Bioresource Technology 245 (2017) 502–510

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Impact of temperatures on microbial community structures of sewage sludge biological hydrolysis

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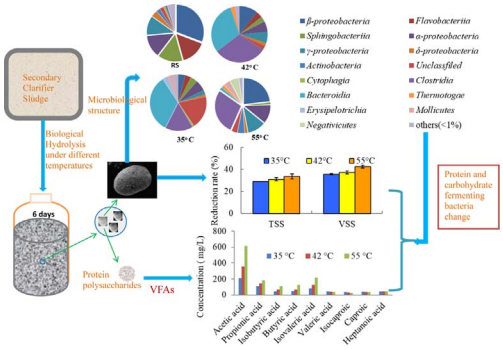
GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Biological hydrolysis
Temperature
Sewage sludge
Illumina MiSeq sequencing
Microorganism community structure

ABSTRACT

This study investigated the biological hydrolysis performance at 35 °C (BH35), 42 °C (BH42), and 55 °C (BH55) and the effect of temperatures on microbial communities of the hydrolyzed sludge. The results showed that the suspended solid reduction, volatile fatty acids (VFA) production, and biogas production increased with the BH temperatures. VFAs produced in the sludge BH included acetic acid, propionic acid, isobutyric acid, butyric acid, and isovaleric acid with the fractions of acetic acid increased with BH temperatures. The Illumina MiSeq sequencing analysis showed that the microbial taxonomic structures of the BH systems varied with BH temperatures. It was found that *Acidaminobacter* at 35 °C, *Proteiniphilum* and *Lutispor* at 42 °C, and *Gelria* at 55 °C were the main protein fermenting bacteria genera, while the carbohydrate fermenting bacteria might belong to the genera of *Macellibacteroides* and *Paludibacter* at 35 °C, *Fronticella* at 42 °C, and *Tepidimicrobium* at 55 °C.

1. Introduction

Anaerobic digestion (AD) of activated sludge has gained increasing interests due to its essential role in reducing carbon footprints of wastewater treatment plants via energy recovery and waste reduction. Biological hydrolysis/acidification (BH) is a widely used pre-treatment process to increase the methanogenic degradation of complex organic compounds for methane production (Ding et al., 2017; Gao et al., 2011; Ucisik and Henze, 2008; Wang et al., 2014; Yang et al., 2015a). Temperature is one of the key BH process parameters that can exert a profound impact on the hydrolysis performance (Chu et al., 2008; Duarte et al., 2015; Liu et al., 2013). Although the effect of BH conditions on the biomass hydrolysis performance have been assessed by a number of studies (Chu et al., 2008; Zhang et al., 2009), knowledge of microbial communities structures in sludge BH systems is far from adequate for understanding sludge BH mechanisms.

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http://dx.doi.org/10.1016/j.biortech.2017.08.143
Received 8 August 2017; Received in revised form 20 August 2017; Accepted 22 August 2017
Available online 24 August 2017

The anaerobic conversion of organics into methane gas involves the hydrolysis, acidogenesis, acetogenesis, and methanogenesis steps (Lee et al., 2014). Hydrolysis and acidogenesis bacteria, or the so-called primary fermenters, catalyze the extracellular hydrolytic degradation of polymers into oligo- or monomers and intracellular conversion of sugars, amino acids, fatty acids and other monomers into fatty acids, lactate, alcohols, etc. Acetogenesis bacteria, or the secondary fermenters, degrade products of the primary fermentation into acetate, H_2, and CO_2. The methanogenesis converts acetogenesis products into methane gas via two main pathways: acetoclastic and hydrogenotrophic CH_4 production (Da Silva et al., 2015). The complete conversion of complex organics to methane relies on syntrophic interactions of primary fermenters, secondary fermenters, and methanogens because mutual metabolic dependencies between different groups of anaerobic microorganisms eliminate the accumulation of intermediate hydrolysis products, making the conversion of complex organics to methane thermodynamically favorable (Schink and Stams, 2013). The syntrophic interactions in the sludge BH systems will depend on the microbial community structures and compositions. Thus, the determination of bacterial and archaeal community structures of BH systems would be of great importance for the understanding of microbial syntrophic interactions and process mechanisms of the sludge biological hydrolysis.

Illumina MiSeq Sequencing is an effective method for the characterization of microbial community structures. Compared to the conventional cultivation and biological molecular methods, Illumina MiSeq Sequencing can generate an enormous number of sequences, providing an excellent platform for the analysis of microorganism communities in wastewater treatment systems (Lin et al., 2016; Sheng et al., 2017; Xie et al., 2016; Zamanzadeh et al., 2016). With the use of Illumina MiSeq sequencing platform, Xie et al. (2016) revealed that the phyla of *Bacteroidetes*, *Proteobacteria*, and *Firmicutes* were dominant in the hydrolysis acidification reactors treating dyeing wastewater. Lin et al. (2016), who investigated the effect of temperature on microbial communities in the anaerobic digestion of organic food waste, revealed that the phyla of *Firmicutes*, *Chloroflexi*, *Bacteoridetes*, and *Actinobacteria* were dominant under mesophilic conditions while the phyla of *Firmicutes*, *Thermotogo*, *Synergistales* dominated under thermophilic conditions.

The objective of this study was to investigate the effect of temperature on microorganism communities in the sludge BH systems. Temperature is one of the most critical process parameters for the sludge BH treatment. It can exert a critical impact on the types of microorganisms, fermentation pathways, and end-products of sludge BH. In this study bench-scale hydrolysis and biochemical methane potential (BMP) tests were carried out to assess the performance of sludge BH at 35 °C, 42 °C, and 55 °C. The high throughput Illumina MiSeq sequencing platform was used to determine the microbial community structures at the BH temperatures tested. Based on the MiSeq sequencing results, the characteristics of microbial community structures, syntrophic interactions and main functional fermenting bacteria under mesophilic and thermophilic BH conditions were discussed.

2. Material and methods

2.1. Sludge source and hydrolysis experiments

The raw sludge (RS) and seed sludge used in the BMP tests were taken, respectively, from the secondary clarifier and the anaerobic digester of Guelph Wastewater Treatment Plant, Ontario, Canada, and were transported to the laboratory within 2 h.

