growing bacteria are cultivated at longer SRTs (>10 days) (Rubio-Loza and Noyola, 2010). Additionally, accumulation of organic acids in the acidogenic reactor aids release of non-crystalline organic polymers for faster sludge degradation. 2-Phase AD could therefore, be operated under higher organic loadings, while achieving better sludge degradation and biogas production than the single-stage AD (Bhattacharya et al., 1996; Rubio-Loza and Noyola, 2010). This had been demonstrated at pilot and full-scale (Ghosh et al., 1995). 2-Phase AD could also improve pathogen destruction (Rubio-Loza and Noyola, 2010) and alleviate foam problems during digester operation (Ghosh et al., 1995).

Despite the many reports on process performance, the underlying microbial community structure involved in phased AD for municipal sludge digestion has rarely been reported. For instance, there were cases when methanogenic activity was detected in the acidogenic reactor but this phenomena had not been adequately explained (Ghosh et al., 1995; Shimada et al., 2011). In-depth microbial consortium characterization studies have often focused on the single-stage AD configuration (Cardinali-Rezende et al., 2012; Shimada et al., 2011; Shin et al., 2010). The acidogenic reactor would, however, be operated in a manner quite dissimilar from the single-stage AD. There were a few studies investigating the methanogenic *Archaea* and bacterial populations in the 2-phase AD system, but the tools used had not yielded clear identification of key microbial populations. Zhang and Noike (1991) had used a cultivation-dependant method which might have biased towards excluding viable but non-culturable microorganism. The key microbial populations were not determined by Merlino et al. (2013), Shimada et al. (2011) and Schievano et al. (2012) due to limited number of (DNA) templates sequenced, in spite of denaturing gradient gel electrophoresis (DGGE) and clone library being employed. 454 pyrosequencing was proposed in this study to include more sequencing reads at faster analysis rate than cloning-based methods. A growing number of studies had recently adopted 454 pyrosequencing analysis on engineered environmental processes (Sundberg et al., 2013).

Previous study had compared the performance of single-stage against 2-phase AD systems for the treatment of sewage sludge (Maspolim et al., 2015). That study found that the volatile solids reduction and methane production were improved in 2-phase system operated at 2 + 10 day hydraulic retention time (HRT). The use of 454 pyrosequencing in this study attempted to resolve the microbial compositions within the two systems and to further understand the microbiological differences between the two systems. The characterization would focus on the determination of hydrolytic, acidogenic, acetogenic, and methanogenic communities in the single-stage and 2-phase reactors.

1. Materials and methods

1.1. Reactor start-up and operation

Two previously described (Maspolim et al., 2015) sets of anaerobic continuously stirred tank reactors (CSTRs) were operated as the single-stage and 2-phase systems, with HRTs of 12, 2 and 10 days for the single-stage, acidogenic and methanogenic reactors, respectively. These reactors were originally inoculated with anaerobic sludge from a local full-scale anaerobic digester treating municipal sludge. The feed was a mixture of primary and secondary sludge, collected from the same plant. During feeding, 36 mL of the sludge slurry was transferred from the feed reservoir into the acidogenic or single-stage reactor. Acidogenic reactor mixed liquor would be transferred into the methanogenic reactor as feed while mixed liquor from the single-stage and methanogenic reactors would be transferred into the effluent reservoir. These operations would be performed with peristaltic pumps every 14 min for 1 min. All reactors were operated at 35°C and pH of the acidogenic and methanogenic reactors was controlled at 5.5 ± 0.3 and 7.0 ± 0.2, respectively, by automatic dosing of 1 mol/L sodium hydroxide or hydrochloric acid. pH of the single-stage reactor could be maintained at pH 7.0 ± 0.2 without manipulation. The acidogenic reactor was maintained at pH 5.5 to optimize hydrolysis and acidogenesis reactions, as previously reported (Elefsiniotis and Oldham, 1994; Ghosh et al., 1995). The feed had 42,300 ± 3600 mg/L total COD; 2500 ± 1000 mg/L soluble COD; 32.1 ± 2.6 g/L TS; 25.7 ± 2.0 g/L VS; and pH 5.9 ± 0.2. Chemical oxygen demand (COD) and solids measurements were performed in accordance with Standard Methods (APHA, AWWA, WPCF, 2005). C2 to C7 volatile fatty acids (VFA) and the biogas volume and content were measured as previously described (Maspolim et al., 2015).

