Investigation of the microbial community structure in enhanced anaerobic sludge digestion

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**INVESTIGATION OF THE MICROBIAL COMMUNITY
STRUCTURE IN ENHANCED ANAEROBIC SLUDGE DIGESTION**

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SCHOOL OF CIVIL AND ENVIRONMENTAL ENGINEERING

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**INVESTIGATION OF THE MICROBIAL COMMUNITY
STRUCTURE IN ENHANCED ANAEROBIC SLUDGE DIGESTION**

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A thesis submitted to the Nanyang Technological University
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All the glory and honor belong to Lord God.

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2. **Y. Maspolim**, Y. Zhou, C. H. Guo, K. K. Xiao, W. J. Ng. (2015) "Determination of the archaeal and bacterial communities in two-phase and single-stage anaerobic systems by 454 pyrosequencing", Journal of Environmental Sciences, Vol. 36, pp. 121-129
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Publications 1, 2, 3 and 4:

The author participated in the planning of the study, reactor operation and laboratory works. Performed all enzymatic and molecular works. The author analyzed the results and is the main writer of the manuscript.

Publications 5, 6, 7 and 8:

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Conferences

1. **Y. Maspolim**, C.H. Guo, B. Zhai, Y. Zhou, X.W. Zhang, J.Y. Wang, W.J. Ng. (2011) "Wastewater treatment plant as an urban eco power station: A preceeding report on two-phase anaerobic process for enhanced digestion of sewage sludge", R3C International Symposium 2011, 14-15 November 2011, Singapore. (Oral Presentation)
2. **Y. Maspolim**, K.K. Xiao, C.H. Guo, B. Zhai, Y. Zhou, T. Goh, W.J. Ng. (2012) "Degradation and microbial profiles in single stage and two phase sludge digestion", Singapore International Water Week: Water Convention 2012, 1-5 July 2012, Singapore. (Poster Presentation)
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Abbreviations

AD	anaerobic digestion
AM	aceticlastic methanogenesis
ARDRA	amplified ribosomal DNA restriction analysis
ARISA	automated ribosomal intergenic spacer analysis
ATP	adenosine triphosphate
bp	base pairs
BNR	biological nutrient removal
BSA	bovine serum albumin
COD	chemical oxygen demand
CSTR	continuous stirred tank reactor
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
EPS	extracellular polymeric substances
FAN	free ammonia nitrogen
FISH	fluorescence in-situ hybridization
FOG	fat, oil and grease
GC	gas chromatography
HM	hydrogenotrophic methanogenesis
HPLC	high performance liquid chromatography
HRT	hydraulic retention time
LCFA	long chain fatty acids
MW	molecular weight
NAD	nicotineamide adenine dinucleotide
OLR	organic loading rate
OTU	operational taxonomic unit

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PLC	programmable logic control
qPCR	quantitative polymerase chain reaction
RDP	ribosomal database project
RFLP	restriction fragment length polymorphism
RI	refractive index
rRNA	ribosomal ribonucleic acid
rtPCR	real-time polymerase chain reaction
SAO	syntrophic acetic acid oxidation
SCOD	soluble COD
SEC	size exclusion chromatography
SRA	sequence read archive
SRT	solid retention time
SSCP	single strand conformation polymorphism
TAN	total ammonia nitrogen
TCOD	total COD
TGGE	temperature gradient gel electrophoresis
TPAD	temperature-phased anaerobic digestion
TRFLP	terminal restriction fragment length polymorphism
TS	total solids
TSS	total suspended solids
TVFA	total volatile fatty acids
UASB	upflow anaerobic sludge blanket
UV	ultraviolet
VFA	volatile fatty acids
VS	volatile solids

VSS volatile suspended solids

WAS waste activated sludge

WCO waste cooking oil

WWTP wastewater treatment plant

Abstract

Sludge is an inevitable by-product in the current wastewater treatment process utilizing activated sludge technology, and its treatment and disposal could account up to 50% of WWTP's operating costs. Optimization of the anaerobic sludge digestion to enhance volatile solids (VS) reduction and energy recovery (via methane production) is critical in the face of global challenges today. Process improvement employing biological methods (two-stage system) is preferred due to its high digestion efficiency and relatively lower operating cost and energy requirement compared to other methods. However, the underlying microbial diversity, its interactions, functions and responses to operational or environmental conditions are not clearly understood. In this study, sludge digestion enhancement efforts are complemented with microbiological molecular tools including denaturing gradient gel electrophoresis (DGGE), quantitative polymerase chain reaction (qPCR) and high throughput 454 pyrosequencing to address this research gap.

Process manipulation by phase separation was able to tolerate the reduction of the system's solids retention time (SRT) from 30 (5 + 25) to 20 (3 + 17) and 12 (2 + 10) days without affecting VS reduction and methane production, but not in the conventional single-stage system. The impairment in the single-stage system was attributed to the reduction of hydrolytic efficiency when it was operated at 12 days SRT, thus confirming sludge hydrolysis as the rate limiting step in anaerobic sludge digestion. Unique fermentative *Clostridiales* and *Flavobacteriales* were detected in the acidogenic reactor throughout the SRTs tested and indicated their contribution to enhanced sludge hydrolysis and acidification in the two-phase anaerobic system. The study also confirmed that the two-phase anaerobic system did not completely separate the acidogenic and methanogenic phases in the two reactors. Methanogenesis can in fact be useful by providing alkalinity through carbon dioxide production and consuming potentially inhibitory acetic acid and

hydrogen in the first reactor. Thus, two-stage anaerobic digestion system is assessed.

Optimization of operating pH in the first reactor of a two-stage system found the optimal sludge hydrolysis and acidification occurred at pH 8, 35°C and 3 days SRT. Solubilization increased with higher pH, where abiotic contribution took the leading role, but pH 9 and above demonstrated low biological activities for enhanced acidification. In addition, the soluble fractions after sludge acidification at pH 9 and above also contained high molecular weight (MW) aromatic substances which were commonly associated with low biodegradability and would be unfavourable for subsequent bioprocess. Moreover, sludge hydrolysis and acidification with pH 8 and 3 days SRT could also be improved with thermophilic (55°C) instead of (35°C) mesophilic operation. Enrichment of fermentative *Bacteria* capable of tolerating mesophilic mild alkaline condition for enhanced sludge solubilization and acidification mainly consisted of various genera of *Bacteroidetes*, *Firmicutes* and *Chloroflexi*. On the other hand, *Firmicutes* and *Tenericutes* were found under the thermophilic mild alkaline condition. Methanogenesis could also proceed between pH 5 and 8 at 35°C and 55°C, under enhanced sludge acidification condition, but required enrichment of specific methanogenic communities, i.e. *Methanocorpusculaceae*, *Methanosarcinaceae* and *Methanobacteriaceae*. Hydrogenotrophic methanogens predominated in anaerobic sludge digestion processes in this study. In addition, syntrophic acetogens, such as the propionic acid oxidizing *Smithella* was only detected in the second stage, but it ensured the removal of propionic acids in the alkaline two-stage system. Temporal microbiological analysis also indicated the influence of deterministic factors (i.e. reactor operating or environmental conditions) to shape the community structures as microbial communities from the same operating conditions were clustered closely over the experimental period.

The operation of stage-1 at pH 8 with 35°C and stage-2 at naturally buffered pH with 35°C achieved 39.8% VS reduction, 44% (total chemical oxygen demand)

TCOD removal and $0.16 \text{ L g TCOD}_{\text{added}}^{-1}$ methane yield. As expected, higher VS reduction, TCOD removal and methane yield were obtained when the stage-1 is adjusted to 55°C , but the improvement was small and the energy balance calculation did not advocate this modification with 20 days system SRT configuration. Nevertheless, the mesophilic-mesophilic alkaline two-stage system gave 28.9%, 18.9% and 27.2% higher VS reduction, TCOD removal and methane yield, respectively, compared to that in the conventional mesophilic single-stage system at 20 days SRT.

Further enhancement to recover more energy by anaerobic co-digestion of sewage sludge with waste cooking oil (WCO) was investigated in mesophilic-mesophilic and thermophilic-mesophilic alkaline two-stage configurations. Both alkaline two-stage systems tolerated up to 35% - 45% of WCO ratio to overall organic loading, but failed when a ratio of 48% - 55% was tested. Microbiological analysis revealed that the alkaline enhanced mesophilic (35°C) and thermophilic (55°C) stage-1s were composed of proteolytic, saccharolytic and lipolytic *Bacteria* from *Firmicutes*, *Bacteroidetes*, *Fusobacteria* and *Proteobacteria* phyla, mainly performing sludge hydrolysis, acidogenesis and WCO hydrolysis; while the stage-2s also conducted all these in addition to sludge acetogenesis, methanogenesis, long chain fatty acids (LCFA), glycerol acetogenesis and methanogenesis. Canonical correspondence analysis revealed that propionic acid oxidizing *Candidatus_Cloacamonas*, LCFA degrading *Syntrophomonas* and hydrogenotrophic methanogen, *Methanoculleus* enriched in the stage-2s were key microorganisms that were strongly correlated to the stability of process performance. 56.6% VS reduction, 61.1% TCOD removal and $0.32 \text{ L g TCOD}_{\text{added}}^{-1}$ methane yield were achieved in the mesophilic-mesophilic alkaline two-stage anaerobic co-digestion system at 35% - 45% of WCO ratio, which was almost double the methane yield as obtained in sludge mono-digestion system.

This thesis presents the development of sludge digestion enhancement strategies, which is complemented with insights into the link between microbial communities

and process performance. The microbiological information in this study can potentially assist future applications of process modelling, optimization, monitoring tools and bioaugmentation strategies.

Chapter 1

Introduction

1.1. Background

Advances in wastewater treatment technology and its implementations have successfully protected the environment and populations worldwide from the detrimental effect of aquatic pollutions. However, conventional biological treatment process generates sludge as an inevitable by-product. Sludge should be treated due to its pathogenic nature and vector attraction potential; and its treatment and disposal can account up to 50% of the operating costs of a wastewater treatment plant (WWTP) (Appels et al. 2008). Sludge treatment by anaerobic digestion (AD) is one of the most commonly used methods in WWTPs due to its ability to efficiently reduce sludge mass with limited environmental impacts, improve sludge quality by reducing its vector attraction potential and produces methane, a renewable source of bioenergy (Tchobanoglous et al. 2003; Appels et al. 2008). Nevertheless, increasing population and rapid urbanization will consequently increase the production of sludge in WWTPs. In addition, current global challenges such as climate change, limited supply of fossil fuels and space in land scarce countries, continue to demand better efficiency of the anaerobic sludge digestion process.

Various anaerobic sludge digestion enhancement methods have been developed to improve the biodegradability, sludge quality, and increase methane production (Carrère et al. 2010). Biological method of enhancement (e.g. two-phase anaerobic system) is preferred in comparison to other methods, such as mechanical, oxidative/chemical or thermal pretreatments, because it does not incur relatively high operating cost and energy, is simpler to operate and maintain; while it is able to effectively improve solids reduction, sludge quality and enhances biogas production (Ariunbaatar et al. 2014).

Normally, the first reactor of a two-phase system is operated at acidic or neutral pH, but recent study revealed that alkaline pH was better for sludge solubilization (Zhang et al. 2010; Liu et al. 2012). However, biological activities were impaired under extremely high pH condition and demand additional chemical costs for neutralization before AD (Li et al. 2012). Therefore, alkaline enhancement method still need to be further investigated to optimize the physicochemical mode of degradation while preserving biological activities. Thermophilic condition had been found to achieve higher sludge solubilization than mesophilic condition (Ge et al. 2011), and should also be evaluated towards further enhancing the alkaline two-stage anaerobic system. Lastly, anaerobic co-digestion of sewage sludge with other wastes containing fat, oil and grease (FOG) can help generate even more methane as a source of renewable energy. The additional methane recovered from anaerobic co-digestion process is expected to reduce reliance on fossil fuel consumption to support WWTP's operations.

It is noted that the terms “two-phase” and “two-stage” have often been used interchangeably (Zhang and Noike 1991; Ghosh et al. 1995; Liu et al. 2006; Merlino et al. 2013; Zamanzadeh et al. 2013). The term “two-phase” should be used to describe a system with two physically separate reactors that conduct separate metabolic functions (i.e. acidogenic and methanogenic reactions), whereas a “two-stage” system refers to a system with two physically separate reactors that conduct the same metabolic functions (Azbar and Speece 2001). Microbiological analysis revealed that there was no true phase separation in a two-phase anaerobic sludge digestion system as both acidogenesis and methanogenesis occur in the two physically separated reactors (Zhang and Noike 1991; Shimada et al. 2011). This thesis will address the traditional acidogenic (acidic or near neutral pH) – methanogenic two reactor system as “two-phase”, and the optimized alkaline two reactor system developed in this study as “two-stage”.

The AD process itself involves complex interactions of different microbial populations, generally known to perform hydrolysis, acidogenesis, acetogenesis

and methanogenesis reactions. Even now, the microbial diversity, interactions and functions in AD process are still regarded as a “black box” due to its complexity (Novaes 1986; Ahring 2003; Rivière et al. 2009; Nobu et al. 2015). Estimation of the microbial diversity found in the public database revealed that only 60% of bacterial and 90% of archaeal diversity have been identified, and their roles in the AD process are still not properly understood (Chouari et al. 2005; Narihiro and Sekiguchi 2007; Rivière et al. 2009; Nelson et al. 2011; Nobu et al. 2015). Fundamentally, conflicting views had been reported, whether microbial community structure in an engineered system is shaped by deterministic (Vanwonterghem et al. 2014) or stochastic factors (Zhou et al. 2013). A better understanding of the microbial community structure in AD can help to improve process performance and stability in the future. For example, the microbial community structure in the inoculum seed sludge is considered to be important in the process performance and stability during perturbation (McMahon et al. 2004; Huang et al. 2014; De Vrieze et al. 2015). Therefore, a better description of the microbial community structure is desirable. Reactor operating and environmental conditions are known to cause microbial community shifts leading to possibly different process performance (Karakashev et al. 2005; Palatsi et al. 2010). This information can potentially assist the development of process modelling or improvement and help identify sensitive metabolic channels as predictive tools to process failure (Angelidaki et al. 2000; Williams et al. 2013). In addition, identification of critical population groups could be useful in designing bioaugmentation strategies for process improvement or recovery when the system fails (Westerholm et al. 2012).

1.2. Problem statement

The microbial ecology of anaerobic digestion system has often been studied and described in the literature, but the link between the microbial community structure and the bioprocess performance, especially in the two-phase or two-

stage AD systems, still remained unclear. It is known that two-phase AD system achieved better sludge reduction and methane production compared to a single-stage system; but the microbiological understanding to the improvement is not clearly understood. Within the two-phase system, the role of the first reactor is critical. Process intensification by operating at higher pH and temperature had been shown to give better sludge hydrolysis and acidogenesis, but the underlying microbiological community structure has also not been clarified. Furthermore, there has been limited study on the significance of biotic and abiotic contributions to the process. Process intensification of the first reactor should also accommodate for methanogenesis which provides alkalinity through carbon dioxide generation and helps consume the potentially inhibitory acetic acids and hydrogen to further acidogenic and methanogenic reactions; thus two-stage AD system is proposed. A better understanding in the underlying microbial community structure and dynamics can assist plant operators to improve the operation and control of AD system, for instance to predict and recover overloading conditions during operation.

1.3. Research objectives

The main objective of this study is to characterize the microbial community structure, dynamic and roles in enhanced anaerobic sludge digestion process and understand its responses to reactor operational or environmental conditions.

The sub-objectives of this study (with their respective scopes) are:

1. To characterize the microbial community structures in the single-stage and two-phase AD system and understand the microbiological contribution to the process improvement of the two-phase system.

- Monitoring of sludge digestion performances in terms of volatile solids (VS) reduction, total chemical oxygen demand (TCOD) removal, methane production) at decreasing SRTs.

- Comparison of the microbial community structures in the single-stage and two-phase anaerobic systems by denaturing gradient gel electrophoresis (DGGE) and quantitative polymerase chain reaction (qPCR).

2. To optimize operating pH of the first reactor for enhanced sludge solubilization and acidogenesis and assess the microbial community and its roles at different pH.

- Evaluation of the extent of solubilization, production of VFA, biogas and other organic components in stage-1 reactors with operating pHs ranging from pH 4 to 11.
- Comparison of molecular weight (MW) distribution of the solubilized organic matter at different pHs.
- Measurement of sludges' hydrolytic enzyme activities at different pHs
- Identification of the microbial community structures acclimated at different pHs by 454 pyrosequencing.

3. To evaluate the effect of temperature of the first reactor operated at mild alkaline condition on sludge solubilization and acidogenesis; and investigate the overall sludge digestion performance and microbial community structures as alkaline two-stage anaerobic systems.

- Proof of concept study for the application of novel alkaline two-stage system to treat sewage sludge, with the first reactor operated at optimal pH, as determined by sub-objective 2.
- Comparison of the extent of sludge solubilization and acidogenesis in the alkaline enhanced first reactor at mesophilic and thermophilic conditions.
- Comparison of the overall sludge digestion performances between conventional single-stage and alkaline two-stage anaerobic systems.
- Temporal characterization of the microbial community structures in the mesophilic-mesophilic and thermophilic-mesophilic alkaline two-stage anaerobic system by 454 pyrosequencing.

4. To investigate the feasibility of anaerobic co-digestion of sludge with lipid-rich substrate (waste cooking oil, WCO) by the alkaline two-stage anaerobic system and identify the key microbial populations at increasing organic loadings.

- Determination of the optimal and limit of WCO mixing ratio for anaerobic co-digestion of sludge with WCO by the alkaline two-stage anaerobic system.
- Monitoring of archaeal and bacterial communities at increasing WCO mixing ratio by 454 pyrosequencing.

1.4. Thesis outline

This thesis consists of 7 chapters, which are highlighted as follows:

Chapter 1: Introduction – An overview of background information and the research objectives of the study.

Chapter 2: Literature review – A comprehensive literature review on sludge treatment, the consideration for AD process with its optimization strategies, and information related to microbiological analysis and current understanding of microbial community structures in AD process. Knowledge gaps are identified and form the focus of this study.