For the BH experiments, the RS was filled into a 1.5 L plastic bottle without addition of seed sludge, flushed with N_2 for 1 min, and then sealed for hydrolysis reactions. The hydrolysis experiments at 35 °C, 42 °C, and 55 °C were carried out in parallel by placing these sludge-filled plastic bottles into shaking incubators at the desired temperatures for six days. The sludge solubilisation caused by the 6-day hydrolysis was assessed by examining the change in the sludge TSS and VSS contents according to the Standard Methods (APHA-AWWA-WEF, 2005). The VFA compositions were measured using an Agilent 5890 gas chromatography (GC) (Agilent Technologies, US) with an Agilent J & W GC column (DB-FFAP 30 m × 0.25 mm) and a flame ionization detector (FID). The injector and detector temperatures were 250 °C and nitrogen was used as the carrier gas. The oven temperature was increased from 80 °C to 120 °C at a rate of 20 °C/min and held at 120 °C for 4 min, and then increased from 120 °C to 220 °C at a rate of 6.1 °C/min and held at 220 °C for 5 min. Volatile fatty acid mix standards (Sigma-Aldrich, Canada) including acetic, propionic, isobutyric, butyric, isovaleric, valeric, 4-methylvaleric (isocaproic), hexanoic (caproic), and heptanoic acids were used for the peak identification and standard curve determination.

2.2. Biochemical methane potential test

The biogas production from the hydrolyzed sludge was assessed using the BMP method. The BMP test procedure followed those of Owen et al.(1979) and Angelidaki et al. (2009) with modifications. Briefly, the main steps included (1) filling 30 mL of the hydrolyzed sludge and the equal volume of seed sludge into a 125 mL serum bottles (American Scientific Products, McGraw Park III); (2) flushing the headspace of the bottles with N_2 for 15 s; and (3) capping the BMP bottles with rubbers and aluminum crimp; and (4) placing the sealed BMP bottles upside down in a shaking incubator (New Brunswick Scientific C25) with the temperature setting at 35 °C. The BMP tests lasted for 10 days and the biogas produced during the BMP test periods was measured using glass syringes (Popper and Sons Inc.) fitted with a disposable needle. BMP bottles only filled with AD seed sludge and MilliQ water were used as the blanks to estimate the biogas generated from the seed sludge. Triplicated BMP bottles were used for each of the hydrolysis temperature conditions and the blanks.

2.3. DNA extraction

The PowerSoil DNA Isolation Kit (MOBIO Laboratories, Inc.) was used to extract DNA from the raw sludge and hydrolyzed sludge. The sludge samples were centrifuged at 10,000*g* for 10 min and the pellets were used for DNA extraction according to the PowerSoil kit manufacture's protocol. The extracted DNA was eluted using 100 μL sterile DNA-Free PCR Grade Water. The extracted DNA was quantified by using the NanoDrop 2000 spectrophotometer (Thermo Scientific, Canada) and stored at −20 °C for further use.

2.4. 16S rDNA gene amplification and Illumina MiSeq sequencing

The primer pairs of 515F (5′-GTGCCAGCMGCCGCGG-3′) and 926R (5′-CCGTCAATTCMTTTGAGTTT-3′) that have been demonstrated to have the high coverages of almost all phyla in metagenomic analyses (Baker et al., 2003) were used to amplify the V4 and V5 regions of bacterial 16S rRNA genes of the extracted DNA. The primer pairs of 519F (5′-CAGCCGCCGCGGTAA-3′) and 915R (5′-GTGCTCCCCCGCCAATTCCT-3′) were used to amplify the 16S rRNA genes of methanogenic archaeal (Wei et al., 2015). The PCR reaction agent consisted of the 1 μL template, 1 μL of 10 mM dNTPs, 1 μL of 10 μM of primers, 1 U of Phusion DNA Polymerase, and 10 μL of 5 × reaction buffer (New England Biolabs, USA). The bacterial and archaeal PCR conditions were programmed as: denaturation at 94 °C for 2 min, followed by 25 cycles for bacteria or 30 cycles for archaea at 94 °C for 30 s, 56 °C for 30 s, extension at 72 °C for 30 s; with a final extension of 72 °C for 5 min.

The Illumina Nextera XT Index kit (Illumina Inc., San Diego. CA, USA) was used to attach 5′ MiSeq adapter and barcode to the amplicons for multiplexing following the manufacture's protocol. The amplicons were purified using the DNA gel extraction kit of AxyPrepDNA (Axygen, China) and quantified by using the FTC-3000™ real-time PCR before

library pooling. The purified multiplexed amplicons were sequenced by the paired-end sequencing (2 × 300 bp reads) on the MiSeq platform using MiSeq Reagent Kit V3 (TinyGene Bio-Tech, Co., Ltd. Shanghai, China).

2.5. Illumina data analysis

All sequences were read and classified according to the barcodes after sequencing on the MiSeq platform. The raw data were reverted as the FASTQ file of the forward or reverse sequences for each sample. The resulting sequences were processed mainly upon software of mothur (version 1.35.1) following the pipeline outline of the MiSeq SOP (Schloss et al., 2009). The remaining sequences after assembling of contigs and filtering of length and ambiguous reads were aligned with the version 119 of SILVA databases (Pruesse et al., 2007). All reads of the archaeal communities were merged and trimmed as bacteria. Shannon index was used for the diversity calculation. The richnesses were described based on the abundance-based coverage estimator (ACE) and species richness estimators (Chao1). The microbiological similarities for the sludge samples obtained under different conditions were calculated by the OTU overlapping in Venn diagram, and the distances of variation of microbiological communities were presented by the principal component analysis (PCA).

3. Results

3.1. Effect of temperatures on the solid solubilization and biogas production

Experiments demonstrated that BH could result in a considerable suspended solid solubilization and VFA production. Fig. 1a–c show the TSS and VSS reductions, VFA concentrations, and VFA fractions at the end of 6-day hydrolysis at 35 °C, 42 °C, and 55 °C. The VSS reductions of 35.4%, 37.3%, and 42.4% and TSS reductions of 28.9%, 31.0%, and 33.5% were achieved in the 6-day hydrolysis at 35 °C, 42 °C, and 55 °C, respectively (Fig. 1a). Accordingly, the VFA concentrations in sludge were increased from the initial concentration of 24.5 mg/L to 666.5 mg/L, 918.4 mg/L, and 1398.5 mg/L in the 6-d hydrolysis at 35 °C, 42 °C, and 55 °C, respectively. VFAs determined by the GC analysis in the hydrolyzed sludge included acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, 4-methylvaleric acid (isocaproic), hexanoic acid (caproic), and heptanoic acid (Fig. 1b). The concentration of acetic acid increased from 210.8 mg/L at 35 °C, 356.1 mg/L at 42 °C, to 615.0 mg/L at 55 °C. The sum of the concentrations of acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, and valeric acid counted for 81.5%, 88.0%, and 92.3% of the total detected VFAs at 35 °C, 42 °C, and 55 °C, respectively. The fractions of acetic, isobutyric, and isovalric acid increased while those of propionic acid, valeric acid, isocaproic, caproic, and heptanoic acid reduced with the increase in the hydrolysis temperature (Fig. 1c). These results indicated that BH temperature not only exerted a positive effect on the sludge solubilization and VFA productions but also affected the compositions of VFAs.