1.2. Nucleic acid extraction

Microbiological samples were obtained 82 days after the start of the anaerobic system operated with 12 days system HRT. It was assumed that the system would then hold representative microbial communities. DNA was extracted immediately after the samples were taken from the reactors. Prior to DNA

extraction, 1 mL of sludge samples was initially washed twice by centrifugation (20,000 *g*, 2 min), decanting and re-suspension in phosphate buffered saline solution. CTAB phenol/chloroform DNA extraction method was employed on 1 mL of sludge samples following the nucleic acid extraction protocol reported by Griffiths et al. (2000).

1.3. DNA amplification and 454 pyrosequencing

Genomic DNA was submitted to Research and Testing Laboratory (RTL, Lubbock, USA) for the bacterial tag-encoded FLX amplicon pyrosequencing protocol described by Dowd et al. (2008), as 16S rRNA gene universal bacterial primer set (357F and 926R, targeting V3–V5 hypervariable region) (Claesson et al., 2010) and 16S rRNA gene universal archaeal primer set (517F and 909R, targeting V4–V5 hypervariable region) (Wang and Qian, 2009) were used for the amplification. The results are deposited into the NCBI sequence read archive database (accession numbers: SRX667738 and SRX667741 to SRX667743).

1.4. Bioinformatics and statistical analysis

The programme Mothur v1.30 was used to process raw data from pyrosequencing, according to Mothur 454 SOP (Schloss et al., 2011). Raw sequences were trimmed to exclude sequences with at least 1 ambiguous base call, more than 1 barcode mismatch, 2 primer mismatch and 8 homopolymeric bases. Sequences were also removed if the average quality score fell below 25 over a 50 bp sliding window. Sequences were then aligned using SILVA bacterial and archaeal database. This is to ensure that bases which were outside the desired range can be excluded. Bacterial and archaeal sequences were screened to only include sequences starting from the same aligned position, with at least half of the expected amplicon length (285 bp for *Bacteria* and 196 bp for *Archaea*). Additionally, a "pre-cluster" function was used to merge sequences with 1 bp difference. UCHIME was employed to detect and remove chimeras from bacterial and archaeal sequences, using database-independent approach to eliminate undesirable artefact. OTUs were clustered at 97% sequence similarity with average neighbouring clustering algorithm. Normalisation of sample size was conducted by the "sub.sample" function in Mothur by resampling the same number of reads for each sample, based on the smallest sample size. Alpha-diversity (Chao1, ACE, Good's coverage, Shannon and evenness index) and beta-diversity (Yue–Clayton dissimilarity index) were both computed using Mothur. Taxonomic classification of the sequences was done with naïve Bayesian classifier method using RDP training set 9 alignment database and taxonomy, with bootstrap of 1000 and confidence threshold of 50% (Claesson et al., 2010; Sundberg et al., 2013). Phylogenetic relationship of the unclassified *Bacteroidetes* was established by selecting the main OTUs represented in the acidogenic reactor (OTUB0001, OTUB0009 and OTUB0032) and selecting closely related sequences with the highest score in Seqmatch, an online RDP web tool. Neighbour-joining trees were subsequently constructed with MEGA5 using Jukes–Cantor algorithm and bootstrapped 1000 times.

2. Results

2.1. Bioreactor performance

The 2-phase and single-stage AD systems were operated in parallel at HRT of 2 + 10 days and 12 days, respectively. The bioreactor performance had been reported previously (Maspolim et al., 2015) where the 2-phase system achieved higher COD removal, VS reduction, and methane yield (40.7% ± 5.7%, 35.5% ± 6.6%, 0.14 ± 0.03 L/g COD_{added}, respectively), compared to the single-stage system (30.8% ± 6.1%, 26.3% ± 6.1%, 0.1 ± 0.01 L/gCOD_{added}, respectively) ($p < 0.05$, $n = 15$). The improvement had then been shown to be associated with reduced particulate COD in the effluent of methanogenic reactor (24,000 ± 1400 mg/L) than in the single-stage reactor effluent (28,500 ± 1500 mg/L) (Maspolim et al., 2015).