Chapter 3: Comparison of single-stage and two-phase anaerobic sludge digestion systems – performance and microbial community dynamics – Comparison of conventional single-stage and two-phase anaerobic configuration as a biological mean to enhance sewage sludge digestion at decreasing SRT. Process performance, in terms of VS reduction, TCOD removal and methane production were reported. The link between process performance and microbial community structures in both anaerobic systems at decreasing SRT

was described and indicated the benefit of operating two-phase anaerobic system.

Chapter 4: The effect of pH on sludge solubilization, acidogenesis and microbial community structures – Optimization of the first reactor for enhanced hydrolysis and acidogenesis from pH 4 to 11 to generate suitable solubilized mixed liquor for methanogenesis in the second reactor. VFA accumulation and molecular weight (MW) distribution of solubilized products were determined to validate the optimized operating pH value. Microbiological analysis determined variations in the archaeal and bacterial communities at various pHs and their role in biotic contribution to solubilization and acidogenesis was discussed.

Chapter 5: Enhanced sludge digestion and the microbial community analysis in alkaline two-stage anaerobic system – Application of the optimal operating pH value established in Chapter 4 as an alkaline two-stage anaerobic system for sewage sludge treatment. Mesophilic and thermophilic conditions were tested in the first reactor to examine its effect on solubilization and acidogenesis and compare the overall alkaline two-stage digestion performances to a conventional single-stage anaerobic system. Temporal microbiological analysis identified key populations involved in the novel systems and their significance to process performance was elaborated.

Chapter 6: Anaerobic co-digestion of sewage sludge with waste cooking oil in alkaline two-stage anaerobic system – The operation of alkaline two-stage anaerobic system for co-digestion of sewage sludge with waste cooking oil (WCO). Increasing ratio of WCO to sewage sludge was fed until the process failed to determine the limit of WCO loading for co-digestion. Process improvement in terms of VS reduction and methane yield were evaluated. Long chain fatty acids

(LCFA) degrading *Bacteria* and methanogenic populations were monitored and correlated with process performances during stable and failed reactor states.

Chapter 7: Conclusions and recommendations – Summary of major findings and their implications, and recommendations for future work.

Chapter 2

Literature Review

This chapter provides the literature review on the use of anaerobic digestion (AD) for sludge treatment, the different strategies of its enhancement and the understanding of the microbial community structures in AD processes. Knowledge gaps will be identified, and form the focus of this study.

2.1. Sludge treatment

Increasing technological advances and application of wastewater treatment processes have improved water quality around the world. However, current wastewater treatment processes utilize the widely used activated sludge technology, which produces sludge as an inevitable by-product. The treatment and disposal of sludge can account up to 50% of the operating costs of a wastewater treatment plant (WWTP) (Appels et al. 2008). Increasing population and rapid urbanization lead to the rise of wastewater treatment plant constructions or upgrades in existing plants, resulting in more sludge being generated (Appels et al. 2008). In addition, current global challenges on the impacts of climate change, limited fossil fuel supply and lack of land space in a country such as Singapore, demand for more efficient WWTPs.

In conventional wastewater treatment process, sludge is generated from the primary, secondary and (sometimes) tertiary treatment processes; respectively called primary, secondary (waste activated sludge, WAS) and tertiary sludge (Tchobanoglous et al. 2003; Appels et al. 2008). The main purpose of sludge treatment is to reduce the putrescible (organic) content in the sludge, which in turn reduce the mass, remove odours and pathogens, and render the sludge suitable for further reuse or disposal. Sludge is commonly treated by alkaline stabilization, composting, aerobic digestion or anaerobic digestion. Treated sludge

is then disposed to landfill, incineration, off shore dumping, or if suitable, reused on agricultural land or stabilized within solid matrices (Tchobanoglous et al. 2003; Bednarik et al. 2004).

Amongst the other options, anaerobic digestion (AD) is the most common method to treat sludge due to its limited environmental impacts, solids reduction efficiency and the potential for energy recovery (Tchobanoglous et al. 2003). AD process biochemically oxidizes organic material in the absence of oxygen to produce carbon dioxide, methane, water, ammonia and sometimes sulphide (Gujer and Zehnder 1983). Compared to aerobic digestion, it consumes less energy, produces methane and is not as easily affected by changing conditions (Lettinga 1995). The low biological sludge production associated with anaerobic microorganisms, while reducing sludge organic content also make AD process more competitive than aerobic digestion. Furthermore, net energy production can be obtained when the methane generated is recovered.

2.2. Anaerobic digestion

AD is a complex biological decomposition of organic matter into biogas, in the absence of oxygen. It is one of the oldest processes in wastewater treatment discipline. The first reported process in the modern world was installed by Louis Mouras in France around 1860 (Dunbar 1908). Many reviews on anaerobic digestion are available and had been collated by Amani, et al. (2010). Generally, AD is understood to consist of four major steps, namely: disintegration or hydrolysis, acidogenesis, acetogenesis and methanogenesis (Gujer and Zehnder 1983; Batstone et al. 2002), which were performed by different microorganisms.

Figure 2.1. The anaerobic digestion model adapted from ADM1 (Batstone et al. 2002). The numbers refer to the biochemical reaction of: (1) Acidogenesis from sugars, (2) amino acids, (3) acetogenesis from LCFA, (4) propionic acid, (5) butyric acid and valeric acid, (6) methanogenesis from acetic acid and (7) hydrogen.

Disintegration and hydrolysis is the first step in AD, where complex organic polymer is broken down into simple monomers, so that it can be taken up by the microbial cells. Major components of the organic polymers are protein, carbohydrate and lipid, in which the respective hydrolysis products are amino acid, monosaccharide, long chain fatty acids (LCFA) and glycerol. Palmitic (C16:0), stearic (18:0) and oleic (18:1) acids are some of the most common LCFA found in organic waste and wastewater processes (Hwu et al. 1998). Depolymerisation of the polymers occurs outside microbial cell, mediated by extracellular enzymes

produced by hydrolytic microorganisms. This step would also yield inert components which cannot be consumed and degraded, such as lignin (Batstone et al. 2002). Various concepts for hydrolysis had been hypothesized: hydrolytic microorganisms secreting enzymes to the bulk liquid (Jain et al. 1992), or the microorganism is adsorbed onto a surface and secrete enzymes in its vicinity (Vavilin et al. 1996), or the combination of both. Hydrolysis becomes the rate-limiting step when the substrate contains large particles with low surface area to volume ratio (Vavilin et al. 1996) or a high polymeric content in the substrate (Noike et al. 2004), such as sludge. The degradability of sludge also worsened when the upstream sludge age was longer; hence, a concern over the extended sludge age required for nutrient removal in activated sludge process (Carrère et al. 2010).

In acidogenesis, the hydrolyzed products are taken up by acidogens and fermented into volatile fatty acids (VFA) or other “electron sink” compounds, such as VFA with longer carbons, alcohol or lactic acid. The formation of “electron sink” compounds occurs when there is excess hydrogen in the environment as the hydrogen utilizing reaction cannot cope with the fast acidogenesis (Cohen et al. 1982). Monosaccharides are fermented via the Embden-Meyerhof-Parnas pathway to pyruvate and then propionic or lactic acids (C3 compounds), or acetyl Co-A to form C2 - C6 compounds (acetic, butyric acids or ethanol) (Batstone and Jensen 2011). However, the most efficient fermentation pathways is to produce acetic acid, carbon dioxide and hydrogen as they would give higher energy yields and can be directly utilized by the methanogens (Gujer and Zehnder 1983). pH may affect the acid species formation during acidogenesis. Horiuchi, et al. (2002) fermented glucose with anaerobic sludge and found that the main acidification product shifted from acetic, butyric acids to acetic, propionic acids when operating pH was adjusted from pH 6 to 8. On the other hand, the fermentation of amino acids is performed by direct oxidation or “Stickland reaction”, where amino acids are degraded as a pair (Ramsay and Pullammanappallil 2001). The “Stickland reaction” produces ammonia, carbon dioxide and a carboxylic acid (with one carbon shorter

than original length of the donor amino acid); and ammonia and a carboxylic acid (with the same carbon chain length as the acceptor amino acid) (Ramsay and Pullammanappallil 2001). Glycerol, which is the product of lipid hydrolysis, is fermented to propionic acid (Angelidaki et al. 2000).

Fermentation products (VFA or alcohols) and LCFA are further converted into acetic acid, hydrogen and carbon dioxide during acetogenesis. **Table 2.1** shows that some acetogenic reactions proceed with a positive Gibbs energy (ΔG°) value, meaning a thermodynamically unfavourable reaction. The concentrations of the products (hydrogen) have to be kept low to make the reaction endergonic (Stams 1994). Nicotineamide adenine dinucleotide (NAD^+), a cellular cofactor, accepts the electron (H^+) during the biological oxidation process. The electron is necessary for energy generation (in the form of adenosine triphosphate, ATP, being cellular energy currency) during oxidative phosphorylation, as the electron is reduced to molecular hydrogen. The NADH could not be re-oxidised into NAD^+ if hydrogen tension is high in the environment (hydrogen partial pressure below 10^{-4} , 10^{-3} and 10^{-4} atm are required for propionic, butyric and palmitic acid oxidations, respectively) (Batstone and Jensen 2011). Therefore, hydrogen scavenging partner is required (Thauer et al. 1977). This situation is also the reason why fermentative bacteria divert electrons into a different metabolic pathway to generate “electron sink” products during acidogenesis (Gujer and Zehnder 1983). Hydrogenotrophic methanogen (carbon dioxide being the electron acceptor) is the preferred syntrophic partner in this situation, but there are also other electron acceptors that may be found in AD processes, such as nitrate reduction, sulfate reduction, ferric reduction and homoacetogenesis (Achtnich et al. 1995; Stams and Plugge 2009). Homoacetogenesis is a reversible reaction and can also form acetic acid from hydrogen and carbon dioxide, but is not common as hydrogenotrophic methanogenesis is more energetically favourable (Schink 1997). Lastly, LCFA is also oxidized into acetic acid and hydrogen, which are then consumed in methanogenesis (Sousa et al. 2009).

Table 2.1. Reactions involved in anaerobic/anoxic syntrophic metabolism with its Gibbs energy values (Thauer et al. 1977; Stams 1994).

Substrate	Reaction	ΔG° (kJ mol ⁻¹)
VFA oxidation		
Propionic acid	$\text{CH}_3\text{CH}_2\text{COO}^- + 3\text{H}_2\text{O} \rightarrow \text{CH}_3\text{COO}^- + \text{H}^+ + 1\text{HCO}_3^- + 3\text{H}_2$	+ 76.1
i-/n-Butyric acid	$\text{CH}_3(\text{CH}_2)_2\text{COO}^- + 2\text{H}_2\text{O} \rightarrow 2\text{CH}_3\text{COO}^- + \text{H}^+ + 2\text{H}_2$	+ 48.1
i-/n-Caproic acid	$\text{CH}_3(\text{CH}_2)_4\text{COO}^- + 3\text{H}_2\text{O} \rightarrow 3\text{CH}_3\text{COO}^- + 2\text{H}^+ + 4\text{H}_2$	+ 96.2
Acetic acid	$\text{CH}_3\text{COO}^- + 2\text{H}_2\text{O} \rightarrow 2\text{HCO}_3^- + 4\text{H}_2 + \text{H}^+$	+ 104.6
Alcohol oxidation		
Ethanol	$\text{CH}_3\text{CH}_2\text{OH} + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{COO}^- + \text{H}^+ + 2\text{H}_2$	+ 9.6
LCFA oxidation		
Palmitic acid (C16:0)	$\text{CH}_3(\text{CH}_2)_{14}\text{CO}_2^- + 14\text{H}_2\text{O} \rightarrow 8\text{CH}_3\text{COO}^- + 14\text{H}_2$	+ 353
Oleic acid (C18:1)	$\text{CH}_3(\text{CH}_2)_7\text{CH}=\text{CH}(\text{CH}_2)_7\text{COO}^- + 16\text{H}_2\text{O} \rightarrow 9\text{CH}_3\text{COO}^- + 15\text{H}_2$	+ 338
Amino acid oxidation		
Alanine	$\text{CH}_3\text{CH}(\text{NH}_2)\text{COOH} + 3\text{H}_2\text{O} \rightarrow \text{CH}_3\text{COO}^- + \text{HCO}_3^- + \text{NH}_4^+ + \text{H}^+ + 2\text{H}_2$	+ 7.5
Leucine	$\text{HO}_2\text{CCH}(\text{NH}_2)\text{CH}_2\text{CH}(\text{CH}_3)_2 + 3\text{H}_2\text{O} \rightarrow \text{CH}_3(\text{CHCH}_3)\text{CH}_2\text{COO}^- + \text{HCO}_3^- + \text{NH}_4^+ + \text{H}^+ + 2\text{H}_2$	+ 4.2
Hydrogen-consuming reactions		
Methanogenesis	$4\text{H}_2 + \text{CO}_2 \rightarrow \text{CH}_4 + 2\text{H}_2\text{O}$	- 135.6
Nitrite reduction	$2\text{NO}_2^- + 2\text{H}^+ + 3\text{H}_2 \rightarrow \text{N}_2 + 4\text{H}_2\text{O}$	- 794.1
Sulphate reduction	$\text{S} + \text{H}_2 \rightarrow \text{H}_2\text{S}$	- 28
	$\text{SO}_4^{2-} + 4\text{H}_2 + \text{H}^+ \rightarrow \text{HS}^- + 4\text{H}_2\text{O}$	- 151.9

The last step in AD, methanogenesis, can be grouped into two types of reaction: hydrogenotrophic methanogenesis (HM) which utilizes formate or hydrogen as electron donor and carbon dioxide as electron acceptor to produce methane and water; and acetoclastic methanogenesis (AM) which cleaves acetic acid into methane and carbon dioxide (Garcia et al. 2000; Demirel and Scherer 2008). It is estimated that AM contributed to around two-thirds of methane production in anaerobic process (Speece 1996; Garcia et al. 2000). There are also other substrates which can be directly used by methanogens but less commonly found, such as carbon monoxide, alcohol, formic acid and other one-carbon methylated compounds (e.g. methylamines, methylmercaptans) (Liu and Whitman 2008). Methanogenesis is considered the rate-limiting step when the substrate contains

easily degradable organics (e.g. glucose, SCFA) due to the slower methanogenic rate compared to the acidogenic reaction rate (Noike et al. 2004).

2.3. Factors affecting anaerobic sludge digestion

There are different factors which can affect AD process due to the involvement of various microorganisms in the same environment. Ensuring a stable operating condition is critical to select for the optimal microbial community and activities dedicated to the digestion process. Some of the key factors are the influent substrate characteristic, solids retention time (SRT), pH, alkalinity and temperature.

The physical and chemical characteristics of a sludge substrate can directly affect the efficiency of AD. Size distribution of sludge particle affects process efficiency, where smaller particle with higher surface area to volume ratio would be more accessible to hydrolytic enzyme (Vavilin et al. 2008). Specific composition of the sludge such as polymeric or monomeric components affects the process, where complex polymers mean slower degradation rate and also possibly, lower extent of degradation. For instance, it is very difficult to degrade lignin, a plant-derived polymer (Stinson and Ham 1995). In general, primary sludge is more degradable than WAS (Ghosh 1987).

The type of products being released is also related to the influent substrate characteristics and affects AD process. Degradation of protein, which formed the main composition of sludge, would yield nitrogenous compounds that lead to ammonia and sulphide production that could affect AD process (Appels et al. 2008). Substrate with easily degradable substrates (e.g. monosaccharides) could be hydrolyzed and acidified quickly, leading to sudden pH drop and inhibition to the methanogens. Substrate with high lipid content can also impair AD process. Despite lipid being easily hydrolyzed into long chain fatty acids (LCFA) and glycerol, lipid and/or LCFA can adsorb onto microbial cells, limiting mass transfer and potentially cause biomass floatation and cell washouts (Hanaki et al. 1981; Hwu et

al. 1998). In addition, LCFA is inhibitory to syntrophic acetogens and methanogens, but if controlled, gives higher methane production. There are other inhibitory compounds which will be described later. The presence of micronutrient (e.g. iron, cobalt, nickel) is also essential in AD process (McCarty 1964), though this is unlikely to affect anaerobic sewage sludge digestion process. Municipal wastewater is known to contain sufficient micronutrients for a stable AD process (Tchobanoglous et al. 2003). The presence of nitrate, nitrite or sulphate in the substrate could also acclimatize the growth of chemoheterotrophic denitrifying (Ruiz et al. 2003) and sulphate reducing bacteria (Muyzer and Stams 2008), which used these as electron acceptor instead of carbon dioxide. These communities could compete with methanogenesis for electrons during AD process, thus aggravating the methane production. Based on theoretical thermodynamics consideration (**Table 2.1**), denitrification would occur more readily than sulphate reduction, and followed by methanogenesis. However, nitrate, nitrite and sulphate concentrations were typically low in municipal wastewater (Tchobanoglous et al. 2003).

SRT is critical in AD to establish the reaction time for optimal microbial degradation of the sludge and influence the reactor volume in sludge digestion process design. **Table 2.2** summarizes the maximum specific growth rate ($\mu_{\max}$) of different microorganisms involved in AD process to indicate the retention time required to cultivate anaerobic microorganisms in a digester, especially the slower-growing methanogenic communities. Miron et al. (2000) established that methanogenesis occurred only above 8 days SRT, whereas SRT below 8 days resulted in acidogenic condition during anaerobic primary sludge digestion. A similar SRT threshold was also observed in mixed primary, secondary and tertiary sewage sludge digestion at 9 days (Nges and Liu 2010). In specific, hydrolysis of carbohydrate and lipid can be improved by increasing SRT from 3 to 15 days, while protein hydrolysis and acidogenesis only effectively occurred above 8 days SRT (Miron et al. 2000). Carbohydrate can be easily acidified with increasing efficiency at higher SRT, but lipid acetogenesis only occurred at above 8 days SRT (Miron et

al. 2000). Therefore, sludge digesters are normally operated with SRT of around 10 to 30 days (Tchobanoglous et al. 2003; Appels et al. 2008).

Table 2.2. The maximum growth rate and optimal growth pH condition of acidogenic, acetogenic and methanogenic reactions.