Sludge treated at different BH temperatures was assessed for biogas production using the BMP method outlined in Section 2.2. Fig. 2 shows that the accumulated biogas produced by sludge hydrolyzed at 35 °C, 42, °C, and 55 °C. As shown in Fig. 2, sludge treated at 42 °C and 55 °C showed a higher biogas production than that treated at 35 °C although no significant difference in biogas production from sludge treated at different temperatures was observed in the first 50 h of the BMP tests.

3.2. Microbial community diversity of bacteria

Microbial communities at different BH temperatures were characterized using the Illumine MiSeq sequencing platform. The average total effective sequencing tags were 62,439 for the RS, and 58,803, 67,348, and 73,988 for the BH35, BH42, and BH55 sludge, respectively.

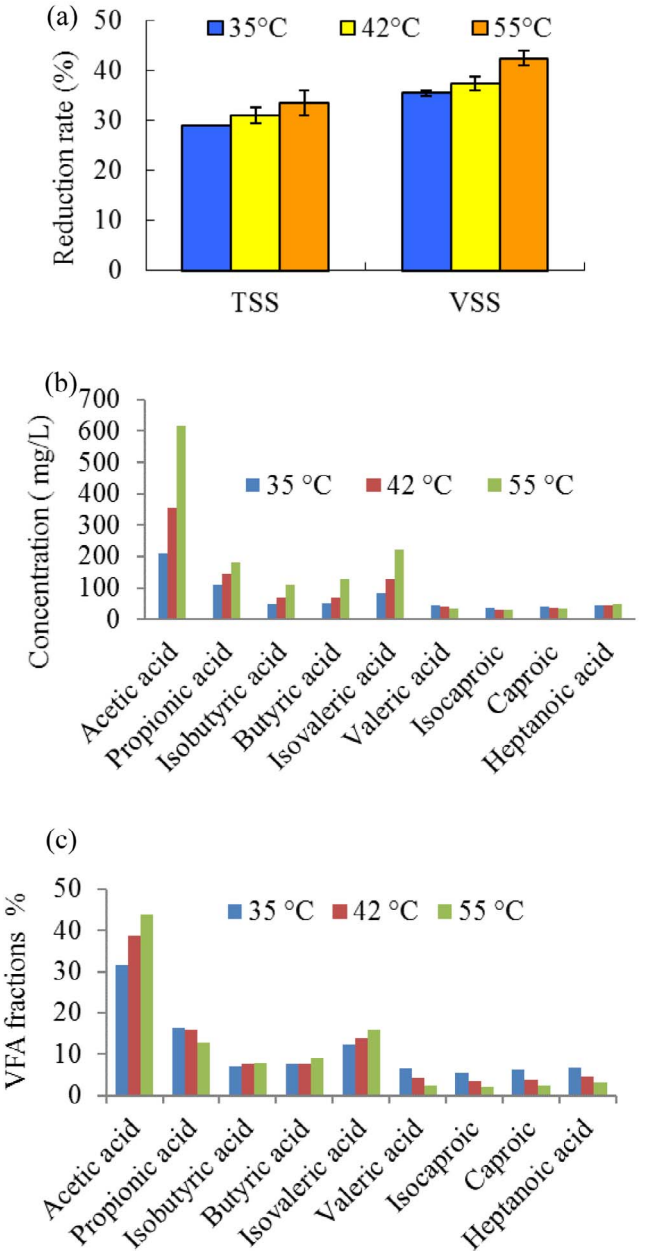

Fig. 1. TSS and VSS reductions (a), VFA concentrations (b), and VFA fractions (c) after 6-day hydrolysis at 35 °C, 42 °C, and 55 °C.

All the effective sequencing tags were clustering at a 97% similarity at the species level, and more than 500 OTUs were obtained for each of the individual samples. The Shannon index of the RS, BH35, BH42, and BH55 sludge was 4.6, 4.5, 4.3, and 4.5 respectively, corresponding to ACE of 602, 707, 675, and 730, respectively, and Chao1 of 606, 708, 694, and 740, respectively. The RS and the BH treated samples all showed the high indexes, suggesting the high bacterial diversities and richness of these sludge samples.

The Venn diagram reflects the similarity of the OTUs of the sludge samples (Fig. 3). The samples tested had a total 380 overlapped OTUs with 468 OTUs overlapped among the sludge hydrolyzed at 35 °C, 42 °C, and 55 °C. The overlapped OTUs between the RS and the hydrolyzed sludge were 473, 470, and 501 for the BH35, BH42, and BH55 sludge, respectively. It is interesting to note that there were more overlapped OTUs between the BH55 and RS sludge than between the RS and the BH35 or BH42 sludge. Similar to the OTU overlap analysis, the Principal Component Analysis (PCA) also indicated that the microbial

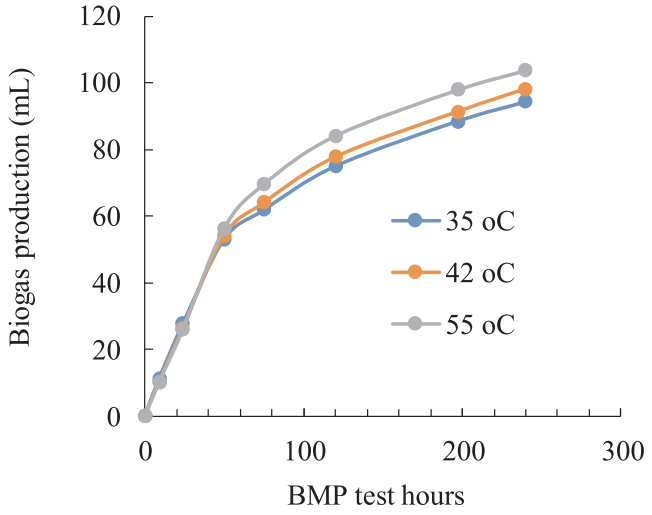

Fig. 2. Biogas production from sludge hydrolysed at 35 °C, 42 °C, and 55 °C during 10-day BMP tests.