As previously reported (Maspolim et al., 2015), an average of 1700 mg COD/L of total VFA was observed in the acidogenic reactor, with propionic acid (42% of total VFA) as the predominant VFA species. The acetic acid concentration in terms of COD in the feed sludge and acidogenic reactor effluent was 700 and 200 mg COD/L, respectively. It was negligible in the methanogenic and single-stage reactors. Based on carbon balance, acetic acid produced was assumed to have been consumed in the acidogenic reactor and converted into methane, contributing to approximately 24% ± 4% of the total methane production in the 2-phase system. Hydrogen, an electron donor for methanogenesis was also not detected in the acidogenic, methanogenic and single-stage reactors. Despite the methanogenic capability observed in the acidogenic reactor (methane yield of 0.03 L/gCOD_{added}), a longer retention time in the methanogenic reactor (10 day HRT), with methane yield of 0.13 L/gCOD_{added}, was required to reduce residual COD in the feed drawn from the acidogenic reactor (2 day HRT).

2.2. 454 pyrosequencing analysis

A total of 14,404 sequences from universal bacterial primer sets and 31,862 sequences from universal archaeal primer sets were obtained. Sequence processing and OTU-based clustering at 97% similarity by Mothur gave each sample 2137 bacterial sequences of at least 253 bp read length and 2660 archaeal sequences of at least 187 bp read length.

The alpha diversity was investigated by Good's coverage, Chao1 and ACE estimation, Shannon and the evenness index (Table 1). At least 391 bacterial OTUs were formed, compared to a maximum of 78 archaeal OTUs in this experiment. Bacterial community was the richest in the feed sludge, and then followed by those in the single-stage, methanogenic and acidogenic reactors, in decreasing order, as indicated by the number of OTUs, Chao1 and ACE indices. Bacterial diversity according to the Shannon index also followed the same trend, reflecting the feed's higher number of unique phylotypes and/or greater community evenness. It was noted that bacterial richness and diversity of the single-stage reactor were only slightly higher than the methanogenic reactor, in terms of number of OTUs observed (469 *vs.* 435) and Shannon's

Table 1 – Alpha-diversity analysis of the bacterial and archaeal community in the feed sludge, single-stage, acidogenic and methanogenic reactors.

Sample	No. of OTUs	Good's coverage	Chao1	ACE	Shannon index	Evenness index
Archaea						
Feed sludge	78	98.6%	132	181	1.46	0.34
Single-stage	64	98.7%	149	266	1.69	0.41
Acidogenic phase	54	99.1%	86	139	1.59	0.40
Methanogenic phase	51	99.1%	178	135	1.36	0.35
Bacteria						
Feed sludge	564	87.0%	997	1305	5.65	0.89
Single-stage	469	89.5%	773	992	5.27	0.86
Acidogenic phase	391	91.8%	596	628	4.72	0.79
Methanogenic phase	435	90.5%	688	921	5.12	0.84

diversity index (5.27 *vs.* 5.12). Similarly, the archaeal diversity by Shannon index was also higher in the single-stage reactor than the other anaerobic reactors.

Beta diversity of the microbial community structures between different samples was evaluated by Yue–Clayton dissimilarity index, and was presented as a dendogram in Fig. 1. It shows that the *Bacteria* in the acidogenic reactor was distantly related (>40% dissimilarity) to samples from the other reactors, whereas the *Bacteria* in the methanogenic and single-stage reactors were more closely related (ca. 20% dissimilarity). However, the archaeal community structures of all samples were closely related, where the furthest dissimilarity was close to 5%.

2.3. *Methanogenic Archaea characterization*

Fig. 2a presents the relative abundance of archaeal genera in the four samples tested. All of the archaeal phylotypes detected were known methanogenic microorganisms. The low dissimilarity between the methanogenic *Archaea* in the three reactors as shown in Fig. 1 could be attributed to the two dominant groups of methanogens equally detected in all samples, namely, *Methanolinea* and *Methanospirillum*, which were of the *Methanomicrobiales* order. Members of the *Methanomicrobiales* order are known to utilise hydrogen/formate and carbon dioxide to produce methane (Liu and Whitman, 2008).

Methanobrevibacter and *Methanosarcina* were detected at higher relative abundance in the acidogenic reactor than in the single-stage reactor. Table 2 shows that the relative abundances of *Methanobrevibacter* and *Methanosarcina* in the acidogenic reactor were 3.8% and 7.7%, respectively, while these were 0.6% and 0% in the single-stage reactor, and 1.0% and 0.5% in feed sludge. *Methanobrevibacter* is also a hydrogenotrophic methanogen, while *Methanosarcina* is capable of using acetate and hydrogen as electron donor (Liu and Whitman, 2008).