Reaction	Substrate	$\mu_{\max}$ (d ⁻¹)	pH	References
Acidogenesis	Cellulose	3.84	5.8 - 7.3	(Lynd et al. 2002)
	Glucose	5.1	4 - 9	(Angelidaki et al. 2000; Li et al. 2008)
	Amino acids	6.38	4 - 11	(Angelidaki et al. 2000; Liu et al. 2012)
Acetogenesis	Propionic acid	0.49	6.4 - 7.4	(Liu et al. 1999)
	Butyric acid	0.67	6.5 - 8.5	(Angelidaki et al. 2000; Wu et al. 2007)
	Oleic acid	0.55	6.3 - 8.1	(Angelidaki et al. 2000)
Methanogenesis	H ₂ + CO ₂	0.17*	6.7 - 8*	(Imachi et al. 2008)
		0.053 ⁺	6.6 - 7.5 ⁺	(Dubach and Bachofen 1985)
		0.139 ⁻	6.5 - 7.7 ⁻	(Dubach and Bachofen 1985)
	Acetic acid	0.6 [#]	5 - 8 [#]	(De Vrieze et al. 2012)
		0.2 [§]	6.5 - 8.5 [§]	(De Vrieze et al. 2012)

* *Methanolinea*'s maximum growth rate and growth pH range.

⁺ *Methanospirillum*'s maximum growth rate and growth pH range.

⁻ *Methanobrevibacter*'s maximum growth rate and growth pH range.

[#] *Methanosarcina*'s maximum growth rate and growth pH range.

[§] *Methanosaeta*'s maximum growth rate and growth pH range.

Different microorganisms require different pHs for optimal growth and activities (**Table 2.2**) and the operating pH of AD process is recommended to be between 6.5 and 7.2 (Kim et al. 2003; Appels et al. 2008). Both pH and alkalinity have long served to be useful operational values to ensure reactor stability (Ripley et al. 1986). Reactor instability is normally associated with imbalanced degradation rates of the acidogenic and methanogenic microorganisms. Acidogens metabolize faster than methanogens, thus VFA accumulation can occur (Gujer and Zehnder 1983; Ghosh 1987). As VFA accumulates, pH will decrease and exacerbate the condition. VFA at acidic pH is in undissociated forms and undissociated acetic acid is known to be the cause of methanogenesis inhibition (Fukuzaki et al. 1990). Ammonia and hydrogen sulphide are also toxic when they are in the undissociated form, at pH

above 7 for ammonia ($pK_a = 9.25$) and at neutral and lower pH for hydrogen sulphide ($pK_a 7.05$) (Chen et al. 2008). Alkalinity represents the ability of the liquor to resist pH change and maintains the stability of AD. The main buffering capacity originates from the dissolved bicarbonate ions of carbon dioxide and VFA. Normally, a stable anaerobic digester would have a total alkalinity of 2000 – 5000 $\text{mg CaCO}_3 \text{ L}^{-1}$ (Tchobanoglous et al. 2003).

Similar to pH, temperature also determines the growth and microbial activities in the digester. AD had been applied in psychrophilic ($<20^\circ\text{C}$) (Kashyap et al. 2003), mesophilic ($20 - 45^\circ\text{C}$) and thermophilic ($45 - 60^\circ\text{C}$) conditions (van Lier et al. 1997; Lu et al. 2008). Overall, sludge solubilization is increased with increasing temperature to thermophilic condition as microbial activity is accelerated and the complex compounds disintegrate. This would lead to higher acidogenesis and thus the overall AD process. Higher microbial decay rate in thermophilic condition also helped in keeping the sludge yield low and destroy pathogens (Buhr and Andrews 1977; Kim et al. 2002).

AD, being a biological process, is sensitive to certain substances which can restrict microbial activities. Speece et al. (2008) differentiated “inhibition, as impairment of bacterial function (commonly reversible)” and “toxicity, as an adverse effect on bacterial metabolism (commonly irreversible)”. These effects are characterized by a reduction in methane production, normally preceded by VFA accumulation and a drop in pH (Speece 1996; Chen et al. 2008). Toxic compounds often target specific mechanism of cellular metabolism, e.g. detergents, aldehydes, nitro-compounds, cyanide, azides, antibiotics or electrophiles (Batstone and Jensen 2011). LCFA can also be considered toxic by nature as it adsorbs onto cellular surfaces and restrict cellular membrane transport mechanism (Hwu et al. 1998). However, Palatsi et al. (2010) demonstrated that this effect is reversible and microbial adaptation step could help recover affected digester. Meanwhile, some common examples of inhibition in AD process include ionic inhibition, product inhibition, weak acid-base inhibition and pH inhibition (Batstone and Jensen 2011). Ionic inhibition occurs as

increasing ionic concentration (e.g. sodium) requires increasing cellular maintenance requirement to regulate the cellular osmotic pressure (homeostasis). Product inhibition occurs when products of a catabolic reaction accumulate until its production becomes thermodynamically unfavourable. As mentioned previously, inhibition by hydrogen accumulation is one such example and impair the acetogenesis reaction in AD. Weak acid-base inhibition occurs when there is excessive passive transport of undissociated acids or bases into the cell (e.g. VFA and ammonia), hence, increasing the cellular maintenance requirement to regulate intracellular pH (homeostasis). This is called the uncoupling theory of inhibition where energy in the form of ATP is required to regulate intracellular pH, instead of biomass formation (Palmqvist and Hahn-Hägerdal 2000). Finally, pH inhibition contributes to weak acid-base inhibition. It also disrupts cellular homeostasis of cellular pH and caused detrimental protein denaturation, which impair essential cellular activities. McCarty (1964) evaluated that inhibitory substances may in fact be stimulatory at low concentrations. This stimulatory benefit will reach a certain point where further rise in concentration causes the rate of biological activity to decrease. **Table 2.3** summarizes the inhibitory compounds and concentrations which are often found in AD process.

Table 2.3. List of inhibitory compounds and concentrations associated with anaerobic sludge digestion process.

Compound	Inhibitory Concentrations	References
VFA	4000 mg acetic acid L ⁻¹ ; 900 propionic acid mg L ⁻¹ ; Undissociated acids: 13 mg L ⁻¹	(Siegert and Banks 2005; Wang et al. 2009; Batstone and Jensen 2011)
LCFA	30 mg oleic acid L ⁻¹ ; 100 mg stearic acid L ⁻¹ ; 30 mg linoleic acid L ⁻¹	(Alosta et al. 2004; Fernández et al. 2005)
Ammonia	1,500 mg total ammonium nitrogen (TAN) L ⁻¹ ; 150 mg free ammonia nitrogen (FAN) L ⁻¹	(Van Velsen 1979)
Sulfide	100 - 800 mg dissolved sulphide L ⁻¹ ; 50 - 400 mg undissociated H ₂ S L ⁻¹	(Chen et al. 2008)
Chloride	6,000 mg Cl ⁻ L ⁻¹	(Appels et al. 2008)
Light metals	3500 mg Na ⁺ L ⁻¹ ; 2,500 mg K ⁺ L ⁻¹ ; 2,500 mg Ca ²⁺ L ⁻¹	(McCarty 1964)
Heavy metals	35 mg L ⁻¹ (iron containing compounds); 180 mg Cr ⁶⁺ L ⁻¹ ; 30 mg Ni ²⁺ L ⁻¹ ; 50 mg Cu ²⁺ L ⁻¹	(Appels et al. 2008)
N-oxides	NO ₃ : 30 mg N-NO ₃ L ⁻¹ ; NO ₂ : 17 mg N-NO ₂ L ⁻¹ ; NO: 0.16 mg N-NO L ⁻¹ ; N ₂ O: 19 mg N-N ₂ O L ⁻¹	(Tugtas and Pavlostathis 2007)

2.4. Sludge digestion enhancement

Sludge is mainly composed of microbial cells or flocs, organic and inorganic matter, which are agglomerated together by microbial extracellular polymeric substances (EPS) and cations, both of which maintain the sludge matrix stability (Frølund et al. 1996; Sheng et al. 2010). According to Nielsen and Jahn (1999), EPS is subdivided into bound EPS (sheaths, capsular polymers, condensed gel, loosely bound polymers and attached organic material) and soluble EPS (soluble macromolecules, colloids and slimes), as shown in **Figure 2.2**. However, there is not yet a standard method to separate each part of the EPS due to its complex structure and characteristics (Sheng et al. 2010). Various intermolecular interactions between the microbial cells have been summarized to establish the stability of EPS (Sheng et al. 2010), including polymer entanglement (Mikkelsen and Nielsen 2001), ion bridging in the EPS and electrostatic interaction (Klausen et al. 2004) and van der Waal's force and hydrogen bonds (Mayer et al. 1999). Disruption to the EPS and cell membranes are required for sludge digestion to proceed, hence, sludge hydrolysis becomes the rate limiting step in anaerobic sludge digestion process (Carrère et al. 2010). Various methods have been investigated to enhance sludge digestion process by increasing the extent of sludge degradation and/or accelerating the rate of degradation. Enhancements by biological and mechanical methods generally increase the rate of degradation, while high intensity chemical and thermal hydrolysis improve the extent of degradation (Carrère et al. 2010).

There are various strategies to enhance sludge digestion by biological method, for example, operating a thermophilic digester, two-phase anaerobic system or bioaugmentation with hydrolytic bacteria. Operating the digester in thermophilic condition is known to increase the microbial activity, accelerate sludge digestion (Siegrist et al. 2002) and increase cellular decay rate to keep the sludge yield low and destroy pathogens (Buhr and Andrews 1977; Kim et al. 2002). Two-phase anaerobic configuration was first studied by Pohland and Ghosh (1971). Acidogenesis and methanogenesis were physically separated as they have different optimum environmental conditions (**Table 2.2**). The cultivation of

Figure 2.2. Simplified sketch of the structure of microbial cells, loosely bound (LB), tightly bound (TB) and soluble EPS, adapted from Nielsen and Jahn (1999) with permission from Elsevier.

acidogens at long SRT and neutral pH to accommodate for methanogens' growth can lead to suboptimal hydrolytic and/or acidogenic activities (Ghosh et al. 1975). Secondly, acid production rates are higher than the utilization rate up to a certain threshold SRT. At a significantly low SRT, the acid accumulation will reach inhibitory levels, leading to digester failure (Ghosh 1987). Phase separation would help ensure acidogens and methanogens to be optimized according to their respective ideal growth condition by adjusting SRT, and ultimately enhance sludge digestion. The accumulation of organic acids in the first phase would lead to a drop in pH value. Both the presence of organic acids and low pH were complemented to help release non-crystalline polymers from the macromolecules, which were subsequently exposed to hydrolytic enzymes and sludge hydrolysis step is enhanced (Xiao and Clarkson 1997; Chen et al. 2007). The terms "two-phase" and "two-stage" had often been used interchangeably (Zhang and Noike 1991; Ghosh et al. 1995; Liu et al. 2006; Merlino et al. 2013; Zamanzadeh et al. 2013). However, "two-phase" should be reserved when the two physically separate reactors conducted separate metabolic functions (i.e. acidogenic and methanogenic reactions) whereas the metabolic functions in the two reactors of "two-stage" system are the same (Azbar and Speece 2001). Bioaugmentation of hydrolytic

bacteria (*Caldicellusiruptor* and *Dictyoglomus*) to improve process performance had also been attempted but did not yield overall improvement in solids reduction and methane production (Nielsen et al. 2007).

Figure 2.3. Operating characteristics of the acidogenic phase of two-phase anaerobic system treating sewage sludge, to convert VS into soluble organics and produce VFA and to convert VFA into biogas (Ghosh 1987) with permission from American Society of Civil Engineers.

Ultrasonic and homogenization pretreatment methods enhance sludge digestion by mechanically disrupting the cell structure and sludge matrix by acoustic cavitation and depressurization, respectively (Tiehm et al. 1997; Barjenbruch and Kopplow 2003). Hydrolytic and acidogenic microorganisms can then easily

Table 2.4. Enhancement methods for sludge digestion.

Sludge type	Operating condition	Performance improvement	References
Biological method			
Mixed sludge	Control: 12 days SRT, 35°C Two-phase: 2 days SRT, 35°C phase-1 + 10 days SRT, 35°C phase-2	48.3% to 54.3% VS reduction (+12.4%)	(Bhattacharya et al. 1996)
Mixed sludge	Control: 24 days SRT, 35°C TPAD: 4 days SRT, 55°C phase-1 + 20 days SRT, 35°C phase-2	32% to 45% VS reduction (+41%) 2.7 to 3.3 STP L CH ₄ d ⁻¹ (+22%)	(Han et al. 1997)
WAS	Control: 2 days SRT, 35°C, 14 days SRT, 35°C TPAD: 2 days SRT, pH 6.6, 65°C phase-1 + 14 days SRT, 35°C phase-2	37% to 48% VS reduction (+30%) 0.1 to 0.15 L CH ₄ g VS _{added} ⁻¹ (+50%)	(Ge et al. 2011)
Primary sludge	Control: 2 days SRT, 35°C, 14 days SRT, 35°C TPAD: 2 days SRT, pH 6.8, 50°C phase-1 + 14 days SRT, 35°C phase-2	+20% VS reduction +25% CH ₄ production	(Ge et al. 2010)
Primary Sludge	Control: 15 days SRT, 55°C TPAD: 2 days SRT, pH 6.4, 70°C phase-1 + 13 days SRT, 55°C phase-2	43% to 55% VSS reduction (+28%) 0.146 to 0.162 mL CH ₄ L ⁻¹ d ⁻¹ (+11%)	(Skiadas et al. 2005)
Mechanical method			
Mixed sludge	Control: 20 days SRT, 37°C Sonication: 5000 kJ kg TS ⁻¹	+8% VS reduction +36% biogas production	(Tiehm et al. 1997)
Mixed sludge	Control: 22.5 days SRT, 29°C - 33°C Sonication: 20kHz, 13.7 W cm ⁻² for 1.5 seconds	+45% biogas production	(Xie et al. 2007)
Mixed sludge	Control: 20 days SRT, 36°C Homogeniser: 600 bar	0.35 to 0.413 L CH ₄ g VS _{added} ⁻¹ (+18%)	(Barjenbruch and Kopplow 2003)
Mixed sludge	Control batch: 37°C Electric focused pulse: 10kW m ⁻³	+100% CH ₄ production	(Salerno et al. 2009)
Oxidation/Chemical method			
Mixed sludge	Control batch Ozonation: 0.1 g O ₃ g COD ⁻¹ (0.08 g O ₃ g TSS ⁻¹)	36 to 68% COD reduction (+89%) 0.11 to 0.22 L CH ₄ gCOD _{added} ⁻¹ (+100%)	(Weemaes et al. 2000)

WAS	Control: 20 days SRT, 35°C Alkaline: 0.04 mol NaOH per 12 g TS for 1 hour (pH 12.2) Control batch: 35°C	35 to 42% VS reduction (+20%) 0.083 to 0.113 L CH ₄ L ⁻¹ d ⁻¹ (+36%)	(Lin et al. 1997)
Mixed sludge	Alkaline: 0.1 mol NaOH per 20 g TS for 30 minutes (pH 10.3)	30.3% to 38.3% VS reduction (+26%)	(Li et al. 2012)
Thermal			
WAS	Control: 20 days SRT, 35°C Thermal: 170°C for 30 minutes Control: 20 days SRT, 37°C	35 to 61% VS reduction (+74%) 0.128 to 0.228 L CH ₄ g VS _{added} ⁻¹ (+78%)	(Bougrier et al. 2006)
WAS	Microwave: 800 W for 3.5 minutes, 80°C (336 kJ kg ⁻¹)	51% to 70% TS reduction (+38%) +50% biogas production	(Appels et al. 2013)
Combination method			
Mixed sludge	Control: 20 days SRT, 35°C Ultrasound-ozone: 20kHz 9000 kJ kg TS ⁻¹ , 0.012 g O ₃ g TS ⁻¹ Control: 25 days SRT, 35°C	37.3% to 45.3% VS reduction (+21.4%) 0.313 to 0.393 L biogas g COD _{added} ⁻¹ (+25.6%)	(Tian et al. 2015)
Mixed sludge	Alkaline-ultrasonic: 0.021 mol KOH per 37 g TS (pH 9) for 1 hour, 20kHz, 7,500 kJ kg TS ⁻¹ Control batch: 37°C	0.082 to 0.127 L CH ₄ g COD _{added} ⁻¹ (+54.9%)	(Kim et al. 2010)
WAS	Ultrasonic thermal: 20 kHz 1W mL ⁻¹ for 1 hour, 80°C for 1 hour Control: 20 days SRT, 35°C	25.8% to 37.8% VS reduction (+46.5%) +13.6% methane production	(Şahinkaya and Sevimli 2013)
WAS	Thermal-alkaline (simultaneously): 130°C, 0.03 mol KOH per 12.5 g TS (pH 10) for 30 minutes Control: 15 days SRT, 37°C	35 to 66% VS reduction (+89%) 0.128 to 0.22 L CH ₄ g VS _{added} ⁻¹ (+72%)	(Bougrier et al. 2006)
WAS	Alkaline-microwave: 0.004 mol NaOH g VSS ⁻¹ (pH 12) for 30 minutes, 600 W for 16 minutes (160C)	43.2% to 58.5% VS reduction (+35.4%) 0.144 L CH ₄ g VS _{added} ⁻¹ to 0.22 L CH ₄ g VS _{added} ⁻¹ (+53%)	(Doğan and Sanin 2009)

consume the resulting solubilized intracellular and extracellular polymers for AD to proceed. Electric focused pulse method sends high voltage (> 20 kV) of electric pulses thousands of times per second and targets cell membrane or cell walls. Under such strong electrical field, pores on the cell membranes will open, leading to cellular swelling and ultimately cell rupture. The strong electric field could also break down sludge aggregates and increase the bioavailability of dissolved and suspended organic materials (Salerno et al. 2009).

Ozone has a strong oxidative property and is able to non-selectively oxidize soluble, particulate, organic or mineral matters (Weemaes et al. 2000). Radical species, such as the hydroxyl radical was also formed during ozonation, to help further enhance the biodegradation process. This way, the sludge matrix is disrupted and cell membrane ruptured. However, ozone will be consumed as organic materials were mineralized into inert materials, in which, the former could have been digested by AD process to produce biogas (Chu et al. 2009).