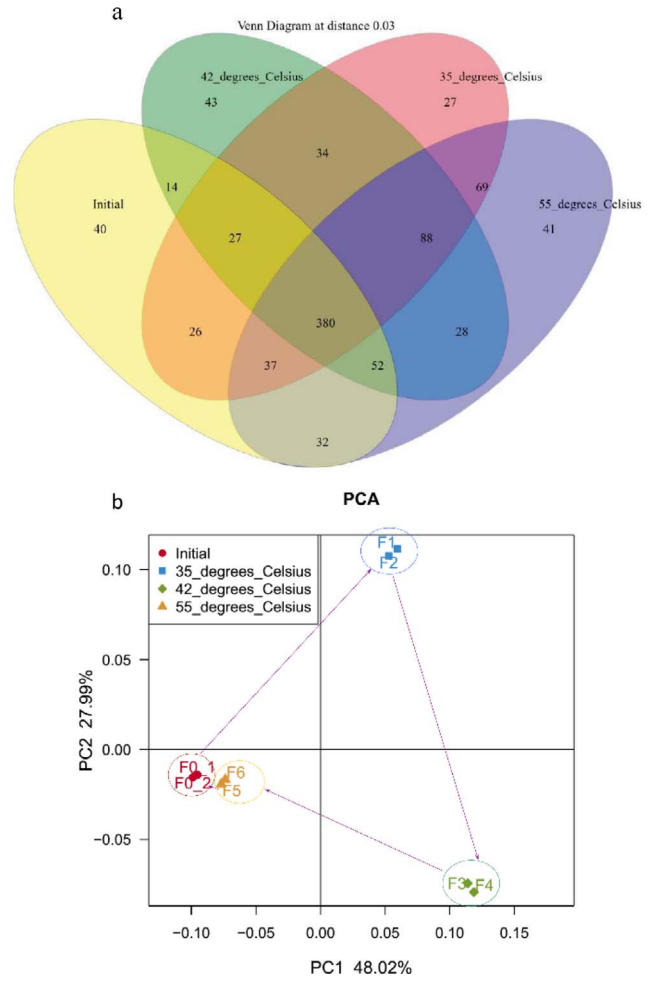

Fig. 3. (a) Bacterial Venn diagram at distance 0.03 and (b) the PCA on the basis of 16S rRNA gene sequencing. F0_1 and F0_2: RS (duplicates); F1 and F2: BH 35 sludge (duplicates); F3 and F4: BH 42 sludge (duplicates); F5 and F6: BH55 sludge (duplicates).

communities of the sludge treated at 55 °C had a higher similarity to the RS than those treated at 35 °C and 42 °C (Fig. 3b).

3.3. Bacterial community structures

Fig. 4a shows the microbial abundances at the phylum level in the RS and sludge treated at different temperatures. For the RS, the dominant phyla were *Proteobacteria* (59.2%) and *Bacteroidetes* (31.6%). The dominant phyla with the BH35 sludge were *Bacteroidetes* (46.8%), *Firmicutes* (19.8%), *Proteobacteria* (14.1%), and *Spirochaetes* (13.3%). Similar to the BH35 sludge, the BH42 sludge contained *Bacteroidetes* (37.9%), *Firmicutes* (30.9%), and *Proteobacteria* (25.2%). The dominant phyla in the BH55 sludge were *Proteobacteria* (47.6%) and *Firmicutes* (34.0%), plus a significant amount of *Actinobacteria* (5.6%), *Tenericutes* (4.1%), and *Bacteroidetes* (4.2%). Both the RS and BH55 sludge showed a high population of bacteria belonging to *Proteobacteria*, which may explain the high similarity in the 16S rRNA gene sequences between the RS and BH55 sludge (Fig. 3b). At the phylum level, *Firmicutes* and *Proteobacteria* populations increased with the BH temperatures, while *Bacteroidetes* decreased dramatically at 55 °C (Fig. 4a–c).

At the class level, the top 5 predominant classes in the RS were *β-proteobacteria*, *Flavobacteria*, *Sphingobacteriia*, *α-proteobacteria*, and *γ-proteobacteria*, which accounted for 30.8%, 14.9%, 14.7%, 13.3%, and 11.2% of total detected OTUs, respectively (Fig. 4b). After the RS had been hydrolyzed at 35 °C for six days, the dominant classes in the sludge changed to *Bacteroidia* (33.3%), unclassified (19.0%), *Clostridia* (15.9%), *α-proteobacteria* (6.3%), and *Spirochaetes* (4.1%) (Fig. 4b), showing that the anaerobic hydrolysis caused a substantial reduction in the population of *β-proteobacteria*, *Flavobacteria*, and *γ-proteobacteria*. The dominant classes with the BH42 sludge were *Clostridia* (30.4%), *Bacteroidia* (30.2%), *β-proteobacteria* (10.7%), *α-proteobacteria* (6.1%), and *γ-proteobacteria* (6.3%). The BH55 sludge was dominated by the class *Clostridia* (28.1%), *β-proteobacteria* (23.2%), *α-Proteobacteria* (11.4%), *γ-proteobacteria* (11.2%), and *Negativicutes* (5.5%). The bacteria associated with the classes of *Clostridia* and *β-Proteobacteria* evidently increased with the increase in temperature. Thus, *Clostridia* was identified as the dominant class of phylum *Firmicutes* in the BH35, BH42, and BH55 sludge and *Bacteroidia* as the dominant class of the phylum of *Bacteroidetes* in the BH35 and BH42 sludge.

Table 1 shows the main families identified for RS, BH35, BH42, and BH55 sludge. Apparently, the family structures of the sludge treated at different temperatures were considerably different. For the BH35 and BH42 sludge samples, around 30% of the identified OTUs belonged to *Rikenellaceae* and *Porphyromonadaceae*, while the fractions of these two families in the BH55 sludge drastically decreased to less than 3%. Also, the family *Spirochaetaceae* that has members to metabolize carbohydrates and amino acids showed a significant presence in BH35 sludge but much less in the BH42 and BH55 sludge. In addition, the families of *Comamonadaceae*, *Ruminococcaceae*, and *Thermoanaerobacteraceae* dominated at 55 °C but only existed as minor groups in the BH35 and BH42 sludge samples.