2.4. *Bacterial community characterization*

Fig. 2b shows the relative abundance of bacterial phyla in the feed sludge, and the single-stage, acidogenic and methanogenic reactors. *Bacteroidetes*, *Firmicutes* and *Proteobacteria* formed the most abundant phyla detected in all the reactors. A closer investigation at class and family levels revealed specific enrichment of certain bacterial groups in the acidogenic reactor, which did not occur in the single-stage reactor (Table 2). The rest of the family classification not included in Table 2 can be found in Table S1. At class level, Table 2 shows that *Bacteroidia*, unclassified (U) *Bacteroidetes* and *Clostridia* were more abundant in the acidogenic reactor than in the single-stage reactor, at 10.5% *vs.* 3.3%, 24% *vs.* 9.2% and 28.5% *vs.* 15.6%, respectively. This enrichment was also observed when the acidogenic community was compared against the community from the methanogenic reactor: *Bacteroidia* at 2.7%, *U_Bacteroidetes* at 12.2% and *Clostridia* at 18.1% in the methanogenic reactor.

Within *Bacteroidia*, *Porphyromonadaceae* was detected at 1.6 to 2.4 times higher relative abundance in the acidogenic reactor compared to the single-stage and methanogenic reactors (Table 2). Another *Bacteroidia* family, *Prevotellaceae*, had relative abundance of 3.7% in the acidogenic reactor, while having less than 0.5% in the single-stage and methanogenic reactor. Members of the *Bacteroidia* are known to possess saccharolytic or proteolytic and acidogenic function (Cardinali-Rezende et al., 2012). In particular, *Porphyromonadaceae* had been identified previously in anaerobic reactors treating municipal solid waste; meanwhile, *Prevotellaceae* are saccharolytic anaerobic bacteria, presumably involved in the acidogenesis of carbohydrates (Downes et al., 2007).

As previously mentioned, *U_Bacteroidetes* was enriched to at least double of those found in the single-stage and methanogenic reactors. It was strongly represented by OTUB0001 (16.1%), OTUB0009 (4.1%) and OTUB0032 (2.2%) in the acidogenic reactor, but none of these OTUs was present in

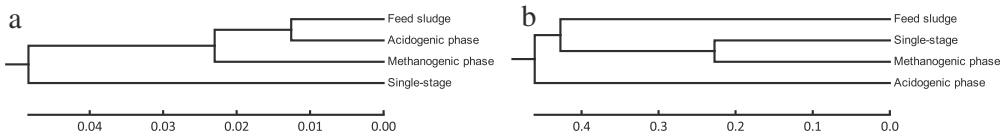

Fig. 1 – Linkage clustering of (a) archaeal and (b) bacterial OTUs from each sample, based on the Yue–Clayton index.

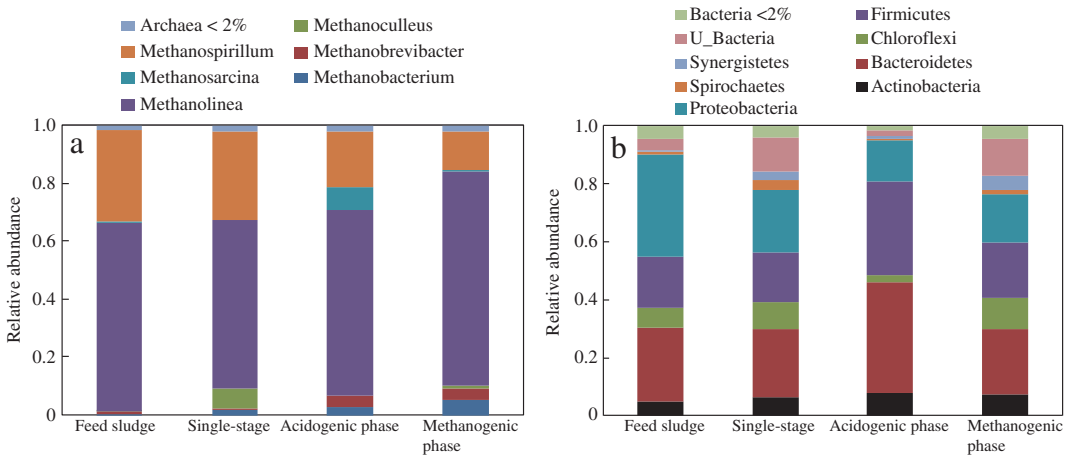

Fig. 2 – Taxonomic classification of archaeal genus (a) and bacterial phylum (b) in the feed sludge, single-stage, acidogenic and methanogenic reactors. Minor taxons with relative abundance of <2% in any sample were grouped together as *Archaea* < 2% and *Bacteria* < 2%, respectively.