Alkaline condition was found to be more efficient than acidic condition to improve sludge solubilization (Chen et al. 2007). Amongst the other alkali, sodium hydroxide is the most effective chemical for sludge enhancement by alkaline pretreatment method (Kim et al. 2003). Alkaline condition induces protein denaturation, disrupts the EPS matrix and transfers organic components (protein and carbohydrate) from the pellet and tightly-bound EPS fractions into the loosely-bound EPS and soluble fractions of sludge, thus, making the organics accessible to microorganisms and extracellular hydrolytic enzymes (Neyens et al. 2004). Cellular lysis occurs by saponification of lipid-based cell membranes and inactivation of cellular viability, which subsequently allow cellular disruption and the release intracellular content. However, higher solubilization mediated by higher alkaline dosage did not necessarily translate to better sludge biodegradability (Li et al. 2012). Li et al. (2012) reported that pH had to be neutralized to less than pH 8 after alkaline pretreatment, but even then, salinity contributed by sodium hydroxide dosage could also impact the subsequent AD efficiency. Refractory

compounds were also generated during extremely high alkaline pretreatment, which impaired anaerobic biodegradability (Penaud et al. 1999). Hence, ideal operating pH value for enhanced sludge solubilization still needs to be further optimized.

Thermal pretreatment method helps in enhancing sludge digestion by disintegrating sludge structure and cell membrane, which lead to the solubilization of organic compounds. Proteins become denatured and the physical structure of sludge changes, which lead to the exposure of more contact sites for hydrolysis (Neyens et al. 2004). It was found that there is no difference in performance between steam and electrical heating, but microwave heating was able to solubilize more biopolymers (Mottet et al. 2009). This might have been due to the polarization of macromolecules during microwave. Similar to ozonation, loss of easily biodegradable substrates could occur and reduce the biochemical conversion potential into biogas. In addition, thermal condition to very high temperature can lead to the formation of refractory matter by Maillard reaction, an agglomeration reaction of carbohydrates and amino acids which led to dark brown colour development to the treated sludge (Carrère et al. 2010). Besides thermal method, combinations of different methods have also been investigated in recent years which led to a synergetic improvement to sludge solubilization and degradability. For example, alkaline condition was initially introduced to weaken cell membrane structure and was subsequently subjected to shear force by ultrasonication. Hence, lower alkaline intensity (pH 9) and lower energy input (7,500 kJ kg TS⁻¹) were sufficient to obtain comparable sludge solubilization to just an individual method (Kim et al. 2010).

It is shown in **Table 2.4** that oxidation, chemical and thermal pretreatment methods demonstrate higher degree of solids reduction and methane production compared to biological or mechanical methods. However, the energy requirement and operating costs related to mechanical, oxidation, chemical, thermal methods, or their combinations are high (Ariunbaatar et al. 2014). Thermal enhancement

methods also involved higher capital costs than mechanical or other methods due to the requirement of ancillary equipment (Carrère et al. 2010). In contrast, enhancement of anaerobic sludge digestion by biological method, via two-stage anaerobic system, would not incur as high cost and energy requirement as the other methods (Ariunbaatar et al. 2014). In addition, the two-stage anaerobic system would be easier to operate and maintain than the other methods. Therefore, further optimization of the two-stage anaerobic system is deemed suitable towards establishing a more efficient sludge treatment method.

Anaerobic co-digestion of sludge with other substrates can also be considered to improve AD process to recover more methane as renewable source of energy. Some organic substrates are unsuitable for anaerobic mono-digestion but could be complemented with anaerobic sludge digestion process. For instance, animal manures or slaughterhouse wastes have nitrogen content and may inhibit methanogenesis; agricultural wastes lack nitrogen or other micronutrients; fat, oil and grease (FOG) gave inhibitory LCFA and lacked essential nutrients for microbial growth (Mata-Alvarez et al. 2014). FOG, in particular, is of interest as lipid contains high biochemical methane potential per gram VS. It could also be obtained from the grit collection chamber in a WWTP, making it a convenient source for co-digestion with sludge (Tchobanoglous et al. 2003) or collected from grease traps or grease interceptors of commercial kitchens (Long et al. 2012). The biogas produced from anaerobic sludge digester would typically meet 20-40% energy requirement of a WWTP, but sludge co-digestion with FOG could contribute to over 50% energy requirement of a WWTP (Long et al. 2012). Anaerobic co-digestion of sludge with FOG was found to improve methane yield from $0.3 - 0.42 \text{ L g VS}_{\text{added}}^{-1}$ in sludge mono-digestion to $0.7 - 1.1 \text{ L g VS}_{\text{added}}^{-1}$ in co-digestion (Mata-Alvarez et al. 2014). Hydrolysis of lipids into LCFA and glycerol could proceed easily, but the accumulation of inhibitory LCFA might lead to process failure (Hwu et al. 1998). Optimal FOG dosage was explored but was found to be variable at 77:23 (Silvestre et al. 2011), 40:60 (Noutsopoulos et al. 2013) or 34.5:65.5 (Wang et al. 2013) ratio of sludge and FOG (VS). Nevertheless, studies had mainly been based on the

conventional single-stage configuration and had not explored to complement sludge digestion enhancement strategies with anaerobic co-digestion processes (Long et al. 2012).

2.5. Microbial community in AD

The biological reactions in anaerobic sludge digestion process rely on the concerted effort of various microorganisms with different physiology, growth requirements and functions; which due to its complexity, has long been considered as a “black box” (Angelidaki et al. 2000). Elucidating the microbial community structure is a critical chapter towards understanding and improving AD process. The microbial population necessary for AD processes can be recognised and appropriately seeded during the start-up of full-scale digester (Huang et al. 2014). The impact of operating and the resulting environmental conditions on the microbial communities, their activities and overall process performance can hence be input towards process modelling or improvement (Angelidaki et al. 2000; Karakashev et al. 2006). Bioaugmentation opportunity can also be explored to aid process stability (Westerholm et al. 2012). Also, sensitive metabolic channels can be exploited to function as predictive tools to process failure (Williams et al. 2013).

2.5.1. Microbiological analysis

Early microbiological studies involved microscopic observation, microbial enumeration, physiological and biochemical property characterization by cultivation methods (Toerien and Hattingh 1969). However, microscopic observations alone were not sufficient to differentiate the wide diversity of microorganisms present. In addition, ideal growth requirement of all microorganisms were not well understood and limited the application of cultivation dependent method. It was estimated that only 1% of all microorganisms can be cultivated by standard methods (Amann et al. 1995).

Recent advances in the development of molecular techniques allow the adoption of rapid cultivation independent methods through phylogenetic study.

Phylogenetic study is defined as the study of evolutionary relationship of organisms in an environment (Woese 1987). The use of 16s ribosomal RNA (rRNA) gene as a genetic marker for taxonomic identification has become the preferred approach in recent years (Stackebrandt and Goebel 1994; Tringe and Hugenholtz 2008). This gene encodes for the small subunit ribosomal RNA essential for the translation of nucleic acid information into functional protein. This genetic marker is highly conserved, yet contain regions of hypervariability (Muyzer et al. 1993) (Stackebrandt and Goebel 1994). Similarity within these hypervariable regions allowed researchers to define the evolutionary relationship, and hence the taxonomy of a particular bacterium. It has to be appreciated that 16s rRNA gene has been consistently used to define taxonomic classification in Bergey's Manual of Systematic Bacteriology; and allowed the classification of prokaryotic organisms into two different domains (*Eubacteria* and *Archaeobacteria*) (Woese 1987). Comparison of the sequenced 16S rRNA fragment with publicly available database (e.g. GenBank and Ribosomal Database Project (RDP)) provides the taxonomic identification for phylogenetic study (Nelson et al. 2011). Evolutionary relationships may vary per taxonomic groups, but generally, two 16s rRNA sequences of 97% similarity or above are considered of the same species (Stackebrandt and Goebel 1994), whereas 95% similarity or above are of the same genus (Ludwig et al. 1998).

Various molecular methods have been developed which targeted 16S rRNA genes in microbial characterization studies (Nocker et al. 2007; Talbot et al. 2008). There are methods which did not involve DNA amplification by polymerase chain reaction (PCR) and also those that did not, for instance dot blotting and fluorescence in-situ hybridization (FISH) (Wagner et al. 2006). These two methods involved hybridization of fluorescent probes with target 16S rRNA sequence of the sample which was subsequently viewed under fluorescent light or fluorescent microscope. Methods that involve PCR amplification carry an inherent amplification bias. However, methods that do not involve PCR amplification cannot be used when the microbial community structure is unknown. This is because

these methods utilized specific probes to target well-defined microbial populations and may not be suitable for global-scale characterization studies.

Methods involving PCR amplification generally aimed to differentiate individual amplified DNA before DNA sequencing for phylogenetic identification. Such methods include the construction of clone library (Sekiguchi et al. 1998), denaturing or temperature gradient gel electrophoresis (D/TGGE), amplified ribosomal DNA restriction analysis (ARDRA) or restriction fragment length polymorphism (RFLP) (McHugh et al. 2004), terminal restriction fragment length polymorphism (TRFLP) (Liu et al. 1997) or single strand conformation polymorphism (SSCP) (Leclerc et al. 2004). Detailed procedures of the above mentioned methods had been reviewed by Talbot, et al. (2008). Compared to the others, D/TGGE is one of the most popular methods investigate microbial community structures in many samples of different time points or operating conditions (Muyzer et al. 1993; Shin et al. 2010). It is simple to operate and microbial dynamics of certain population can be visually detected. On the other hand, the clone library construction method is time consuming and the others provided poor specificity and resolution of the microbial community structures (Nocker et al. 2007; Talbot et al. 2008).

The principles in D/TGGE analysis is as follows: as the PCR products progress through the electrophoretic gel, double-stranded DNA is separated into single-strand over the entire length except the GC-rich clamp, creating a “butterfly-shaped” molecule. This separation depends on the molecular weight of DNA (higher GC content has higher molecular weight) and the strength of denaturing agents (formamide and urea for DGGE, and higher temperature for TGGE). D/TGGE aims to create a partial denaturation where such structure with “butterfly shape” will migrate slower compared to its native form. Thus, separation of PCR products containing different GC content can be achieved and documented. The separated bands of PCR product are then extracted from the electrophoretic gel, purified and

sequenced for identification. However, recent advances in technology has brought about advanced high throughput DNA sequencing technology.

High throughput sequencing technology (e.g. 454 pyrosequencing, Illumina MiSeq or Ion Torrent) and its decreasing cost have pushed their application into the field of environmental engineering (Loman et al. 2012). The main benefit is due to its ability to conduct massively parallel sequencing effort at one time while the traditional Sanger sequencing could only process one DNA sequence at a time. Even with an automated system, Sanger sequencing can only produce 192 sequences while pyrosequencing 400,000 sequences, making it less laborious, faster and cheaper (Hugenholtz and Tyson 2008). One drawback to the high throughput sequencing technology is the shorter read length compared to Sanger sequencing. Nevertheless, 454 pyrosequencing is capable of generating around 400 – 500 bp of sequence, reported to be sufficient for genus classification using the RDP database (MacLean et al. 2009; Claesson et al. 2010). As a comparison, Ion Torrent and Illumina MiSeq yielded only about 100 and 150 bp, respectively (Loman et al. 2012). Therefore, a more comprehensive microbial assessment of the sample by 454 pyrosequencing can be obtained at higher sequencing reads, faster and possibly lower cost compared to using DGGE. In an amplicon 454 pyrosequencing, sample DNA is initially attached with genetic marker-specific primers (e.g. 16s rRNA gene) and adaptor primers. This adaptor primer connects the sample DNA to streptavidin beads. One bead will only carry 1 specific DNA template which is amplified by emulsion PCR. The beads carrying amplified DNA is then transferred to a picotiter plate where many beads, each carrying clonal DNA can be sequenced in parallel. The bases are detected by sequencing-by-synthesis technique, measuring the release of inorganic pyrophosphate by chemiluminescence (Morozova and Marra 2008). This high throughput method also improves the workflow for microbial genomic study. Whole genome construction of an important microorganism (Iacono et al. 2008) and metagenomics study (Jaenicke et al. 2011) had already been attempted with success using 454 pyrosequencing.

Figure 2.4. Workflow for DGGE (A), amplicon 454 pyrosequencing (B) and qPCR (C) analyses.

End-point detection PCR which has been previously discussed, only gives qualitative data. In contrast, real-time PCR (rtPCR) or quantitative PCR (qPCR) provides quantitative data. It is based on real-time measurement of fluorescence intensity, which increases proportionally to the amount of DNA amplicons during amplification (Kim et al. 2013). SYBR Green I and TaqMan chemistry are the two assays commonly used in qPCR. SYBR Green I dye binds non-specifically to the minor groove of double stranded DNA and emit fluorescence with 1000 fold greater intensity than when unbound (Wittwer et al. 1997). Due to the nature of its non-specific binding, the amplification products should be checked by melting curve analysis to visualize unwanted DNA amplicon based on its melting temperature. TaqMan system is more specific compared to SYBR Green I system that it required an additional oligonucleotide (a TaqMan probe) between the forward and reverse primers, possessing a reporter dye at the 5' end and a

quencher dye at the 3' prime end (Yu et al. 2005). An intact TaqMan probe's reporter fluorescence will be absorbed by the quencher due its close proximity. The measurement of the fluorescence occurred when the reporter and quencher of a TaqMan probe is degraded and excised by DNA polymerase activity during extension. Hence real-time monitoring of the fluorescence indicates the amount of amplicons being generated.

Figure 2.5. Amplification curve of qPCR plotting fluorescence intensity (y-axis) against cycle number (x-axis) (Ahmed 2005) with permission from International Institute of Anticancer Research.

Theoretically, a sample with higher number of template DNA achieved saturation with fewer cycles than a sample with fewer template DNA (Kim et al. 2013). Therefore, a threshold cycle (C_t) value can be estimated which indicated the number of cycles required to exceed a chosen threshold within the exponential phase of amplification (**Figure 2.5**). This C_t value can then be used to construct a standard curve against a known DNA concentration for quantification. Concentrations of the standard DNA concentration can then be converted into copy number concentration using the following equation (Kim et al. 2013):

$$\text{DNA (copy mL}^{-1}\text{)} = \frac{6.02 \times 10^{23} \text{ (copy mol}^{-1}\text{)} \times \text{DNA concentration (g mL}^{-1}\text{)}}{\text{DNA length (bp)} \times 660 \text{ (g mol}^{-1} \text{ bp}^{-1}\text{)}} \quad (2.1)$$

where Avogadro's number is 6.02×10^{23} copy ml⁻¹ and the average molecular weight of one DNA base pair is 660 g mol⁻¹ bp⁻¹.

2.5.2. Microbial diversity in anaerobic digestion

Considerable advances in our understanding of microbial ecology and processes in biological treatment processes, and the availability of advanced molecular tools, present opportunities to have a better understanding of the role of microorganisms and their function contributions to the AD process. **Table 2.5**, **Table 2.6** and **Table 2.7** summarize a list of various microorganisms and their functions according to protein, carbohydrate and lipid hydrolysis, acidogenesis, the acetogenesis and methanogenesis reactions in AD process. The tables do not contain a complete list of microorganism but present genera commonly found in anaerobic digesters. The degradation of an organic substance occurs as microorganisms aimed to derive energy (Thauer et al. 1977). This involves biological oxidation of organic compound by fermentative bacteria to produce ATP (cellular energy currency) through substrate level phosphorylation or oxidative phosphorylation (Thauer et al. 1977). Hence, hydrolytic microorganisms are acidogenic in nature. A bacterial species can also degrade different substrates. For instance, proteins are hydrolyzed into amino acids and oligosaccharides. So, some proteolytic microorganism would also be capable of degrading carbohydrate, e.g. *Clostridia* and *Bacillus* (**Table 2.5**).

Table 2.5. Some of the genera associated with hydrolysis and acidogenesis of proteins, carbohydrates, lipids and alcohols.

Substrate	Phylum	Genus	References
Protein	<i>Bacteroidetes</i>	<i>Bacteroides</i>	(Hazlewood et al. 1981)
		<i>Proteiniphilum</i>	(Chen and Dong 2005)
	<i>Firmicutes</i>	<i>Bacillus</i>	(Toerien 1967)
		<i>Caloramator</i>	(Tarlera et al. 1997)
		<i>Clostridium</i>	(Jain and Zeikus 1989)
		<i>Coprothermobacter</i>	(Etchebehere et al. 1998)
		<i>Sedimentibacter</i>	(Nelson et al. 2011)
	<i>Synergistetes</i>	<i>Aminobacterium</i>	(Baena et al. 1998)
		<i>Anaerobaculum</i>	(Menes and Muxí 2002)
		<i>Cloacibacillus</i>	(Ganesan et al. 2008)
		<i>Synergistes</i>	(Kumar et al. 2008)
Carbohydrate	<i>Actinobacteria</i>	<i>Propionibacterium</i>	(Riedel and Britz 1993)
		<i>Bifidobacterium</i>	(Dong et al. 2000)
	<i>Bacteroidetes</i>	<i>Bacteroides</i>	(O'Sullivan et al. 2005)
		<i>Paludibacter</i>	(Ueki et al. 2006)
		<i>Prolixibacter</i>	(Holmes et al. 2007)
		<i>Parabacteroides</i>	(Tan et al. 2012)
	<i>Chloroflexi</i>	<i>Anaerolina</i>	(Sekiguchi et al. 2003)
		<i>Caldilinea</i>	(Sekiguchi et al. 2003)
	<i>Firmicutes</i>	<i>Acetivibrio</i>	(Khan 1980)
		<i>Bacillus</i>	(Combet-Blanc et al. 1995)
		<i>Butyrivibrio</i>	(Sewell et al. 1988)
		<i>Clostridium</i>	(Yang et al. 1990)
		<i>Lactobacillus</i>	(Shin et al. 2010)
		<i>Lactococcus</i>	(Tsushima et al. 2010)
		<i>Ruminococcus</i>	(Miller et al. 2000)
		<i>Saccharofermentans</i>	(Chen et al. 2010)
	<i>Proteobacteria</i>	<i>Brachymonas</i>	(Hiraishi et al. 1995)
	<i>Synergistetes</i>	<i>Anaerobaculum</i>	(Menes and Muxí 2002)
Lipids	<i>Firmicutes</i>	<i>Clostridium</i>	(Cirne et al. 2006)
		<i>Thermosyntropho</i>	(Svetlitsnyi et al. 1996)
	<i>Proteobacteria</i>	<i>Pseudomonas</i>	(Ramani et al. 2010)
Alcohol	<i>Firmicutes</i>	<i>Acetobacterium</i>	(Eichler and Schink 1984)
		<i>Saccharofermentans</i>	(Chen et al. 2010)
		<i>Tepidanaerobacter</i>	(Sekiguchi et al. 2006)
	<i>Proteobacteria</i>	<i>Desulfovibrio</i>	(Bryant et al. 1977)
		<i>Pelobacter</i>	(Schink 1984)
	<i>Thermotogae</i>	<i>Thermotogoa</i>	(Balk et al. 2002)

Table 2.6. Some of the genera associated with acetogenesis from carbon dioxide, propionic, butyric and long chain fatty acids.