The fractions of OTUs of unclassified genera were 32.8%, 60.1%, 51.0%, and 33.4% for the RS, BH35, BH42, and BH55 sludge, respectively. It is interesting to note that the percentages of unclassified genera in the BH sludge decreased significantly with the increase in the hydrolysis temperature. For the identified genera, *Flavobacterium*, *Zoogloea*, *Haliscomenobacter*, and *Novosphingobium* counted for 12.2%, 9.6% 4.2%, and 4.0% of the total OTUs, respectively, in the RS (Fig. 4c). The dominant bacterial genera in the sludge switched to *Macellibacteroides* (7.5%) and *Acidaminobacter* (5.6%) after being hydrolyzed at 35 °C for six days. *Lutispora* (9.8%), *Albidiferax* (4.3%), and *Proteiniphilum* (4.5%) were the three predominant genera identified in the BH42 sludge. For the BH55 sludge, the genera of *Gelria* (7.2%), *Albidiferax* (5.6%), *Simplicispira* (4.4%), and *Hialoplasma* (4.0%) were identified as the dominant groups. Overall, the identified bacteria genera structures with the RS, BH35, BH42, and BH55 sludge were very

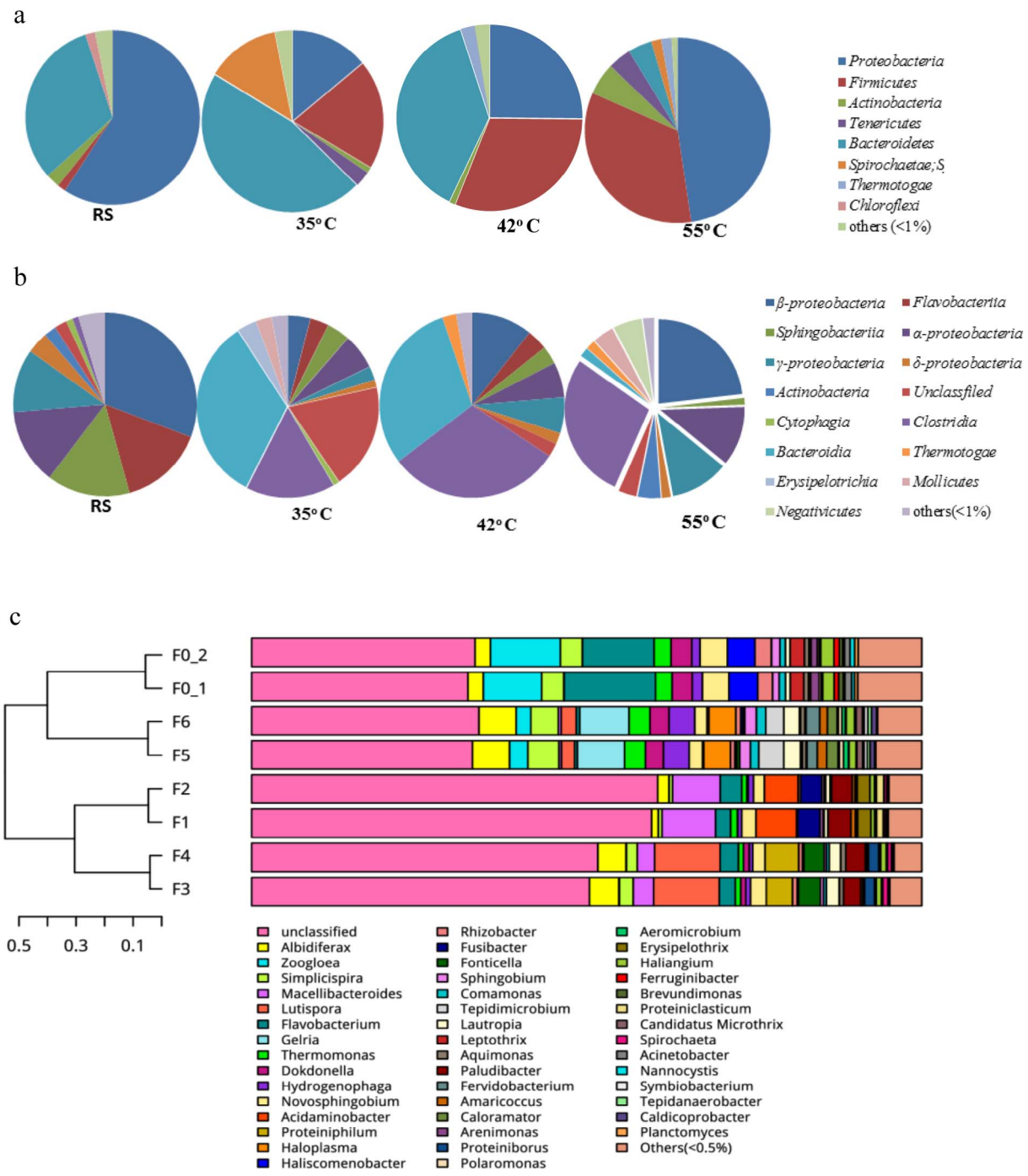

Fig. 4. Microbial community structures at the phylum (a), class (b), and genus (c) levels for the initial, BH35, BH42, and BH55 sludge. F0_1 & F0_2: RS (duplicates); F1 & F2: BH35 sludge (duplicates); F3 & F4: BH 42 sludge (duplicates); F5 & F6: BH55 sludge (duplicates).

different, reflecting the variance in the functional hydrolysis bacteria under different hydrolysis temperature conditions.

3.4. Archaeal diversities and communities

Fig. 5 shows the fractions of various archaeal classes, families, and genera in the BH35, BH42, and BH55 sludge. *Methanomicrobia* and *Methanobacteria* were identified to be the dominant classes for all the tested sludge but the distributions of these two classes were different in the sludge treated at different temperatures. The class of *Methanomicrobia* accounted for 84% of the total archaeal OTUs for the BH35 sludge, 40% for the BH42 sludge, and 19% for the BH55 sludge, while *Methanobacteria* accounted for 14% for the BH35 sludge, 56% for the BH42 sludge, and 80% for the BH55 sludge. At the family level, *Methanocorpusculaceae*, *Methanobacteriaceae*, and *Methanosarcinaceae* accounted for 81%, 14.3% and 2.2% of the archaeal OTUs, respectively, in the BH35 sludge; and *Methanobacteriaceae* and *Methanosarcinaceae* accounted for 56.0% and 38.3%, respectively, in the BH42 sludge. As the temperature increased from 42 °C to 55 °C, the percentage of *Methanobacteriaceae* increased to 80%, while *Methanocorpusculaceae* and *Methanosarcinaceae* were down to 11.6% and 7.0%, respectively.