the single-stage reactor (Table S2). Phylogenetic tree of closely related 16S rRNA sequences to OTUB0001, OTUB0009 and OTUB0032 retrieved from RDP database indicated that they were affiliated with sequences obtained from anaerobic processes treating coking wastewater, as well as sequences from anaerobic digester sludge in Hokkaido and a biogas reactor in China (Fig. 3). Some of the other reference partial 16S rRNA sequences retrieved were also related to sequences from rumen *Bacteria*, hence, possibly related to the digestion of cellulosic matter. Lastly, *Ruminococcaceae* of *Clostridia* was the other predominant family in the acidogenic reactor, making up to 22.8% out of 28.5% *Clostridia* class relative abundance (Table 2). *Ruminococcaceae* had been strongly associated with degradation of insoluble particulate organic

Table 2 – Relative abundance of archaeal phylum *Euryarchaeota*, bacterial phyla *Bacteroidetes*, *Firmicutes*, *Deltaproteobacteria* and *Synergistetes* to main family/genus level (>2% relative abundance) in the feed sludge, single-stage, acidogenic and methanogenic reactors.

Phylum	Class	Genus/family	Feed sludge	Single-stage	Acidogenic phase	Methanogenic phase
Euryarchaeota	***Methanobacteria***		**1.3%**	**2.4%**	**6.7%**	**9.4%**
		Methanobacterium	0.3%	1.7%	2.9%	5.2%
		Methanobrevibacter	1.0%	0.6%	3.8%	4.2%
	Methanomicrobia		**98.7%**	**97.0%**	**93.3%**	**89.4%**
		Methanoculleus	0.0%	7.0%	0.0%	0.9%
		Methanolinea	65.3%	58.2%	64.2%	73.9%
		Methanosarcina	0.5%	0.0%	7.7%	0.3%
		Methanospirillum	31.5%	30.5%	19.2%	13.4%
Bacteroidetes	***Bacteroidia***		**9.6%**	**3.3%**	**10.5%**	**2.7%**
		Porphyromonadaceae	8.1%	3.3%	5.3%	2.2%
		Prevotellaceae	1.2%	0.0%	3.7%	0.4%
	Flavobacteria		**3.3%**	**7.2%**	**1.7%**	**6.1%**
		Flavobacteriaceae	3.0%	0.6%	1.7%	0.5%
		U_Flavobacteriales	0.3%	6.6%	0.0%	5.7%
	Sphingobacteria		**8.0%**	**4.0%**	**1.8%**	**1.6%**
		Chitinophagaceae	5.1%	2.1%	1.0%	0.5%
		Cytophagaceae	1.4%	0.1%	0.4%	0.4%
	U_Bacteroidetes		**4.1%**	**9.2%**	**24.0%**	**12.2%**
Firmicutes	***Clostridia***		**15.3%**	**15.6%**	**28.5%**	**18.1%**
		Clostridiaceae_1	3.4%	0.7%	1.1%	0.8%
		Peptostreptococcaceae	0.5%	1.4%	0.7%	1.0%
		Ruminococcaceae	8.0%	10.2%	22.8%	9.8%
Proteobacteria	***Deltaproteobacteria***		**3.7%**	**6.3%**	**0.9%**	**7.5%**
		Syntrophaceae	0.3%	4.0%	0.0%	5.9%
		Syntrophorhabdaceae	0.0%	1.3%	0.0%	0.5%
Synergistetes	***Synergistia***		**0.5%**	**3.1%**	**0.9%**	**5.1%**
		Synergistaceae	0.5%	3.1%	0.9%	5.1%
U_Bacteria			**4.0%**	**11.4%**	**2.1%**	**12.9%**

Characters in bold represent the sub-total of the defined class.