Substrate	Phylum	Genus	References
Carbon dioxide	Firmicutes	<i>Acetobacterium</i>	(Balch et al. 1977)
		<i>Clostridium</i>	(Braun et al. 1981)
	Thermotogae	<i>Thermotoga</i>	(Balk et al. 2002)
Propionic acid	Firmicutes	<i>Desulfotomaculum</i>	(Plugge et al. 2002)
		<i>Pelotomaculum</i>	(de Bok et al. 2005)
	Proteobacteria	<i>Desulfovibrio</i>	(Roy et al. 1986)
		<i>Smithella</i>	(Liu et al. 1999)
		<i>Syntrophobacter</i>	(Harmsen et al. 1998)
Butyric acid	Firmicutes	<i>Syntrophomonas</i>	(McInerney et al. 1981)
		<i>Syntrophothermus</i>	(Sekiguchi et al. 2000)
		<i>Thermosyntropha</i>	(Svetlitsnyi et al. 1996)
	Proteobacteria	<i>Syntrophus</i>	(Jackson et al. 1999)
LCFA	Firmicutes	<i>Syntrophomonas</i>	(Roy et al. 1986)
	Proteobacteria	<i>Syntrophus</i>	(Jackson et al. 1999)

Table 2.7. Some of the genera associated with methanogenesis.

Substrate	Family	Genus	References
Acetic acid	<i>Methanosaetaceae</i>	<i>Methanosaeta</i> (<i>Methanothrix</i>)	(Patel and Sprott 1990)
Acetic acid, formic acid, CO ₂ methyl-amines, alcohol	<i>Methanosarcinaceae</i>	<i>Methanomethylovorans</i>	(Jiang et al. 2005)
		<i>Methanosarcina</i>	(Kenealy and Zeikus 1982)
Formic acid, CO ₂ , methyl-amines	<i>Methanobacteriaceae</i>	<i>Methanosphaera</i>	(Bouallagui et al. 2004)
	<i>Methanococcaceae</i>	<i>Methanococcus</i>	(Touzel and Albagnac 1983)
	<i>Methanobacteriaceae</i>	<i>Methanobacterium</i>	(Henson and Smith 1985)
Formic acid, CO ₂ alcohol	<i>Methanomicrobiaceae</i>	<i>Methanogenium</i>	(Zellner et al. 1990)
	<i>Methanospirillaceae</i>	<i>Methanospirillum</i>	(Patel et al. 1976)
Formic acid, CO ₂	<i>Methanomicrobiaceae</i>	<i>Methanoculleus</i>	(Zellner et al. 1998)
		<i>Methanolinea</i>	(Imachi et al. 2008)
		<i>Methanosphaerula</i>	(Cadillo-Quiroz et al. 2009)
	<i>Methanobacteriaceae</i>	<i>Methanobrevibacter</i>	(Savant et al. 2002)
		<i>Methanothermobacter</i>	(Hori et al. 2006)
	Unclassified	<i>Thermogymnomonas</i>	(Xia et al. 2012)
	Thermoplasmatales		
	Unclassified subphylum	WSA2 group	(Chouari et al. 2005)
	Phylum Crenarchaeota	Class <i>Thermoprotei</i>	(Nelson et al. 2011)

A meta-analysis to examine all publicly available 16S rRNA genes recovered from anaerobic digesters was reported for data up to May 2010 by Nelson et al. (2011) with a total of 16,519 bacterial and 2,869 archaeal sequences. Rarefaction curve analysis showed that bacterial sampling reached saturation at phylum level and it was at family level for archaeal sampling. This shows that *Bacteria* were only well characterized up to phylum level, while it was at family level for *Archaea*. There is still much to be discovered in regards to the microbial diversity in anaerobic digesters.

2.5.3. Linking microbial community to process performance

Large-scale microbial community analysis had been performed in full-scale digesters to obtain a global overview of the microbial diversity. The last step in AD, methanogenesis is known to be performed only by microorganisms from the domain *Archaea* (Nelson et al. 2011). A large-scale archaeal community analysis on 44 digesters from eight different countries treating different substrates found aceticlastic *Methanosaeta* and hydrogenotrophic *Methanobacterium* to be the most frequently identified genera (Leclerc et al. 2004), and that process configurations influenced the diversity of methanogens. A study investigating 6 different digesters treating different substrates (McHugh et al. 2003) and another investigating 9 full-scale digesters treating only sewage sludge (Rivière et al. 2009) also found *Methanosaeta* to be the predominant methanogen. This is in agreement with previous estimation that AM contributed to around two-thirds of methane production in anaerobic process (Speece 1996; Garcia et al. 2000). However, different observations were found in full-scale sewage sludge digester study by Chouari et al. (2005) and Kim et al. (2013). HM by a novel lineage, the WSA2 group, and *Methanomicrobiales* dominated the archaeal community, respectively. The difference in environmental conditions might have led the variation of predominant methanogens. Karakashev et al. (2005) characterized the methanogenic communities in 15 full-scale anaerobic digesters and found a correlation between ammonia, VFA concentrations and the predominant methanogen. *Methanosarcina* dominated when ammonia and VFA concentrations

were high and *Methanosaeta* dominated when ammonia and VFA concentrations were low (Karakashev et al. 2005). In addition, high ammonia, VFA concentrations (Lü et al. 2013), temperature (Hao et al. 2010) and low dilution rate (Shigematsu et al. 2004) were found to enhance the syntrophic acetic acid oxidation with hydrogenotrophic methanogenesis (SAO-HM) pathway conducted by the *Methanomicrobiales* order, *Methanothermobacter*, *Methanobacterium* or *Methanoculleus*. This preference could be attributed to the tolerance of SAO and HM microorganisms to such environmental stresses (Angelidaki and Ahring 1993; Westerholm et al. 2012).

Hydrolytic, acidogenic and acetogenic reactions are typically performed by microorganisms from the domain *Bacteria* (Nelson et al. 2011). Large-scale study by Rivire et al. (2009) attempted to define a core group of AD bacterial community and found *Chloroflexi*, *Betaproteobacteria*, *Bacteroidetes* and *Synergistetes* to be predominant in most digesters. 454 pyrosequencing bacterial analysis of 7 full-scale digesters revealed *Proteobacteria*, *Bacteroidetes*, *Firmicutes* and *Chloroflexi* to be the most abundant phyla (Lee et al. 2012). These were consistent with the meta-analysis investigation conducted by Nelson et al. (2011). The bacterial phyla most frequently identified in the meta-analysis were *Proteobacteria*, *Firmicutes*, *Bacteroidetes* and *Chloroflexi*. There were also 24 minor bacterial phyla, which might have played a significant role in the AD process, such as *Synergistetes*, *Planctomycetes* and *Actinobacteria*. Matching the bacterial phyla with literature data revealed that *Deltaproteobacteria* consisted of syntrophic acetogenic microorganisms (**Table 2.6**) (Nelson et al. 2011; Stams et al. 2012). *Bacteroidetes* and *Firmicutes* were associated with hydrolysis and acidogenesis of proteins, carbohydrates and lipids (Vos et al. 2009; Krieg et al. 2010). In fact, *Bacteroidetes* and *Firmicutes* had been found in the presence of higher hydrolytic activities to break down organic particulates (Walker et al. 2008; Regueiro et al. 2012). Meanwhile, *Chloroflexi* was mainly associated with hydrolysis and acidogenesis of carbohydrates (Ariesyady et al. 2007). This is especially important as sludge hydrolysis is the rate limiting step in the anaerobic

sludge digestion process. Regueiro et al. (2012) also found that hydrolytic and methanogenic activities depended on the microbial community structure but not so significantly for acidogenic activities. This implied that a strict assemblage of microbial communities for hydrolysis and methanogenesis steps might be required for optimum digestion performance, while acidogenesis is more versatile and does not require an equivalent strict requirement of microbial community structure.

The role of microbial community in the operation of conventional single-stage digester had also been much evaluated. The characteristics of influent substrate can influence the microbial diversity and ultimately, the digestion efficiency (Sundberg et al. 2013; Ziganshin et al. 2013). During start-up, the microbial composition of the inoculum and the shift to acclimated community were linked to the process performance (Huang et al. 2014; De Vrieze et al. 2015). Higher levels of methanogenic populations were found to be started successfully than a digester with lower level of methanogens (McMahon et al. 2004). Several literatures had also described the archaeal and bacterial community shifts during the start-up of an anaerobic digester (Liu et al. 2002; McMahon et al. 2004; Kobayashi et al. 2009; Shin et al. 2010).

Historically, a microbial community structure is thought to be shaped by deterministic factors such as interspecies interactions (competition, syntrophy, predation, etc.) and niche differentiation, hence the community structure will converge to a single pattern (Chave 2004). This is called the niche based theory. However, this theory cannot completely explain the presence of rare taxon in a very diverse environment. Another alternative theory, the neutral theory, disregards differences between species at the same trophic level and that similar environmental conditions can give rise to many natural patterns by stochastic factors, such as birth, death, dispersal. Recent studies suggested that the anaerobic process is governed by neutral theory. Fernández et al. (1999) examined temporal microbial dynamics of methanogenic reactor fed with glucose over 605 days. That study found microbial successions of *Methanobacterium*, *Spirochaetes*

domination in the first period and *Methanosarcina*, *Methanobacterium*, with sequential replacement of *Eubacterium*, *Propionibacterium* and unknown genus domination in the second period. This microbial succession did not result in functional variation and ultimately, stable process performance was still observed. Stochastic assembly of community structure was also observed by Zhou et al. (2013) when 14 identical replicates of microbial electrolysis cell system gave rise to different microbial community structure with different ecosystem function and different process performance. However, such extreme stochastic variation might apply to a community which was introduced to a novel environment, whereas an inoculum which had been preconditioned to the new habitat would be more predictable (Pagaling et al. 2014). Microbial monitoring of anaerobic digester treating cattle manure with 3 identical digester replicates revealed that stochasticity had minor role in the microbial community assembly and that the microbial community structure was reproducible and converged after a temperature disturbance (Luo et al. 2015). A study consisting of 3 replicate digesters treating α -cellulose by Vanwonterghem et al. (2014) also supported deterministic rather than stochastic factor in microbial dynamics under controlled condition. Nevertheless, perturbations (e.g. temperature, pH, organic loading) to the process operation strongly influence the microbial community assembly and dynamics in anaerobic digesters (Karakashev et al. 2005; Huang et al. 2014; Jang et al. 2014; Li et al. 2015; Luo et al. 2015). Often times, the difficulty in fully comprehending microbial community structure is due to the resolution of microbial fingerprinting technique, sampling frequency of the reactor and the spatial consideration during sampling, which would consume a lot of time and resources (Wagner et al. 2006; Cabrol et al. 2012). High throughput sequencing shows promises to revolutionize microbial community characterization studies to overcome these limitations, compared to the earlier generation of molecular tools (involving PCR, Sanger sequencing and DNA fingerprinting) (Werner et al. 2011; Sundberg et al. 2013; Zhou et al. 2013; Luo et al. 2015).

On the contrary, microbial characterization studies have rarely been performed under sludge digestion enhancement conditions. The nature of substrate, environmental conditions under sludge digestion enhancement conditions are expected to produce different microbial community structures compared to a conventional single-stage system. Despite improved digestion performance, thermophilic digester was found to have lower diversity compared to its mesophilic counterpart (Sekiguchi et al. 1998). Sludge digestion in TPAD system with phase-1 at 50, 60 and 65°C showed enrichment of *Thermotogae* to *Lutispora* and *Coprothermobacter*, and microbial diversity decreased as temperature and hydrolysis efficiency increased (Pervin et al. 2013). Here, the community shift can be attributed to the operating temperature of phase-1 in TPAD system whereas the community structure in a mesophilic two-phase system was attributed to the populations in the influent feed sludge (Pervin et al. 2013). A digester with low diversity might be disadvantageous as lower diversity implied lower functional redundancy in the digester to maintain process stability during perturbations (Merlino et al. 2013). There had been several others investigating the microbial community in mesophilic two-phase anaerobic system. Zhang et al. (1991) and Raskin et al. (1995) reported that the microbial community in a single-stage system was not simply the sum of populations found in the acidogenic and methanogenic phases of the two-phase system. Instead, different populations were observed between the two systems (Shimada et al. 2011). In addition, sludge pretreatment strategies by electric focused-pulse and microaeration had been found to increase microbial diversity and were associated with the improved digestion performance (Zhang et al. 2009; Lim et al. 2013). Microbial variations had also been observed between conventional single-stage digester and digester fed with ozone and thermal-alkaline pretreated sludges (Yan et al. 2009; Kim et al. 2013). It could be seen that different sludge digestion enhancement methods affected microbial communities differently, but the roles, interactions and significances of the bacterial and archaeal communities under sludge enhancement methods had not been clearly understood.

2.6. Concluding remarks

Increasing global concerns over the impacts of climate change, limited fossil fuel supplies and land space encourage the optimization of sludge digestion enhancement strategy by innovative biological method to minimize operating cost/resources, while leveraging on improved digestion performance. Reactor performance of the two-phase anaerobic digestion process, which optimizes the biological activities of the hydrolytic/acidogenic and methanogenic microorganisms, has been studied in depth but still can be expanded by optimization of the operating pH, temperature and SRT. The literature also indicated that two-stage is better than two-phase AD system, and needs to be further evaluated. Complementing such biological method of digestion enhancement with anaerobic co-digestion of sludge with FOG is rarely explored and also provides an avenue towards reducing reliance on fossil fuel to power WWTP's operations. As anaerobic sludge digestion depends on the concerted effort of a variety of microorganisms, understanding the microbial community structure and its functions become critical. Despite the wealth of information on microbial diversity in AD process, the link between microbial communities, interaction and functions remain complex and poorly understood, especially under sludge digestion enhancement conditions. Fundamentally, there are still conflicting views whether deterministic or stochastic factors strongly shape a microbial community structure. The use of high throughput sequencing, such as 454 pyrosequencing provides the opportunity to elucidate the microbial community structure with greater efficiency than before.

Chapter 3

Comparison of Single-Stage and Two-Phase Anaerobic Sludge Digestion Systems - Performance and Microbial Community Dynamics

3.1. Introduction

Sludge is an inevitable by-product in biological wastewater treatment plants, employing the conventional aerobic activated sludge process or other bioprocesses. Anaerobic digestion (AD) has commonly been applied to reduce the solids content, the pathogenic and vector attraction potential, while recovering renewable source of energy in the form of methane. AD involves a series of steps, namely hydrolysis, acidogenesis, acetogenesis and methanogenesis, performed by wide range of microorganisms (Angelidaki et al. 2000). Optimization of the AD process to achieve more efficient sludge stabilization by biological enhancement method led to the use of phased anaerobic system (Ghosh et al. 1995). Physical separation of the AD process into its acidogenic and methanogenic phases can help alleviate "souring" problem associated with the mismatched specific activities of the fast-growing acidogens and slower-growing methanogens (Ghosh et al. 1995). In addition, the accumulation of organic acids with concurrent drop in pH value could help release non-crystalline polymers from macromolecule, and accelerate hydrolysis (Xiao and Clarkson 1997; Chen et al. 2007). Process improvements in terms of solids and pathogen reduction, biogas production and foaming alleviation, compared to the conventional single-stage system have been reported previously (Ghosh et al. 1995; Bhattacharya et al. 1996).

Understanding the complex interactions of microorganisms involved in AD process is of interest for better process control and optimization. Previous studies had focused on elucidating the microbial populations in single-stage anaerobic sludge digestion process (Raskin et al. 1995; Rivire et al. 2009; Shin et al. 2010), but little

was understood in two-phase configuration (Zhang and Noike 1991; Shin et al. 2010).

The potential to shorten solids retention time (SRT) and increase organic loading rate (OLR) at the same time, while still maintaining sludge digestion performance via two-phase system is welcomed. This would allow reduction of reactor size and hence, the footprint of the treatment plant. Microbial characterization of two-phase microbial community treating sewage sludge had been performed using clone library technique (Shimada et al. 2011); but, the microbial dynamics under decreasing the system SRT is unknown.

Therefore, the purpose of this study is to evaluate the performance of two anaerobic sludge digestion systems - single-stage and two-phase configurations, operated at decreasing SRT. The consequent microbial community dynamics would then be correlated with the process parameters.

3.2. Materials & Methods

3.2.1. Reactor operation

In this study, two anaerobic systems, i.e. the single-stage and two-phase systems were operated in parallel (**Figure 3.1**). The single-stage system was a single 50 L working volume digester vessel. The two-phase system had a 7.5 L acidogenic reactor (phase 1) preceding a 42.5 L methanogenic reactor (phase 2). The reactor operating conditions are summarized in **Table 3.2**. The working volumes of both systems were adjusted as SRT was reduced from 30 (5 + 25) to 20 (3 + 17) days, and finally to 12 (2 + 10) days. As the SRT was reduced, the OLR increased from 1.5 to 2.2 and 3.5 g TCOD L⁻¹ d⁻¹, respectively. The reactors were operated as continuous stirred tank reactor (CSTR), so SRT = hydraulic retention time (HRT). Temperature of all reactors was controlled at 35 °C. pH of the acidogenic reactor was optimized at pH 5.5 ± 0.2 (Elefsiniotis and Oldham 1994; Ghosh et al. 1995), while the single-stage and methanogenic reactors were controlled at pH 7.0 ± 0.2.

pH was maintained by automated dosing of 1 M hydrochloric acid or 1 M sodium hydroxide, if required. Sewage feed sludge was a mixture of municipal primary and secondary sludge collected from Ulu Pandan municipal water reclamation plant in Singapore. All reactors were seeded with anaerobic sludge collected from the same plant, treating the same mixed sewage sludge. The feed sludge's characteristics were $42,300 \pm 3,600 \text{ mg TCOD L}^{-1}$; $2,500 \pm 1,000 \text{ mg SCOD L}^{-1}$; $32.1 \pm 2.6 \text{ g TS L}^{-1}$; $25.7 \pm 2.0 \text{ g VS L}^{-1}$; and $\text{pH } 5.9 \pm 0.2$. Feed sludge was collected weekly and stored at 4°C before use.