At the genus level, the predominant archaea grew at BH35 were *Methanocorpusculum* (81.2%), *Methanobrevibacter* (7.2%), *Methanobacterium* (6.8%), and *Methanosarcina* (1.7%). For BH42, the genera of *Methanobrevibacter*, *Methanosarcina*, and *Methanobacterium* increased to 37.9%, 37.2%, and 18.3%, respectively, while the genus of

Table 1
Phylogenetic family structures of the RS, BH35, BH42, and BH55 sludge (OTUs fraction > 1%).

Family	Relative abundance (%)			
	RS	35 °C	42 °C	55 °C
Rikenellaceae	< 1	17.2	14.2	< 1
Porphyromonadaceae	< 1	13.2	15.6	< 1
Sphingomonadaceae	6.4	2.8	2.8	3.9
Ruminococcaceae	< 1	2.7	6.3	9.1
Saprospiraceae	9.0	2.7	2.5	< 1
Comamonadaceae	12.7	3.0	8.1	16.7
Flavobacteriaceae	12.3	2.7	2.6	< 1
Xanthomonadaceae	9.0	2.0	5.3	9.9
Rhodobacteraceae	2.6	1.0	< 1	3.4
Rhodocyclaceae	15.7	< 1	< 1	2.8
Haliangiaceae	1.8	< 1	< 1	4.0
Gracilibacteraceae	< 1	< 1	10.1	2.1
Thermotogaceae	< 1	< 1	2.4	1.8
Lachnospiraceae	< 1	< 1	3.1	< 1
Burkholderiaceae	< 1	< 1	1.8	2.4
Thermoanaerobacteraceae	< 1	< 1	< 1	7.2
Intrasporangiaceae	< 1	< 1	< 1	1.8
Spirochaetaceae	< 1	13.1	< 1	1.7
Erysipelotrichaceae	< 1	3.5	< 1	< 1
Cytophagaceae	1.2	1.0	< 1	< 1
Unclassified	14.0	26.9	16.9	19.6

Methanocorpusculum was almost disappeared. For BH55, *Methanothermobacter* accounted for 58.0% while the genera of *Methanocorpusculum* (11.5%), *Methanobrevibacter* (9.9%), *Methanobacterium* (11%), and *Methanosarcina* (7.0%) were largely suppressed. Overall, *Methanocorpusculum* flourished at 35 °C; *Methanobrevibacter* and *Methanosarcina* well grew at 42 °C; and *Methanothermobacter* became dominant at 55 °C.

4. Discussion

4.1. Impact of biological hydrolysis temperature on microbial structures

The experimental data obtained in this study showed the impact of temperatures on the BH of sewage sludge. The sludge BH at 55 °C enhanced the reduction of suspended solid (TSS and VSS) and production of VFA, in agreement with the previous studies reported by Zhang et al. (2009), who showed that the VSS reduction by thermophilic fermentation was greater than that by mesophilic fermentation. These notable processing changes of hydrolyzed sludge at different BH temperatures were further linked to the change in the bacterial and archaeal structure by Illumina MiSeq sequencing. Taxonomic identification showed the shifts of microbiological structures with the BH35, BH42, and BH55 sludge at different levels in present work. Most of the microbiology was clearly classified to the family level, which revealed the significant

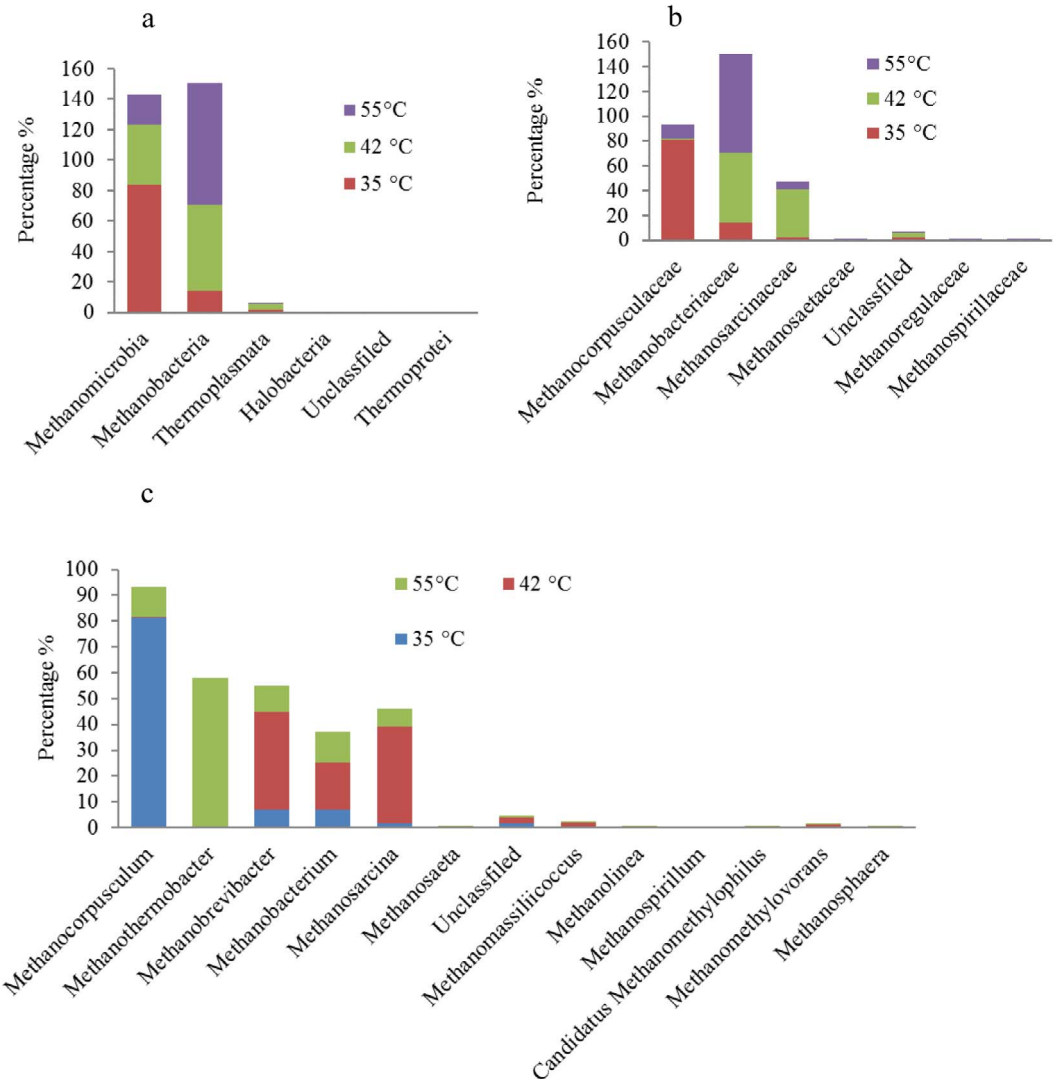

Fig. 5. Archaea compositions at the class (a), family (b), and genus (c) levels in the BH sludge hydrolysed at 35 °C, 42 °C, and 55 °C.

difference in the microbial communities between the mesophilic (BH35 & BH 42) and thermophilic (BH55) hydrolysis systems.