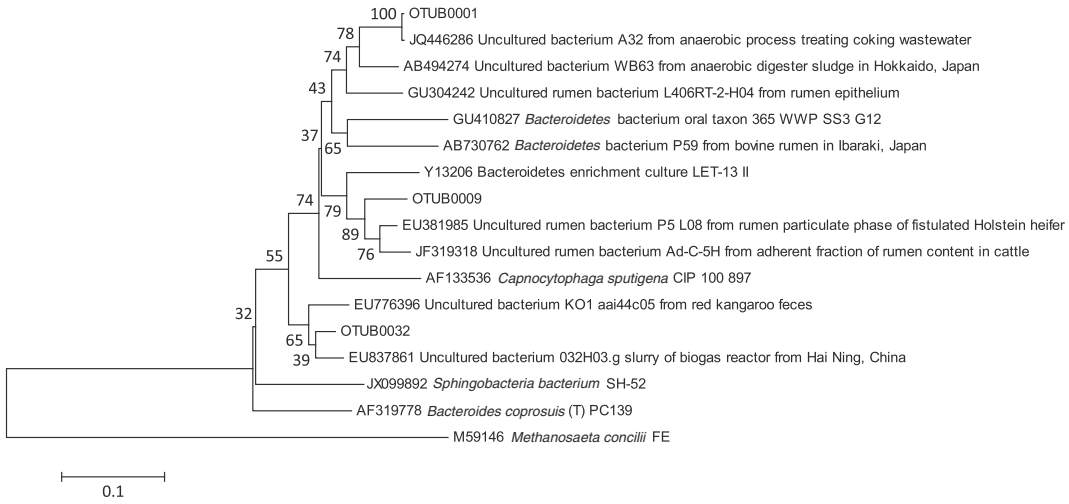

Fig. 3 – Neighbour-joining tree illustrating the phylogenetic relationship of predominant *U_Bacteroidetes* OTUs (OTUB0001, OTUB0009 and OTUB0032) found in the acidogenic reactor.

substrates in human gut in a previous study (Walker et al., 2008).

On the other hand, relative abundance of acetogenic syntrophic microorganisms such as *Syntrophaceae*, *Syntrophorhadaceae* (both of *Deltaproteobacteria*) and *Syntergistaceae* of *Synergistia* was considerably lower in the acidogenic reactor than in the single-stage reactor, at 0% *vs*. 4%, 0% *vs*. 1.3% and 0.9% *vs*. 3.1%, respectively (Table 2). *Syntrophaceae* and *Syntrophorhabdaceae* grow optimally at neutral pH, and formed syntrophic relationship with their hydrogenotrophic partners to oxidise short and long-chain fatty acids (Stams et al., 2012). Most *Synergistia* had proteolytic activity and could ferment amino acids to acetic, propionic or butyric acid. Their activity is known to be enhanced in the presence of hydrogen-utilising methanogens and has been often found in anaerobic digesters treating proteinaceous waste (Vartoukian et al., 2007).

3. Discussion

A previous study treating sewage sludge had reported that the 2-phase system gave higher COD and VS reductions, and methane production compared to the single-stage system, at 12 day HRT (Maspolim et al., 2015). This had also been confirmed in other studies (Bhattacharya et al., 1996; Ghosh et al., 1995). The improvement was attributed to better particulate degradation in the 2-phase system, presumably performed by specific hydrolytic *Bacteria*. Therefore, the involvement of acclimated hydrolytic and acidogenic consortium within the 2-phase system, particularly in the acidogenic reactor, is important to the improved sludge digestion performance. In addition, activity of syntrophic microorganisms and methanogenic *Archaea* was also essential to ensure the complete biodegradation of organic intermediates (*e.g.*, amino acids, propionate, acetate, *etc.*) into methane.

It was noted that the archaeal community in the feed sludge and all anaerobic reactors was predominated by the hydrogenotrophic *Methanolinea* (Fig. 2a). Anaerobic microorganisms must have developed in the feed sludge and could influence the microbial community in the anaerobic reactors. The activity of hydrogenotrophic methanogenesis pathway was confirmed as all anaerobic reactors reported negligible levels of hydrogen (<0.01 mol%). Similar molecular studies had demonstrated hydrogenotrophic methanogens to be prevalent during the sludge digestion process (Kim et al., 2013; Raskin et al., 1995; Shin et al., 2010).