Figure 3.1. Schematic diagrams of the single-stage (A) and two-phase (B) anaerobic sludge digestion systems.

3.2.2. Analytical methods

Samples were collected twice weekly from all reactors. Chemical oxygen demand (COD), total solids (TS), total suspended solids (TSS), volatile solids (VS), volatile suspended solids (VSS) were measured in accordance with the Standard Methods (APHA 2005). Soluble fraction was prepared by centrifuging sludge sample at 13,000 xg for 5 minutes before filtering through a 0.45 µm nylon membrane. Filtration through 0.2 µm nylon membrane was applied for the preparation of VFA samples. 0.9 mL of VFA samples were acidified with 0.1 mL of 10% formic acid in before immediate analysis. Volatile fatty acids (VFA) and biogas compositions were measured using gas chromatography (GC) (7890A, Agilent, USA) as described by Huang et al. (2014). Biogas volume was measured with a thermal-based gas flowmeter (McMillan, USA), fitted on the reactor set. Non-soluble COD was calculated by subtracting total COD with soluble COD. Non-soluble COD signifies organic substances that cannot be filtered through the 0.45 µm filter, such as particulate organics. Student's t-test was calculated in Microsoft Excel (Analysis ToolPak), as statistical analysis of the TCOD removal, VS reduction and methane production between the two reactor configurations ($p < 0.05$ was considered as statistically significant).

The extent of solubilization was calculated as described by Ge et al. (2011), which was expressed as

$$\text{Extent of sludge solubilization \%} = \frac{\text{COD}_{\text{CH}_4} + \text{COD}_{\text{So}} - \text{COD}_{\text{Si}}}{\text{COD}_{\text{Ti}} - \text{COD}_{\text{Si}}} \times 100\% \quad (3.1)$$

where COD_{CH_4} = methane produced per day as mg COD, based on 0.38225 L $\text{CH}_4 \cdot \text{g COD}^{-1}$ at 25°C (temperature during biogas volume measurement); COD_{Si} = soluble COD concentration of inlet; COD_{So} = soluble COD concentration of outlet; COD_{Ti} = total COD concentration of inlet.

TCOD removal and VS reduction were calculated based on mass balance, which is expressed as:

$$\text{Reduction \%} = \frac{C_{in} - C_{out}}{C_{in}} \times 100\% \quad (3.2)$$

where C_{in} = Concentration of inlet; C_{out} = Concentration of outlet.

3.2.3. DNA extraction and storage

To ensure maximum deoxyribonucleic acid (DNA) extraction efficiency, the sludge sample was initially diluted with phosphate buffered saline (PBS) solution, pH 7.5 to TS concentration below 2 g L⁻¹. 1 mL duplicates of the sample were then washed twice with 1X PBS solution by centrifugation (20,000 xg for 2 minutes) and resuspended with the same volume. Genomic DNA was subsequently extracted using an automated DNA extraction kit (MagNA Pure, Roche Diagnostic GmbH, Germany) following the manufacturer's recommended protocol and then stored at -20 °C.

3.2.4. DGGE and phylogenetic identification

Bacterial and archaeal community structures were profiled by targeting the 16S ribosomal ribonucleic acid (rRNA) gene. Genomic DNA was initially amplified using universal bacterial and archaeal primers with GC clamps, as listed in **Table 3.1** (Shin et al. 2010). The PCR mixture was prepared using Taq-&GO™ Ready-to-use PCR Mix kit (MP Biomedicals, USA): 4 µL of Taq-&GO™ premix (5X), 1 µL of each 10 µM primer, 12 µL of PCR-grade water and 2 µL of genomic DNA. Touch-down PCR was done as described by Shin et al. (2010): (1) initial denaturation at 94°C for 10 minutes, (2) 20 cycles of 94°C denaturation for 30 seconds, 65-55°C annealing (reducing temperature by 0.5°C per cycle) for 30 seconds, 72°C elongation for 1 minute, (3) additional 15 cycles of 94°C denaturation for 30 seconds, 55°C annealing for 30 seconds, 72°C elongation for 1 minute, and (4) final elongation at 72°C for 7 minutes. Denaturing gradient gel electrophoresis (DGGE) was performed using DCode system (Bio-Rad, USA) with 8% (w/v) acrylamide, where

Table 3.1. List of primers and probes used in DGGE and qPCR.

Target group	Function	Primer / probe identification	Sequence	<i>E. coli</i> number target region
Universal <i>Bacteria</i> with GC clamp	Forward primer with GC clamp	GCBAC338F	CGCCC GCCGC GCGCG GCGGG CGGGG CGGGG GCACG GGGGG- ACTCC TACGG GAGGC AG	338 – 354
	Forward primer with GC clamp	GCBAC338F	CGCCC GCCGC GCCCC GCGCC CGTCC CGCCG CCCCC GCCCG- ATTAG ATACC CSBGT AGTCC	
Universal <i>Archaea</i> with GC clamp	Forward primer with GC clamp	GCARC787F	ATTAG ATACC CSBGT AGTCC	787 – 806
Universal <i>Bacteria</i>	Forward primer	BAC338F	ACTCC TACGG GAGGC AG	338 – 354
	Taqman probe	BAC516P	TGCCA GCAGC CGCGG TAATA C	516 – 536
	Reverse primer	BAC805R	GACTA CCAGG GTATC TAATC C	785 – 805
Universal <i>Archaea</i>	Forward primer	ARC787F	ATTAG ATACC CSBGT AGTCC	787 – 806
	Taqman probe	ARC915P	AGGAA TTGGC GGGGG AGCAC	915 – 934
	Reverse primer	ARC1059R	GCCAT GCACC WCCTC T	1044 – 1059
<i>Methanobacteriales</i>	Forward primer	MBT857F	CGWAG GGAAG CTGTT AAGT	857 – 875
	Taqman probe	MBT929P	AGCAC CACAA CGCGT GGA	929 – 946
	Reverse primer	MBT1196R	TACCG TCGTC CACTC CTT	1179 – 1196
<i>Methanomicrobiales</i>	Forward primer	MMB282F	ATCGR TACGG GTTGT GGG	282 – 299
	Taqman probe	MMB749P	TYCGA CAGTG AGGRA CGAAA GCTG	749 – 772
	Reverse primer	MMB812R	CACCT AACGC RCATH GTTTA C	812 – 832
<i>Methanosarcinaceae</i>	Forward primer	MSC380F	GAAAC CGYGA TAAGG GGA	380 – 397
	Taqman probe	MSC492P	TTAGC AAGGG CCGGG CAA	492 – 509
	Reverse primer	MSC811R	TAGCG ARCAT CGTTT ACG	811 – 828
<i>Methanosaetaceae</i>	Forward primer	MST702F	TAATC CTYGA RGGAC CACCA	702 – 721
	Taqman probe	MST753P	ACGGC AAGGG ACGAA AGCTA GG	753 – 774
	Reverse primer	MST846R	CCTAC GGCAC CRACM AC	846 – 862
Methanococcales	Forward primer	MCC495F	TAAGG GCTGG GCAAG T	495 – 510
	Taqman probe	MCC686P	TAGCG GTGRA ATGYG TTGAT CC	686 – 707
	Reverse primer	MCC813R	CACCT AGTYC GCARA GTTTA	813 – 832

the 100% denaturant contained 7 M urea and 40% (v/v) formamide. DGGE was run on 30-60% denaturing gradient to resolve the bacterial profiles, whereas another DGGE was run on 40-70% gradient to resolve the archaeal profiles. The gels were run at 85 V for 14 h in TAE buffer (1X). The gel was subsequently stained with ethidium bromide and documented using GelDoc™ XR+ system (BioRad, USA).

Bands of interest were cut and eluted in 50 µL of nuclease-free water overnight. 2 µL of eluted DNA solution was amplified using the same universal bacterial and archaeal primers without the GC clamps. PCR amplification was slightly modified where only 20 cycles were used and the annealing temperature was set at 55°C. PCR products were purified, before cloned into pGEM-T Easy vector (Promega, USA) and sequenced. Resulting sequences were compared against reference database in the GenBank and RDP. Neighbour-joining trees were then constructed with MEGA5 software using Jukes-Cantor algorithm and bootstrapped 1,000 times. The sequences were archived in the NCBI GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) with the accession KR869980 – KR870009.

3.2.5. qPCR

Quantitative polymerase chain reaction (qPCR) was performed using the primers/Taqman probe sets targeting 16S rRNA genes, specific for *Bacteria*, archaeal orders *Methanobacteriales*, *Methanomicrobiales*, and archaeal families *Methanosarcinaceae*, *Methanosaetaceae*, as listed in **Table 3.1** (Yu et al. 2005). PCR mixture was prepared using LightCycler480 TaqMan Probe Master kit (Roche Diagnostics GmbH, Germany): 10 µL of TaqMan Probe Master premix (2X), 1 µL of each 10 µM primer, 2 µL of 2 µM Taqman probe, 4 µL of PCR-grade water and 2 µL of genomic DNA. A two-step PCR amplification was performed using LightCycler 480 (Roche Diagnostics GmbH, Germany), as described by Yu, et al. (2005): (1) initial denaturation at 95°C for 10 minutes and (2) 45 cycles of 94°C denaturation for 10 seconds and 60°C simultaneous annealing and elongation for 30 seconds. Standard curves for the qPCR analysis were prepared as described by Yu et al. (2005) using standard plasmids listed in the **Appendix A**. The calibrated number of

DNA copies per gram VSS would give an estimated measure of quantitative abundance.

3.3. Results

3.3.1. Reactor performance

Both single-stage and two-phase systems were operated for 402 days at 3 different SRTs. **Table 3.2** summarizes the operating parameters and performances. Student's t-test was used to evaluate the VS reduction, TCOD removal and methane production between the single-stage and 2-phase systems and these were only significantly different at 12 days SRT ($p < 0.05$, $n = 16$). At 30 and 20 days SRT, the single-stage system achieved around 39% TCOD removal and 32% VS reduction, before they decreased to 30.8% and 26.3%, respectively, at 12 days SRT. On the other hand, the two-phase system's performances could be maintained at 40.7% - 42.3% TCOD removal and 31.6% - 35.5% VS reduction, even when the SRT was decreased. The average methane production gradually increased due to increasing OLR, ranging from 9.4 to 11.7 and 15.6 L d⁻¹ at 30, 20 and 12 days SRT in the single-stage system, respectively. On the contrary, average methane production in the two-phase system was similar at 30 and 20 days SRT (10.0 and 12.7 L d⁻¹, respectively); but was higher at 12 days SRT (20.6 L d⁻¹) compared to its single-stage counterpart.

The acidogenic reactor contributed 20% - 24% of all the methane generated in the two-phase system (**Table 3.2**). Despite methanogenesis being commonly known to proceed between pH 6.6 and 7.8, previous researchers have also reported methanogenic activity in acidic condition (down to pH 4 - 5.3) (Taconi et al. 2008). However, methanogenic contribution in a similar acidogenic reactor (pH 5.8) was only 7% (Ghosh et al. 1995) and negligible in another study (pH 4.3 to 6.2) treating primary sludge (Elefsiniotis and Oldham 1994). The biogas composition in the single-stage and methanogenic reactors was 64% - 67% methane, 30% - 34% carbon dioxide, while the acidogenic reactor was 48% - 49% methane and 48% -

Table 3.2. Summary of reactor operating parameters and performances in the single-stage and two-phase anaerobic systems (average $\pm$ standard deviation).

		Single-stage			Two-phase system		
SRT (days)		30	20	12	30	20	12
pH			7.0 $\pm$ 0.2			-	
OLR TCOD (g TCOD L ⁻¹ d ⁻¹)		1.5 $\pm$ 0.2	2.2 $\pm$ 0.2	3.5 $\pm$ 0.3	1.5 $\pm$ 0.2	2.1 $\pm$ 0.3	3.5 $\pm$ 0.3
OLR VS (g VS L ⁻¹ d ⁻¹)		0.9 $\pm$ 0.1	1.3 $\pm$ 0.1	2.1 $\pm$ 0.2	0.9 $\pm$ 0.1	1.3 $\pm$ 0.1	2.1 $\pm$ 0.2
Soluble COD (mg SCOD L ⁻¹)		490 $\pm$ 50	620 $\pm$ 100	460 $\pm$ 70			
Non-soluble COD (mg COD L ⁻¹)		27,300 $\pm$ 1,500	25,600 $\pm$ 2,200	28,500 $\pm$ 1,500			
TCOD removal (%)		38.6 $\pm$ 5.1	39.2 $\pm$ 6.5	30.8 $\pm$ 6.1	42.3 $\pm$ 5.6	40.9 $\pm$ 4.7	40.7 $\pm$ 5.7
VS reduction (%)		31.7 $\pm$ 6.4	32.2 $\pm$ 5.5	26.3 $\pm$ 6.1	31.8 $\pm$ 6.9	31.6 $\pm$ 4.8	35.5 $\pm$ 6.6
Total VFA (mg COD L ⁻¹)		26 $\pm$ 18	20 $\pm$ 12	18 $\pm$ 10			
Methane production (L d ⁻¹)		9.4 $\pm$ 1.2	11.7 $\pm$ 1.5	15.6 $\pm$ 2.0	10.0 $\pm$ 1.5	12.7 $\pm$ 0.9	20.6 $\pm$ 1.9
Methane yield (L g TCOD _{added} ⁻¹)		0.13 $\pm$ 0.04	0.12 $\pm$ 0.01	0.10 $\pm$ 0.01	0.16 $\pm$ 0.02	0.14 $\pm$ 0.02	0.14 $\pm$ 0.03
Methane yield (L g VS _{added} ⁻¹)		0.22 $\pm$ 0.08	0.2 $\pm$ 0.01	0.16 $\pm$ 0.01	0.27 $\pm$ 0.04	0.22 $\pm$ 0.01	0.22 $\pm$ 0.04
Biogas composition							
	CH ₄ (%)	66 $\pm$ 1	64 $\pm$ 1	65 $\pm$ 1			
	CO ₂ (%)	32 $\pm$ 1	34 $\pm$ 1	34 $\pm$ 1			
	N ₂ (%)	1 $\pm$ 1	2 $\pm$ 1	1 $\pm$ 1			
		Acidogenic phase			Methanogenic phase		
SRT (days)		5	3	2	25	17	10
pH			5.5 $\pm$ 0.2			7.0 $\pm$ 0.2	
OLR TCOD (g TCOD L ⁻¹ d ⁻¹)		9.2 $\pm$ 1.0	14.3 $\pm$ 1.6	20.9 $\pm$ 2.0	1.7 $\pm$ 0.2	2.2 $\pm$ 0.1	3.5 $\pm$ 0.2
OLR VS (g VS L ⁻¹ d ⁻¹)		5.3 $\pm$ 0.6	8.7 $\pm$ 0.7	12.8 $\pm$ 1.2	1.0 $\pm$ 0.08	1.3 $\pm$ 0.08	2.2 $\pm$ 0.2
Soluble COD (mg SCOD L ⁻¹)		4,800 $\pm$ 1,000	4,400 $\pm$ 700	2,900 $\pm$ 510	440 $\pm$ 50	580 $\pm$ 110	450 $\pm$ 70
Non-soluble COD (mg COD L ⁻¹)		37,600 $\pm$ 3,500	33,400 $\pm$ 2,000	32,200 $\pm$ 2,200	25,800 $\pm$ 1,100	24,600 $\pm$ 960	24,000 $\pm$ 1,400
TCOD removal (%)		7.5 $\pm$ 6.8	11.4 $\pm$ 6.0	15.4 $\pm$ 6.0	37.6 $\pm$ 5.4	33.3 $\pm$ 2.8	29.9 $\pm$ 4.7
VS reduction (%)		9.4 $\pm$ 6.7	12.9 $\pm$ 5.0	12.5 $\pm$ 3.7	24.7 $\pm$ 5.2	21.5 $\pm$ 4.0	26.3 $\pm$ 6.6
Total VFA (mg COD L ⁻¹)		2,706 $\pm$ 546	2,471 $\pm$ 258	1,667 $\pm$ 410	32 $\pm$ 24	44 $\pm$ 17	94 $\pm$ 62
Methane production (L d ⁻¹)		2.4 $\pm$ 0.4	3.1 $\pm$ 0.6	4.1 $\pm$ 0.4	7.8 $\pm$ 1.4	9.6 $\pm$ 0.8	16.5 $\pm$ 2.1
Methane yield (L g TCOD _{added} ⁻¹)		0.04 $\pm$ 0.01	0.03 $\pm$ 0.01	0.03 $\pm$ 0.002	0.13 $\pm$ 0.02	0.11 $\pm$ 0.01	0.13 $\pm$ 0.02
Methane yield (L g VS _{added} ⁻¹)		0.06 $\pm$ 0.02	0.05 $\pm$ 0.01	0.04 $\pm$ 0.002	0.22 $\pm$ 0.03	0.19 $\pm$ 0.02	0.20 $\pm$ 0.03
Biogas composition							
	CH ₄ (%)	48 $\pm$ 2	48 $\pm$ 2	49 $\pm$ 2	67 $\pm$ 1	67 $\pm$ 1	66 $\pm$ 3
	CO ₂ (%)	48 $\pm$ 3	50 $\pm$ 1	48 $\pm$ 2	30 $\pm$ 1	32 $\pm$ 1	30 $\pm$ 3
	N ₂ (%)	4 $\pm$ 2	2 $\pm$ 1	3 $\pm$ 2	2 $\pm$ 2	1 $\pm$ 1	3 $\pm$ 4

50% carbon dioxide. There was low hydrogen partial pressure found in all the anaerobic reactors ($< 0.01\%$). This indicated active hydrogenotrophic methanogenesis in the acidogenic reactor.

Figure 3.2 presents the extent of solubilization and its compositions in the acidogenic reactor at different SRTs. Decreasing the SRT from 5 to 2 days did not influence the extent of sludge solubilization, where 6 - 7% was achieved. However, it could be seen that methanogenesis increased as the TVFA contribution reduced. The other component of solubilized sludge might have been derived from substances such as proteins, carbohydrates or inert materials (Batstone et al. 2002).