Firmicutes, *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* accounted for more than 90% of the total identified OTUs in the BH35, BH42, and BH55 sludge. These phyla were also identified as the dominant bacteria phyla, by other researchers, in the wastewater hydrolysis acidification reactors (Xie et al., 2016), the anaerobic sewage sludge co-digestion reactors (Yang et al., 2016), and the conventional anaerobic digesters (Nelson et al., 2011). Considering the complexity of microbiology in wastewater treatment systems, the descriptions of bacterial roles at class and family levels were widely accepted. For example, the classes of *Clostridia* and *Bacteroidia* that were found as dominant bacteria in the present study were previously depicted by Yang et al. (2015b) as dominant classes in *Geobacillus* sp. G1 hydrolysis of ultrasonic pretreated wasted activated sludge (WAS). *Clostridia* contained many types of fermentative organisms that produce hydrogen along with acetate, butyrate, ethanol, etc. (Arreola-Vargas et al., 2013), while *Bacteroidia* were reported playing a vital role in decomposing solid wastes and generating organic acids (Wong et al., 2013). In this study, the classes of *Clostridia* and *Bacteroidia* accounted for total fractions of 49.3% and 60.6% of the identified OTUs in the BH35 and BH42 sludge, respectively. It can be speculated that bacteria associated with these classes played a major role in the mesophilic sludge solubilization and VFA production, while the class of *Clostridia* that counted for 28.1% of total OTUs in the BH55 sludge should play a major role in the sludge thermophilic fermentation. At the family level, the family *Rikenellaceae* associated with the order *Bacteroidales* was identified as a major bacterial family (17.3%) in BH35. The members of *Rikenellaceae* are anaerobic bacteria that usually use carbohydrates or proteins (Su et al., 2013). This study also showed that the family *Ruminococcaceae* increased sharply with the increase of the BH temperature. It was reported that bacteria associated with the *Ruminococcaceae* family can grow rapidly on cellulose and hemicellulose (Ali Shah et al., 2014). The considerably increased *Ruminococcaceae* population at 55 °C might suggest that the degradation of cellulose components of sewage sludge can be enhanced by thermophilic BH, which could, to some extent, contribute to the increased VSS reduction and VFAs productions at 55 °C.

4.2. Impact of biological hydrolysis temperature on protein and carbohydrate fermenting bacteria

The complex organics in sludge BH can be degraded by primary and secondary fermentative bacteria (Schink and Stams, 2013). This study revealed that the hydrolysis temperature could exert significant impact on the types of fermentative bacteria in the sludge BH systems. Table 2 summarized the main genera identified at 35 °C, 42 °C, and 55 °C that may contain typical protein and carbohydrate fermenting bacterial species, most of which were isolated from wastewater treatment systems. The main protein fermenting bacteria could be associated with the genera of *Acidaminobacter* at 35 °C, *Lutispora* and *Proteiniphilum* at 42 °C, and *Gelria*, *Tepidimicrobium*, and *Lutispora* (2.0%) at 55 °C. All of these genera excepting *Proteiniphilum* belong to the order of *clostridials*. The physiology characteristics of these identified protein fermenting genera were illustrated in Table 2 based on those of the isolated species reported previously by other researchers.

The dominant carbohydrates fermenting bacteria also varied in the BH35, BH42, and BH55 sludge (Table 2). The genus *Macellibacteroides* was determined to be the main carbohydrate degrading bacteria in the BH35 sludge. The isolated species of *Macellibacteroides* can utilize a broad range of mono- and disaccharides as electron donors with the main fermentation products to be lactate, acetate, butyrate and isobutyrate from glucose metabolism (Jabari et al., 2012). Other carbohydrates fermenting bacteria detected in BH35 sludge could be associated with the genera of *Paludibacter* and *Fusibacter* (Ueki et al., 2006; Ravot et al., 1999). For the sludge hydrolysis at 42 °C, the main

Table 2
Summarized main genera in the BH 35, BH42, and BH55 and their previous reported species for proteins or carbohydrate fermenting.

Phylum/order/genus	Percentage	CF	PU	Products	Isolated species	Isolation source
Firmicutes/Clostridiales/Acidaminobacter	BH35 (5.6%)		X	Acetate, propionate	*A. hydrogenoformans* (Stams and Hansen, 1984)	Black mud
Bacteroidetes/Bacteroidales/Macellibacteroides	BH35 (7.5%)	X		Lactate, acetate, butyrate and isobutyrate	*M. fermentans* (Jabari et al., 2012)	Upflow anaerobic filter treating abattoir wastewater
Firmicutes/Clostridiales/Fusibacter	BH35 (3.2%)	X		Acetate, butyrate, CO_2 and H_2	*F. paucivorans* (Ravot et al., 1999)	Water sample from an oil-producing well
Bacteroidetes/Bacteroidale/Paludibacter	BH35 (3.2%)	X		Propionate and acetate	*P. propionicigenes* (Ueki et al., 2006)	Rice plant residue in anoxic rice-field soil
Bacteroidetes/Bacteroidales/Proteiniphilum	BH42 (4.5%)		X	Acetate and NH_3	*P. acetatigenes* (Chen and Dong, 2005)	Brewery wastewater UASB
Bacteroidetes/Bacteroidales/Lutispora	BH42 (9.8%) BH55 (2.0%)		X	Propionate and isovalerate	*L. thermophile* (Shiratori et al., 2008)	Anaerobic thermophilic methanogenic bioreactor treating artificial solid wastes
Firmicutes/Thermoanaeroba-cterales/Gelria	BH55 (7.2%)	X	X	Propionate, H_2, NH_4^+, CO_2 from glutamate and proline; acetate, propionate, CO_2 and H_2 from sugars	*G. glutamica* (Plugge et al., 2002)	Propionate-oxidizing methanogenic enrichment culture
Firmicutes/Clostridiales/Tepidimicrobium	BH55 (3.2%)	X	X	Acetate, ethanol, butyrate, H_2 and trace amount of propionate	*T. xylanilyticum* (Niu et al., 2009)	Thermophilic anaerobic digester treating municipal solid waste and sewage
Firmicutes/Clostridiales/Fonticellla	BH42 (3.2%)	X		Formate, acetate, ethanol and CO_2	*F. tunisiensi* (Fraj et al., 2013)	Water sample of a hot spring