Common bacterial phyla associated with the AD process were detected in both the single-stage and 2-phase systems, namely *Actinobacteria*, *Chloroflexi*, *Bacteroidetes*, *Firmicutes* and *Proteobacteria* (Fig. 2b) (Nelson et al., 2011; Sundberg et al., 2013). These phyla were normally associated with the hydrolysis, acidogenesis and acetogenesis reactions in AD. The microbial diversity by Shannon's index was much lower in the acidogenic reactor, but the microbial diversity in the methanogenic reactor was not much different compared to the single-stage reactor. Hence, it is indicative that the latter two possessed similar variety of metabolic capability (Merlino et al., 2013). In terms of their taxonomic identity, there were obvious differences between the acidogenic and single-stage reactor, while the consortia in the methanogenic reactor closely resembled those in the single-stage reactor.

3.1. Methanogenic population in the acidogenic reactor

Methanogenesis is generally accepted to proceed at pH between 6.6 and 7.8, and at SRT above 8 days (Appels et al., 2008), which was not the operating condition of the acidogenic reactor. Nonetheless, the acidogenic reactor in this study achieved methane yield of 0.03 $L{\cdot}gCOD_{added}^{-1}$, albeit lower than in the single-stage and methanogenic reactors. This study found that 24% of total 2-phase system methane production was from the acidogenic reactor, while the value in a similar acidogenic reactor (HRT 3 days) was only 7% (Ghosh et al., 1995), and was negligible in another study (pH 4.3 to 6.2) treating primary sludge (Elefsiniotis and

Oldham, 1994). Taconi et al. (2008) successfully acclimated a methanogenic population under acidic condition down to pH 4.0–5.3, but the microbial community was not characterized. 454 pyrosequencing analysis confirmed the presence of methanogens in the acidogenic reactor and revealed higher relative abundance of *Methanosarcina* and *Methanobrevibacter* in the acidogenic reactor compared to the other reactors (Fig. 2a). *Methanosarcina* and *Methanobrevibacter* had been previously identified to persist and predominate under acidic pH conditions (Savant et al., 2002; Steinberg and Regan, 2011). Savant et al. (2002) isolated hydrogenotrophic *Methanobrevibacter acidurans* from an acidogenic digester (pH 5) treating alcohol distillery wastewater which contained 8000 to 10,000 mg/L of VFA. Similarly, *Methanosarcina* had been found to be predominant in an acidic digester (pH 5) experiencing shock glucose loads (Steinberg and Regan, 2011). These were similar operating conditions with the acidogenic reactor in this study and hence resulting in enrichment of *Methanosarcina* and *Methanobrevibacter* in the acidogenic reactor (pH 5.5). The decrease in acetic acid concentration from the feed sludge to the acidogenic reactor effluent indicated acetic acid utilisation to produce methane in the acidogenic reactor. There was also negligible hydrogen in the acidogenic reactor indicating its utilisation as electron donor for methanogenesis by hydrogenotrophic methanogens. In the AD process, hydrogen was generated *via* acetogenesis as longer chained fatty acids were degraded into acetic acid (Appels et al., 2008).

The pH inhibition of methanogens was also closely related with the VFA concentrations, due to its speciation as inhibitory undissociated fatty acids at low pH (Appels et al., 2008). Xiao et al. (2013) had compared acetic acid utilisation for methane production by cultures from acidogenic and methanogenic reactors treating municipal sludge. It was found that the methanogenic population which persisted in the acidogenic reactor was able to tolerate higher acetic acid concentration than that in the methanogenic reactor. This ability might have been attributed to the enrichment of *Methanosarcina* sp. within the acidogenic reactor, which was known to survive at pH 5–8 and up to 15,000 mg COD/L acetate concentration (De Vrieze et al., 2012). These results indicated that the enrichment of suitable acidophilic methanogens (*i.e.*, *Methanosarcina* and *Methanobrevibacter*) enhanced methanogenic activity in the acidogenic reactor, which reached 24% of total methane production in the overall 2-phase system in this study.

Active methanogenesis within the acidogenic reactor, as demonstrated in this study, could consume acetate and control the acetate concentration to manageable levels under high organic loading situations. Conversion of acetate into carbon dioxide and methane contributed to the reactor's alkalinity which counteracted significant pH drop, ultimately better ensuring stability of the system. Hence, it is recommended to maintain methanogenesis in the acidogenic reactor (Zhang and Noike, 1991).