Figure 3.2. The average extent of solubilization based on Eq. (3.1) and its compositions in the acidogenic reactor of the two-phase anaerobic system at 5, 3 and 2 days SRT.

Table 3.3 presents the distribution of VFA in the feed sludge and acidogenic reactor. The most abundant VFA species in the acidogenic reactor was propionic acid, followed by isovaleric, butyric and acetic acids. The acetic acid concentration in the acidogenic reactor was lower than in the feed sludge as most of the acetic acid was converted into methane. As indicated previously, the TVFA in the acidogenic reactor decreased as the SRT was reduced (**Table 3.3**). However, calculating the sum of the COD reduced and residual VFA in the acidogenic reactor resulted in values of 27.5, 28.4 and 36.6 g COD for the 5, 3 and 2 days SRTs, respectively. These values represented the amount of substrate COD converted into residual VFA and biogas in the acidogenic reactor. The total amount of substrate COD converted into VFA and biogas were at least maintained as SRT was decreased, and showed that acidogenesis was not adversely affected. This was also supported in **Figure 3.2**, where the contribution of methane and TVFA to sludge solubilization, at 4 - 5%, was not reduced at decreasing SRT.

Table 3.3. Concentrations of total VFA and its species in the feed sludge and acidogenic reactor at various SRTs (average $\pm$ standard deviation).

	Feed sludge	Acidogenic phase		
SRT (days)		5	3	2
TVFA (mg COD L ⁻¹)	1,806 $\pm$ 501	2,706 $\pm$ 546	2,471 $\pm$ 258	1,667 $\pm$ 410
Acetic acid (mg COD L ⁻¹)	707 $\pm$ 194	83 $\pm$ 36	117 $\pm$ 25	201 $\pm$ 77
Propionic acid (mg COD L ⁻¹)	513 $\pm$ 121	1,382 $\pm$ 207	1,330 $\pm$ 180	744 $\pm$ 256
Isobutyric acid (mg COD L ⁻¹)	78 $\pm$ 19	221 $\pm$ 69	181 $\pm$ 25	112 $\pm$ 29
Butyric acid (mg COD L ⁻¹)	200 $\pm$ 99	246 $\pm$ 142	147 $\pm$ 36	122 $\pm$ 32
Isovaleric acid (mg COD L ⁻¹)	155 $\pm$ 39	469 $\pm$ 102	404 $\pm$ 52	254 $\pm$ 68
Valeric acid (mg COD L ⁻¹)	117 $\pm$ 54	251 $\pm$ 93	195 $\pm$ 34	153 $\pm$ 38

3.3.2. Archaeal and bacterial community profiles

DGGE and subsequent phylogenetic identification of partial 16S rRNA genes were performed to reveal the microbial diversity and dynamics in the single-stage and two-phase anaerobic systems. Microbial characterizations of *Archaea* (**Figure 3.3A**) and *Bacteria* (**Figure 3.3B**) were conducted when the anaerobic processes were stable and had undergone at least 4 SRT cycles. A total of 30 bands from both

Figure 3.3. DGGE gel of archaeal (A) and bacterial communities (B) in the feed, seed sludge and anaerobic reactors at various SRTs.

DGGE gels with respective bacterial- and archaeal-specific amplicons were sequenced and the nearest taxonomic identification from the database were identified (**Table 3.4**). Phylogenetic trees were also constructed for the identified *Archaea* (**Figure 3.4**) and *Bacteria* (**Figure 3.5**).

Table 3.4. Archaeal and bacterial identification of the DGGE band sequences.

Band(s)	Closest related sequence	Order	% similarity
A1-5	<i>Methanospirillum hungatei</i> (NR_074177.1)	<i>Methanomicrobiales</i>	98-100
A6-10	<i>Methanolinea mesophila</i> (AB447467.1)	<i>Methanomicrobiales</i>	97-98
A11-12	<i>Methanoregula formicicum</i> (NR_102441.1)	<i>Methanomicrobiales</i>	97
A13-14	<i>Methanoculleus receptaculi</i> (NR_043961.1)	<i>Methanomicrobiales</i>	99
A15	<i>Methanosarcina barkeri</i> (NR_025303.1)	<i>Methanosarcinales</i>	99
B1-2	<i>Cloacibacterium normanense</i> (NR_042187.1)	<i>Flavobacteriales</i>	100
B3	<i>Enhydrobacter</i> sp. (JF792358.1)	<i>Pseudomonadales</i>	97
B4	Uncultured <i>Bacteroidetes</i> bacterium in sludge AD (CU919567.1)	<i>Flavobacteriales</i>	99
B5	Uncultured <i>Ruminococcaceae</i> bacterium from wetlands (JX505387.1)	<i>Clostridiales</i>	98
B6	Uncultured <i>Fluviicola</i> sp. from membrane bioreactor biofilm (GU257757.1)	<i>Flavobacteriales</i>	99
B7	<i>Smithella propionica</i> (NR_024989.1)	<i>Syntrophobacterales</i>	96
B8	Uncultured bacterium in AD treating microcystis bloom (GU559846.1)	<i>Clostridiales</i>	89
B9	Uncultured bacterium in AD treating coking wastewater (JQ446286.1)	<i>Flavobacteriales</i>	99
B10	<i>Saccharofermentans acetigenes</i> (AY949857.1)	<i>Clostridiales</i>	98
B11	<i>Clostridiaceae</i> bacterium in AD treating cattle waste (AB298771.2)	<i>Clostridiales</i>	99
B12	Uncultured bacterium from Evry sludge digester (CT574084.1)	<i>Flavobacteriales</i>	97
B13	Uncultured <i>Firmicutes</i> bacterium in full-scale AD treating sludge (CU925891.1)	<i>Clostridiales</i>	99
B14	Uncultured <i>Bacteroidetes</i> in full-scale AD treating sludge (CU925607.1)	<i>Flavobacteriales</i>	99
B15	Uncultured <i>Clostridiales</i> bacterium treating swine manure (JN173177.1)	<i>Clostridiales</i>	97

Figure 3.3A shows that the archaeal communities of different reactors were similar in all samples tested, irrespective of the SRT. Two methanogenic orders were identified by the phylogenetic analysis, namely *Methanomicrobiales* and *Methanosarcinales* orders (**Table 3.4**). The bands A1 to A11 appeared in the feed, seed sludge and the 3 anaerobic reactors on all SRTs tested. These bands were found to be closely related to *Methanospirillum hungatei*, *Methanolinea mesophila*

and *Methanoregula formicicum*. These species were of *Methanomicrobiales* order and utilized hydrogen or formic acid as electron acceptor to produce methane (Demirel and Scherer 2008). A13 and A14 appeared in the single-stage and methanogenic reactors when the system SRT was reduced to 20 and 12 days. A13 and A14 were closely related to *Methanoculleus receptaculi*, another species of *Methanomicrobiales* order. A15 was affiliated to *Methanosarcina barkei* which seemed to decrease in intensity over reducing SRTs in the methanogenic reactor. *Methanosarcina* tend to be more flexible and can utilise a wider range of substrates, such as acetic acid, hydrogen and carbon dioxide, methanol and methylamines (Demirel and Scherer 2008).

Figure 3.4. Neighbour-joining tree presenting the archaeal phylogenetic affiliation to the DGGE band sequences.

The DGGE gel in **Figure 3.3B** shows distinct bacterial communities in the feed, seed sludge and the anaerobic reactors. However, the acidogenic reactor bacterial communities did not change significantly as the SRT was reduced. There were also bands which consistently appeared in all the samples as well as those which were distinct in a particular reactor. For instance, B3 to B6 appeared in all samples, while B9, B12 and B15 were observed only in the acidogenic reactor. Phylogenetic identification demonstrated that *Clostridiales*, *Flavobacteriales*, *Pseudomonadales* and *Syntrophobacteriales* were the four bacterial orders identified in the anaerobic reactors **Figure 3.5**. Unfortunately, many of the bacterial sequences could not be affiliated to cultured, classified microorganism. B3 was closely related (97%) to *Enhydrobacter* species of *Pseudomonadales* order (Table 3.4). B4 and B6 matched (99%) uncultured *Bacteroidetes* bacterium in sludge AD and uncultured *Fluviicola* sp. from membrane bioreactor biofilm which were of *Flavobacteriales*. B5 was related (98%) to uncultured *Ruminococcaceae* bacterium identified in wetlands of the *Clostridiales*.

Of those bands only found in the acidogenic reactor, B9, B12 were closely related to uncultured bacterium identified in AD process treating coking wastewater and full-scale sludge AD process, respectively (**Table 3.4**). These sequences clustered within *Flavobacteriales* class (**Figure 3.5**). B15 had 97% maximum identity affiliation to uncultured *Clostridiales* bacterium identified in AD process treating swine manure (**Table 3.4**). Additionally, B1 and B2 were present in the acidogenic reactor as well as the feed sludge. Both sequences matched (100%) *Cloacibacterium normanense*, a *Flavobacteriales*, previously isolated from an influent sewage, capable of hydrolysing starch and gelatin (Allen et al. 2006).

There were also bands which were found to be present only in the single-stage and methanogenic reactors. B7 was closely related (96%) to *Smithella propionica*, a known syntrophic acetogen, which cooperated with hydrogenotrophic microorganisms, such as the hydrogenotrophic methanogen to convert propionic acid into acetic acid (Liu et al. 1999). *Clostridiales* and *Flavobacteriales* orders could also be found in these anaerobic reactors. B10 and B13 were affiliated to

Figure 3.5. Neighbour-joining tree presenting the bacterial phylogenetic affiliation to the DGGE band sequences.

Saccharofermentans acetigenes (98%) and uncultured *Firmicutes* bacterium in full-scale AD treating sludge (99%), respectively, which were both clustered within the *Clostridiales*. *Saccharofermentans acetigenes* was isolated from an anaerobic reactor, capable of fermenting saccharides (Chen et al. 2010). Lastly, B14 was closely related (99%) to uncultured *Bacteroidetes* (*Flavobacteriales*) in a full-scale AD treating sludge, which came under the *Flavobacteriales* order.

3.3.3. Microbial quantitative analysis by qPCR

The quantitative microbial dynamics over different SRTs were assessed by qPCR. The qPCR analysis targeted universal *Bacteria* and four methanogenic orders and families commonly found in anaerobic sludge digestion process (Shin et al. 2010).

Figure 3.6 shows the average quantification of universal bacterial and methanogenic communities during each SRT when the process was stable and had undergone at least 4 SRT cycles. The average bacterial 16S rRNA genes of the feed sludge reduced from 1×10^{10} to c. 5×10^9 DNA copies mg VSS⁻¹ in the single-stage and methanogenic reactors regardless of the SRT, indicating cell lysis as microbial cells decayed during the AD process. A quantitative community shift was observed following inoculation with the seed sludge into single-stage, acidogenic and methanogenic reactor biomass at 30 days SRT. However, there was no obvious shift to either the bacterial or methanogenic communities as the SRT was reduced. Average total methanogens in the seed sludge (3.6×10^8 DNA copies mg VSS⁻¹) was lower than in the single-stage and methanogenic reactors (c. 6×10^8 DNA copies mg VSS⁻¹), but was slightly higher than the feed sludge and acidogenic reactor (1×10^8 DNA copies mg VSS⁻¹) (**Figure 3.6A**).

Figure 3.6 shows that hydrogenotrophic *Methanomicrobiales* dominated in all samples, between 80 – 90% in abundance to all the methanogens. The results also demonstrated that the mean 16S rRNA gene concentration of *Methanomicrobiales* (**Figure 3.6B**) and *Methanosaetaceae* (**Figure 3.6C**) declined as the seed sludge (3×10^8 and 9×10^6 DNA copies mg VSS⁻¹, respectively) evolved to the acidogenic reactor biomass (ca. 1×10^8 and 1×10^6 DNA copies mg VSS⁻¹, respectively), while, the mean 16S rRNA gene concentration of *Methanobacteriales* (**Figure 3.6B**) and

Figure 3.6. Quantification of the 16S rRNA gene copy concentrations in the feed sludge, seed sludge and anaerobic reactors by qPCR for: *Bacteria* and total methanogens (A); *Methanobacteriales* and *Methanomicrobiales* (B); and *Methanosaetaceae* and *Methanosarcinaceae* (C).

Methanosarcinaceae (Figure 3.6C) increased as the seed sludge (3×10^6 and 2×10^5 DNA copies mg VSS⁻¹, respectively) formed the acidogenic reactor biomass (c. 1×10^7 and 1×10^6 DNA copies mg VSS⁻¹, respectively). *Methanobacteriales*,

Methanomicrobiales (**Figure 3.6B**) and *Methanosaetaceae* (**Figure 3.6C**) were in the similar range for both the single-stage and the methanogenic reactors (3×10^7 , 5×10^8 and 1×10^7 DNA copies mg VSS⁻¹, respectively). Only the mean 16s rRNA gene concentration of *Methanosarcinaceae* (**Figure 3.6C**) was found to be one order of magnitude higher in the methanogenic reactor (3×10^7 DNA copies mg VSS⁻¹) than any other samples.

3.4. Discussion

This study compared the application of single-stage and two-phase anaerobic digestion systems to treat sewage sludge. The same working conditions in terms of using the same anaerobic seed sludge inoculum, feed sludge substrate and temperature were applied to both configurations. The effect of decreasing SRT which consequently increased the OLR was evaluated. The competitive advantage of using the two-phase configuration at the shortened SRT of 12 days was shown to maintain volatile solids reduction and biogas production.

Stable TCOD removal, VS reduction and methane yield could be maintained at the 30, 20 and 12 days SRT and OLR from 1.5 to 3.5 g TCOD L⁻¹ d⁻¹ in the two-phase system (**Table 3.2**). On the contrary, the single-stage system could not maintain the same performance once the SRT was decreased from 20 to 12 days and OLR increased from 2.2 to 3.5 g TCOD L⁻¹ d⁻¹. To determine if methanogenesis was the limiting factor in the single-stage reactor, residual TVFA in both the single-stage and methanogenic reactors were compared, but both were found to be low (< 100 mg COD L⁻¹), irrespective of the SRT. VFA was directly consumed to produce biogas here and implied that methanogenesis was not restricted in the single-stage reactor (**Table 3.2**). However, higher mean non-soluble COD concentration was found in the single-stage (28,500 mg COD L⁻¹) than the methanogenic reactor (24,000 mg COD L⁻¹) at 12 days SRT ($p < 0.05$, $n = 16$) (**Table 3.2**). The measure of non-soluble COD implies to the organic particulate fractions of the sample. Hydrolysis of particulate organics, which require longer reaction (Mahmoud et al.

2004), seemed to be retarded in the single-stage reactor at 12 days SRT. These results showed that the two-phase AD system could tolerate shorter SRT (12 days) and higher organic loadings than the single-stage AD configuration, by maintaining the sludge hydrolytic efficiency.

This improved performance in the two-phase system could be due to chemically and biologically induced reasons. By maintaining the acidogenic reactor at pH 5.5, protein may lose its conformational structure and be denatured, as the protein was protonated (Neyens et al. 2004); or break glycosidic linkages of carbohydrates and deform their tertiary structure. All this, and disruption to the extracellular sludge matrix, would leave the biodegradable polymers exposed for extracellular enzymes to hydrolyze (Sheng et al. 2010). Some biological cells themselves may lose their viability and turgor pressure under acidic condition, hence cell lysis may also occur (Neyens et al. 2004). Secondly, the physical separation of phases in the two-phase system may enhance the activity of hydrolytic/acidogenic bacteria as their growth condition was optimized under the shortened SRT and pH 5.5 (Ghosh 1987). Acidogens were known to have maximum specific growth rate ($\mu_{\max}$) of 5.1 d⁻¹, while methanogens at 0.6 d⁻¹ (Angelidaki et al. 2000). Cultivating the acidogens under extended SRT to accommodate the methanogens in a single-stage configuration might be disadvantageous, as acidogens would then lose their optimal microbial activity under this stationary growth phase (Ghosh 1987).

Microbial community analysis by DGGE was performed to evaluate the microbial community significance between phase separated and single-stage systems. It was found that the seed sludge, feed sludge, single-stage and two-phase anaerobic reactors showed distinct bacterial fingerprints (**Figure 3.3B**), but similar archaeal fingerprints **Figure 3.3A**. First of all, a core bacterial group was observed in all the anaerobic reactors (B3 – B6), which were *Flavobacteriales*, *Clostridiales* and *Enhydrobacter*-related *Bacteria* (**Table 3.4**) and could be associated with fermentative (acidogenic) functions (Sneath et al. 1986; Kirchman 2002; Lynd et al. 2002). Other acidogenic *Flavobacteriales* and *Clostridiales* species (B10, B13 and

B14) were also identified in the single-stage and methanogenic reactors. The phyla *Bacteroidetes*, *Proteobacteria* and *Firmicutes* which contained *Flavobacteriales*, *Pseudomonadales* and *Clostridiales* orders, respectively, formed the most abundant taxonomy identified in the full-scale AD process (Rivière et al. 2009), and concurred with the findings in this study. The accumulation of propionic acid in the acidogenic reactor could be explained by the absence of B7, a sequence closely matching the syntrophic propionic acid oxidizer *Smithella propionica*. The growth condition in the acidogenic reactor was unfavourable for *Smithella propionica*, as it was not able to grow below pH 6.3 (Liu et al. 1999). Nevertheless, the overall two-phase AD system was not affected as *Smithella propionica* was detected in the methanogenic reactor, consuming propionic acid to < 0.1 mM.

The microbial differentiation between the reactors was more obvious in the acidogenic reactor, where B9, B12 and B15 of *Flavobacteriales* and *Clostridiales* were detected (**Figure 3.3B**). Even though they could not be affiliated to known species, members of these orders were known to be chemoheterotrophs, and would most likely be involved in the hydrolysis and acidogenesis of organic matters (Kirchman 2002; Lynd et al. 2002). The uncultured *Flavobacteriales* of B9 seemed to play a more important role as suggested by its thick band intensity in all SRTs tested in the acidogenic reactor. Since B9, B12 and B15 only appeared in the acidogenic reactor, these bacteria were enriched and maximized their metabolic potential upon cultivation in the acidogenic reactor at 2 to 5 days SRT with pH 5.5. Improved overall sludge digestion stability and performance of the two-phase compared to single-stage system might be attributed to this bacterial community shift.