Note: CF: carbohydrate fermenting; PU: Protein user; "X" means that the genus identified specie has this function.

carbohydrate fermenting bacteria were likely associated with the genera of *Fronticella* (3.2%), *Macellibacteroides* (3.2%), and *Paludibacter* (2.8%). For BH55, dominant bacteria *Gelria* and *Tepidimicrobium* that can use both protein and carbohydrate were not found in BH 35 and BH42, implying that thermophilic selected bacteria can exert a strong function in degradation of complex organics. In addition, some facultative bacteria, *Simplicispira*, *Hydrogenophaga* and *Thermomonas*, which can use nitrate as the electron acceptor (Gao et al., 2011; Kampfer et al., 2005; Mergaert et al., 2003), were found at a significant amount in BH55, likely related to the relatively high NO_3^--N concentrations (~30 mg/L) in the secondary sludge used in this study.

It is worth noting that bacteria associated with the genera of *Macellibacteroides*, *Paludibacter*, *Fonticellla*, and *Tepidimicrobium* were reported to be able to utilize sugars from the hydrolysis of cellulose and hemicellulose, including xylose, arabinose, cellobiose, and mannose, etc. This may imply that these carbohydrates fermenting bacterial could play an important role in degrading cellulose and hemicellulose materials to VFAs (Table 2).

4.3. Microbial syntrophic interactions

The protein- and carbohydrate-fermenting bacteria identified in the BH systems could produce a wide range of VFAs from fermentation of amino acids and sugars (Table 2). The VFA analysis confirmed that acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, 4-methylvaleric acid (isocaproic), hexanoic acid (caproic), and heptanoic acid were produced in the BH systems. Methanogens can only utilize acetate, H_2, CO_2, formate, etc. for methane production. Some secondary fermentation processes will degrade fatty acids with chains longer than 2 carbon atoms, alcohols longer than one carbon atoms, and branched-chain VFAs, and aromatic VFAs to acetate for methane production (Schink and Stams, 2013). However, most of the secondary fermentation processes are only thermodynamically favorable at low H_2 partial pressures, e.g. $< 10^{-5}$ bar. Thus, the presence of hydrogen-consuming bacteria could be critical for the secondary fermentation reactions to be carried on in the BH systems. In this study, the main hydrogen-consuming bacteria could be hydrogenotrophic methanogens, including *Methanocorpusculum* in the BH35 sludge, *Methanobrevibacter* and *Methanosarcina* in the BH42 sludge, and *Methanothermobacter* in the BH55 sludge. The methanogens associated with *Methanocorpusculum*, *Methanobrevibacter* and *Methanothermobacter* were all hydrogenotrophic methanogens that produce methane from CO_2 and H_2 (Nakamura et al., 2013; Sun et al., 2015), while members of *Methanosarcina* can use acetate and reduce CO_2 with H_2 for methane production (Leahy et al., 2017). In addition to hydrogenotrophic methanogens, other H_2-utilizing bacteria identified in the BH systems in this study could include *Fusibacter* (Ravot et al., 1999) with the BH35 sludge and *Tepidimicrobium* (Niu et al., 2009) with the BH55 sludge. Bacteria associated with these genera could reduce thiosulfate using H_2 as electron donor (Escoffier et al., 1998). In the BH42 and BH55 sludge around 4.3%–5.6% OTUs were identified associated with the genus of *Albidiferax*. This genus contains Fe (III) and Mn (IV) reducing bacteria that could also be potential H_2 consumers.

Acidaminobacter and *Gelria* identified in the BH35 and BH55 sludge could be syntrophic partners with hydrogen-consuming bacteria. Stams and Hansen (1984) reported that the rate of the conversion of glutamate to acetate by *Acidaminobacter hydrogenoformans* was greatly enhanced in the mixed cultures with sulfate-reducing bacterium of *Desulfobulbus propionicus* or H_2 utilizing *Methanospirillum hungatei*. The bacteria associated with *Gelria* was reported to co-operate with the hydrogenotrophic *Methanobacterium thermautotrophicum* to degrade glutamate into propionate, H_2, NH_4, and CO_2 and ferment sugars to acetate, propionate, CO_2, and H_2 (Plugge et al., 2002).

The VFA analysis showed that the total fractions of VFAs longer than 2 carbon atoms were 68.7% for BH35, 61.2% for BH42, and 56.0% for BH55, showing a positive impact of hydrolysis temperatures on the conversion of organic to acetic acid. It is well-known that thermophilic conditions favor the growth of hydrogenotrophic methanogens. The presence of hydrogenotrophic methanogens could be critical to the effective conversion of complex organics to acetate and other methanogen usable organics although methane production is not a primary objective of the BH. Thus, the higher acetate fractions obtained at 55 °C could be, to some extent, related to the enhanced syntrophic interactions between different groups of organisms.

5. Conclusions

The results showed that the volatile suspended solids (VSS) reduction, volatile fatty acids (VFA) production, and biogas production increased with the increase in the BH temperature. The Illumina MiSeq sequencing analysis revealed: *Bacteroidetes* (46.8%), *Firmicutes* (19.8%), *Proteobacteria* (14.1%), and *Spirochaetes* (13.3%) dominated in the BH35 sludge; *Bacteroidetes* (37.9%), *Firmicutes* (30.9%), and *Proteobacteria* (25.2%) dominated in the BH42 sludge, and *Proteobacteria* (47.6%) and *Firmicutes* (34.0%) dominated in the BH55 sludge. The microbial structure analysis showed that the dominant protein and carbohydrate fermenting bacterial and hydrogen consuming methanogens varied in the sludge hydrolyzed at 35 °C, 42 °C and 55 °C.

Acknowledgements

The authors wish to thank Ontario Centres of Excellence (OCE), The Natural Sciences and Engineering Research Council of Canada (NSERC), and GE Water & Process Technologies for support. Special thanks to Guelph Wastewater Treatment Plant for providing sludge samples.

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