3.2. Unique hydrolytic and acidogenic bacterial populations in the acidogenic reactor

The acidogenic reactor was operated at pH 5.5 and 2 day HRT to create favourable growth condition for the faster-growing acidogenic bacteria (Rubio-Loza and Noyola, 2010). 454 pyrosequencing analysis confirmed that this operational condition created distinct bacterial populations compared to those found in the single-stage and methanogenic reactors (Fig. 1). Its low richness and diversity compared to samples from the other reactors (Table 1) also indicated this operational condition selected for the growth of specific microorganisms. This was largely driven by the enrichment of bacterial families *Porphyromonadaceae*, *Prevotellaceae*, *Ruminococcaceae* and the unclassified *Bacteroidetes* (Table 2). These microorganisms would have been transferred from the acidogenic into the methanogenic reactor, but their relative abundance was then reduced. Hence, neutral pH and longer HRT > 10 days were not favourable for growth of these microorganisms in the methanogenic reactor (Rubio-Loza and Noyola, 2010). Considering the close similarity of the bacterial communities in the single-stage and methanogenic reactors (Fig. 1), it could be deduced that the crucial microorganisms responsible for the enhanced sludge digestion performance in the 2-phase AD system would likely be the above-mentioned *Bacteria* (Table 2). Their metabolic activity in natural and engineered systems also supported their participation in hydrolysis and acidogenesis, which could have been cultivated for enhanced particulate sludge destruction in this 2-phase system (Cardinali-Rezende et al., 2012; Downes et al., 2007; Regueiro et al., 2012; Walker et al., 2008).

3.3. Syntrophic microbial association in phased AD system

Interspecies hydrogen transfer is a critical metabolic pathway required for the oxidation of thermodynamically challenging substrates, such as propionic, butyric acids, long chain fatty acids and some species of amino acids during the AD process (Stams et al., 2012; Vartoukian et al., 2007). As the predominant methanogen was found to be hydrogen-utilising methanogen in this study, the oxidation of acetic acid into hydrogen and carbon dioxide (ΔG^0 = +104 kJ/mol) also has to be considered. Previous studies argued that phase separation of AD process interrupted the syntrophic balance of anaerobic microorganism and that accumulation of metabolites (*e.g.*, VFA) in the acidogenic reactor was detrimental to the bacterial diversity in the methanogenic reactor (Merlino et al., 2013; Raskin et al., 1995). These would ultimately impair the overall 2-phase system performance. This is true for the acidogenic reactor of this study. As mentioned previously, the bacterial richness and diversity according to number of OTUs and Shannon index were much less in the acidogenic reactor, but not much different between the single-stage and methanogenic reactors. The shortened 2 day HRT and pH 5.5 created an unfavourable environment for the acetogenic syntrophic microorganisms, such as for *Syntrophaceae*, *Syntrophorhabdaceae* and *Synergistaceae* (Table 2). However, these syntrophic microorganisms were able to survive in the methanogenic reactor and there was negligible accumulation of propionic and butyric acid present in both the single-stage and methanogenic reactors to prove their activity, as shown in the previous study (Maspolim et al., 2015). The methanogenic reactor was also predominated by hydrogen-utilising *Methanolinea* which provided the syntrophic partner to alleviate hydrogen accumulation. It is, thus, important to ensure the appropriate

operational HRT and pH of the methanogenic reactor for the cultivation of syntrophic microorganisms. Studies done by Zhang and Noike (1991) and Shimada et al. (2011) also found that interspecies hydrogen transfer was not compromised and did not cause worsening performance of the 2-phase system. These conflicting hypotheses might be explained by the types and concentration of inhibitory compounds synthesised within the acidogenic reactor (*e.g.*, phenols, alcohols, ketones or amines) due to operation under acidic condition as observed by a previous study (Schievano et al., 2012), which could have been detrimental to the growth of syntrophic microorganisms in the methanogenic reactor. Further work is required to define an optimum acidogenic reactor operation to mitigate the production of potentially inhibitory compounds without affecting its hydrolytic and acidogenic activity.

4. Conclusions

This study provided microbiological insights into the 2-phase anaerobic system. The knowledge helped further understanding of the anaerobic digestion (AD) process in order to improve the 2-phase system operation. For example, it was found that methanogenesis, which could help regulate reactor alkalinity, could be sustained in the acidogenic reactor. The acidogenic reactor was also able to enrich specific hydrolytic and acidogenic *Bacteria*, leading to better overall VS destruction than in the single-stage system. However, strict growth conditions were required to cultivate acetogenic syntrophic microorganisms which were unable to survive in the acidogenic reactor, but did so in the methanogenic reactor.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jes.2015.02.017.

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