Acetic acid is widely regarded to be the most important VFA species in the AD process (Angelidaki et al. 2000), but was not the major VFA in this acidogenic reactor (**Table 3.3**). Acetic acid could be consumed directly by aceticlastic methanogens, or converted into hydrogen and carbon dioxide by syntrophic acetic acid oxidizing *Bacteria*, before hydrogenotrophic methanogenesis occurs

(Angelidaki et al. 2000). Despite the detection of methanogens in all samples, methanogens' quantities in the feed sludge and acidogenic reactor were still much lower than in the single-stage and methanogenic reactors (**Figure 3.6A**), indicating that methanogenesis was still optimized in the single-stage and methanogenic reactor conditions. The most abundant methanogenic order in all the anaerobic reactors, including in the feed sludge, was the hydrogenotrophic *Methanomicrobiales* (**Figure 3.6**), suggesting the importance of hydrogenotrophic methanogenesis as the main metabolic pathway in this anaerobic sludge digestion process. Other studies had previously revealed *Methanomicrobiales* to be the most abundant methanogenic group in a sludge AD process by dot-blot hybridisation and qPCR (Raskin et al. 1995; Shin et al. 2010; Kim et al. 2013). DGGE analysis (**Figure 3.3A**) also supported this observation as most of the high-intensity archaeal bands present in all samples were identified to be *Methanomicrobiales* (**Table 3.4**), closely related to *Methanospirillum hungatei* and *Methanolinea mesophila*.

DGGE analysis for archaeal communities showed that there was no obvious shift in the methanogens in the acidogenic reactor (**Figure 3.3A**), whereas qPCR found that *Methanosarcinaceae* and *Methanobacteriales* increased by almost one log from the start to when the acidogenic reactor had stabilised, and another acetoclastic methanogenic family, *Methanosaetaceae* decreased by close to one log (**Figure 3.6**). *Methanosaeta* sp. grow in the pH range of 6.5 to 8.5, while *Methanosarcina* sp. between pH 5 and 8; and could tolerate higher acetate concentration (up to 250 mM acetate) than *Methanosaeta* (De Vrieze et al. 2012). DGGE analysis correspondingly found a band sequence which was closely related to *Methanosarcina barkeri*, while no band was found for *Methanosaetaceae*. The band representing *Methanosarcina* appeared weakly in the acidogenic reactor but more strongly in the methanogenic reactor samples (**Figure 3.3A**), as confirmed by the qPCR data (**Figure 3.6C**). *Methanosarcina barkeri* had been reported to utilize a wide range of substrates (e.g. acetic, formic acids, alcohol, methylamines, carbon dioxide) while *Methanobacteriales* mainly utilized hydrogen or formic acid

(Demirel and Scherer 2008). It is possible that the acidic pH and shortened SRT in the acidogenic reactor generated such compounds as by-products, which were then transferred to the methanogenic reactor and stimulated the proliferation of *Methanosarcina barkeri*. Additionally, both *Methanosarcina* and *Methanobrevibacter* had also been previously found to persist and predominate in acidic pH conditions (Savant et al. 2002; Steinberg and Regan 2011). These results indicated the contribution of *Methanobacteriales* and *Methanosarcinaceae* for the atypical methanogenesis reaction in the acidogenic reactor. However, no *Methanobacteriales* species was identified by the DGGE analysis, suggesting that DGGE may not be sensitive enough to detect less abundant but important microbial groups. The presence and activity of methanogenic communities in the acidogenic reactor and the presence of acidogenic bacteria in the methanogenic reactor confirmed that complete phase separation (acidogenesis and methanogenesis) could not be achieved in a two-phase system treating sewage sludge. Active methanogenesis within the acidogenic reactor, as demonstrated in this study, could consume acetic acid and control its concentration to manageable levels under high organic loading situations. Conversion of acetic acid into carbon dioxide and methane could contribute to reactor alkalinity to counteract significant pH drop, ultimately better ensuring stability of the system. Hence, it is not necessary to completely eliminate methanogenesis in the acidogenic reactor (Zhang and Noike 1991).

The effect of decreasing SRT in AD process could be drastic and impair sludge digestion performance. However, shortening the SRT is also advantageous in reducing the size of reactor volume, especially in places with limited land space or when upgrading existing anaerobic digesters. Fluctuations in the influent substrate volume could also affect process SRT. Too short SRT would wash out slow-growing microorganisms with growth rates shorter than the operating SRT. For instance, B9 decreased in intensity as the SRT was reduced from 3 to 2 days in the acidogenic reactor, suggesting that this bacterium may have been washed out at the shorter SRT of 2 days (**Figure 3.3B**). Fortunately, the reduction of this bacterial species did

not seem to affect the overall two-phase system digestion performance. Secondly, the increase in OLR associated with SRT reduction could also create an unfavourable condition for the sensitive methanogenic populations, as more inhibitory compounds could accumulate in the reactor. Such situation is normally associated with the accumulation of VFA in the anaerobic reactor whereby the conversion of acetic acid into methane is retarded (Speece 1996). However, VFA concentrations in the single-stage and the methanogenic reactor in this study were negligible, which indicated that both systems' methanogenic populations could tolerate the increase in OLR. Evaluation of the methanogens' quantity by qPCR also showed that methanogens were not affected by the decreasing SRT. The presence of methanogens in the feed sludge may have partially helped in maintaining the reactors' methanogenic population and preserve methanogenesis (**Figure 3.3A**). Thirdly, reducing the SRT could also impair the degradation of particulate compounds which require extended reaction time to complete. This was the case in the single-stage system at 12 days SRT (**Table 3.2**). On the other hand, the two-phase system's process performance could still be maintained possibly due to the enrichment of chemoheterotrophic bacterial communities (B9, B12 and B15) in the acidogenic reactor. There was no significant change in the bacterial and archaeal communities in the acidogenic reactor as the SRT was reduced (**Figure 3.3**). Consequently, the extent of solubilization in the acidogenic reactor was not adversely affected as the SRT was reduced from 5 to 2 days (**Figure 3.2**). This implied that the acidogenic reactor selected for a specialized community structure which could tolerate the SRT reduction from 5 to 2 days, and contributed to the stability of process performances in the two-phase anaerobic system. The results also further indicated that sludge hydrolysis was the rate-limiting step in the anaerobic sludge digestion process (Miron et al. 2000; Appels et al. 2008; Vavilin et al. 2008).

3.5. Conclusion

A two-phase anaerobic system was able to maintain TCOD removal, VS reduction and methane production when the SRT was decreased up to 12 days, but not in the conventional single-stage system. The latter was due to the decrease in non-soluble COD reduction efficiency in the single-stage system at 12 days SRT. Hence, sludge digestion enhancement should be targeted at the rate-limiting sludge hydrolysis step. Microbial community analysis revealed distinct microbial profiles in the single-stage, acidogenic and methanogenic reactors, which generally remained unchanged as the SRT was decreased. The enrichment of hydrolytic and acidogenic *Flavobacteriales* and *Clostridiales* only in the acidogenic reactor could possibly explain the better stability of sludge digestion performance in the two-phase over the single-stage system at 12 days SRT. Methanogenic activity in the acidogenic reactor does not need to be completely eliminated as it could help regulate alkalinity in acidogenic reactor, and may have been attributed by the enrichment of *Methanobacteriales* and *Methanosarcinaceae*. A two-stage AD system is hence considered to improve sludge digestion further. In addition, DGGE analysis might not be sensitive enough to identify less abundant but important microbial groups, such as *Methanobacteriales* detected by qPCR in this study. Advanced molecular technique (e.g. 454 pyrosequencing) with higher resolution than DGGE is recommended to obtain greater insights into the microbial community structure in anaerobic processes.

Chapter 4

The Effect of pH on Sludge Solubilization, Acidogenesis and the Microbial Community Structures

4.1. Introduction

Sludge solubilization or hydrolysis has been recognised to be the rate-limiting step in anaerobic sludge digestion (Miron et al. 2000; Appels et al. 2008) and thus, its optimization is pursued. Recent studies have assessed the impact of pH on waste activated sludge (WAS) solubilization and acidogenesis in batch configuration between pH 4 and 12 at 21°C (Chen et al. 2007), 28°C (Jie et al. 2014), 35°C and 55°C (Zhang et al. 2009; Zhang et al. 2010). Alkaline conditions were consistently reported to be more efficient than acidic and neutral conditions for sludge solubilization, and the highest solubilization was found at the highest pH value tested (Chen et al. 2007). However, increased solubilization of sludge does not necessarily translate into a better AD process (Kim et al. 2013). Acidification was found to reach the maximum at pH 10 at 21°C and 28°C after 8 to 10 days (Chen et al. 2007; Jie et al. 2014). Zhang et al. (2009) reported that the sludge acidification in 35°C yielded the highest VFA production at pH 9 after 5 days and even higher VFA concentrations in 55°C at pH 8 after 9 days. Extreme pH condition might be detrimental to the biological activity of acidogenic *Bacteria*. Furthermore, acidic or alkaline conditions at pH 2.5, 9.8 and 11.8 were known to affect the molecular weight (MW) of soluble compounds released from the sludge (Xie et al. 2014), and these properties could influence the biodegradability of the solubilization products (Eskicioglu et al. 2006; Kim et al. 2013). The biotic contribution to sludge solubilization and the solubilization products' characteristics when operating at milder pH (pH 4 – 11) is not well understood.

Little is known of the hydrolytic and acidogenic microorganisms driving the sludge solubilization and acidification reactions at different pHs. Microbiological studies

had been limited to using DGGE (Jie et al. 2014), clone library and FISH analyses (Zhang et al. 2010) that could not clearly establish a proper link between the roles of microbial populations at different pHs to the process performance. Correlation of the identified microorganisms with their possible functions would provide valuable insights into the underlying biological reactions. This study will utilize 454 pyrosequencing where higher number of sequencing reads can be obtained compared to clone library and DGGE analyses. This way, the analytical confidence level becomes higher and a more detailed representation of the microbial community structure can be obtained (Pervin et al. 2013; Sundberg et al. 2013).

This chapter aims to define the optimal operating pH for enhanced sludge solubilization and acidogenesis in the first reactor, as well as evaluate the characteristics of the solubilization products at different pHs. The microbial communities established at different pHs would be elucidated and linked with the sludge acidification performances. Batch experiments had demonstrated that the major portion of WAS acidification at 35°C was achieved within 3 to 4 days (Zhang et al. 2009; Zhang et al. 2010). Hence, SRT of 3 days at 35°C is chosen for this study.

4.2. Materials and Methods

4.2.1. Reactor operation

Semi-continuous reactors were operated in 1 L glass bottles with 0.9 L working volume. 0.3 L of sludge was withdrawn from the reactor and replaced with 0.3 L of feed sludge daily to make SRT of 3 days. Reactors were incubated in temperature controlled shaker at 35°C and 120 rpm. One reactor was operated without pH adjustment serving as control, and eight other reactors were adjusted to pH 4, 5, 6, 7, 8, 9, 10 and 11 using 5 M hydrochloric acid or 5 M sodium hydroxide. The pH adjustments were done throughout the experiment, twice every day with 12 hour intervals. However, a fixed pH value could not be maintained. The pH variations and chemical dosage are presented in **Table 4.1**. The reactors were initially seeded

with anaerobic sludge treating sewage sludge collected from Ulu Pandan water reclamation plant, Singapore, and purged with nitrogen to eliminate oxygen in the headspace. The feed sludge is municipal mixed sewage sludge, composed of primary and secondary sludge from the same plant. The feed sludge characteristics were 25.4 ± 3.0 g TS L⁻¹; 20.3 ± 2.5 g VS L⁻¹; $37,200 \pm 2,800$ mg TCOD L⁻¹; $2,000 \pm 400$ mg SCOD L⁻¹; and pH 5.8 to 6.0.

Table 4.1. The pH variations and chemical dosage of the reactors (average $\pm$ standard deviation).

Target pH of reactor	pH variations	Chemical dosage
No control	5.8 - 6.3	-
pH 4	3.9 - 4.3	0.05 ± 0.01 g HCl g TS feed ⁻¹
pH 5	4.9 - 5.1	0.01 ± 0.00 g HCl g TS feed ⁻¹
pH 6	5.8 - 6.2	0.01 ± 0.00 g NaOH g TS feed ⁻¹
pH 7	6.5 - 7.2	0.03 ± 0.02 g NaOH g TS feed ⁻¹
pH 8	7.1 - 8.2	0.07 ± 0.02 g NaOH g TS feed ⁻¹
pH 9	8.2 - 9.2	0.1 ± 0.02 g NaOH g TS feed ⁻¹
pH 10	9.2 - 10.2	0.14 ± 0.02 g NaOH g TS feed ⁻¹
pH 11	10.2 - 11.2	0.17 ± 0.02 g NaOH g TS feed ⁻¹

4.2.2. Sludge fractioning protocol

Sludge fractioning protocol was performed to extract the extracellular polymeric substances (EPS) and measure extracellular hydrolytic enzymatic activity. To date, there is not yet standard method to separate individual parts of EPS, but a method described by Yu et al. (2008) can give a close approximation to separate the pellet, tightly-bound EPS, loosely-bound EPS, and slime and soluble EPS fractions. During the fractionation, the sediments were resuspended to the same original volume in phosphate buffered saline solution (PBS), pH adjusted according to the pH of their respective reactors. The bulk solution after 2,000 xg centrifugation for 15 minutes at 4°C is classified as the slime and soluble EPS fractions. The sediment is then resuspended in PBS and subsequently centrifuged at 5,000 xg for 15 minutes and 4°C. The resulting bulk solution is classified as loosely bound EPS fractions. The sediment is then resuspended again in PBS and sonicated at 80 – 120 W for 10 minutes, before being centrifuged at 18,000 xg for 20 minutes at 4°C. The resulting

bulk solution is classified as tightly bound EPS and the sediment is resuspended in PBS to form the pellet fraction. The tightly-bound EPS, loosely-bound EPS and slime and soluble EPS fractions were filtered through 0.45 μm nylon filters to remove particulates before measuring extracellular enzymatic activities. All EPS fractions were subsequently stored at 4°C before further use.

4.2.3. Analytical methods

The chemical analyses and equation to calculate the extent of sludge solubilization were described in the analytical methods of **Chapter 3**.

Soluble protein and carbohydrate concentrations were measured using Lowry-Folin and phenol-sulphuric acid methods, respectively (Feng et al. 2009). The approximate COD conversion factors for carbohydrate (glucose) was 1.07 mg COD.mg glucose⁻¹ and protein (bovine serum albumin, BSA) was 1.5 mg COD.mg BSA⁻¹ (Feng et al. 2009). In this study, daily biogas volume produced was collected in tedlar gas bags and measured with a wet gas meter (Ritter, Germany). The average values were calculated after the reactors had achieved steady-state from the 37th day.

Amylase and protease activities were measured to represent extracellular enzymatic activities produced by microorganisms to hydrolyze carbohydrate and protein. These enzymatic tests were performed to all the sludge fractions. Amylase activity was analyzed with enzymatic assay of α -amylase (Sigma-Aldrich, USA) using 3, 5-dinitrosalicylic acid colorimetric method with soluble starch (S2630, Sigma-Aldrich, USA) as substrate and 30 minutes incubation. Protease activity was analyzed with universal protease activity assay (Sigma-Aldrich, USA) using Folin's & Ciocalteu's phenol colorimetric method with casein (C7078, Sigma-Aldrich, USA) as substrate and 60 minutes incubation.

Size exclusion chromatography (SEC) was performed using high-performance liquid chromatography (HPLC) (Agilent, USA) following methods described by Tian et al. (2014). Refractive index (RI) and ultraviolet (UV) light at 254 nm were used as detectors. The use of RI detector allowed universal visualization of materials as

long as sufficient concentrations were present. UV detector at 254 nm allowed for specific visualizations of aromatic compounds such as proteins and humic acids (Yang et al. 2013). The molecular weight (MW) was calibrated using polyethylene glycol and polyethylene oxide standards with MW of 500, 70, 4, 0.6 and 0.1 kDa. A linear relationship could be derived (Tian et al. 2014) and was expressed as

$$\text{Log (molecular weight)} = 9.8223 - 0.6748 \times (\text{retention time}) \quad (4.1)$$

4.2.4. DNA extraction and quantitative PCR (qPCR)

Genomic DNA was extracted from the seed sludge, feed sludge and reactors after 47 and 77 days of the reactor operation. Before extraction, sludge samples were washed twice by centrifugation at 10,000 xg for 5 minutes, discarding the supernatant, and resuspension in PBS. PowerSoil DNA isolation kit (MoBio, Singapore) was used to extract the DNA in duplicates, following the manufacturer's recommended protocol. DNA extraction efficiency was checked and found to be optimized at around 10 mg TS, and hence, 0.5 mL of sludge sample was used for each DNA extraction. Extracted DNA was then stored at -20°C before further analysis.

Quantitative PCR (qPCR) was performed using primers and TaqMan probes targeting the 16S rRNA gene of universal *Bacteria* and universal *Archaea*, as described in the materials and methods in **Chapter 3**.

4.2.5. Amplicon 454 pyrosequencing and the bioinformatics analysis

The extracted genomic DNA was sent to Research Testing Laboratory (RTL, USA) for bacterial tag-encoded FLX amplicon 454 pyrosequencing as described by Dowd et al. (2008). 16S rRNA gene universal bacterial primer set (357F and 926R, targeting V3-V5 hypervariable region) (Muyzer et al. 1993; Muyzer et al. 1995; Sim et al. 2012) and 16S rRNA gene universal archaeal primer set (517F and 909R, targeting V4-V5 hypervariable region) (Burggraf et al. 1997; Baker et al. 2003; Lay et al. 2013) were used for the PCR amplification (**Table 4.2**). The sequences were archived in NCBI Sequence Read Archive (SRA) with the accession SRP055486 (<http://www.ncbi.nlm.nih.gov/genbank/>). The program Mothur v 1.33.3 was used

to prepare the raw data for analysis in accordance with Mothur 454 SOP (Schloss et al. 2011). Raw sequences obtained from RTL were initially trimmed to exclude sequences with at least 1 ambiguous base calls, more than 1 barcode mismatch, 2 primer mismatch, 8 homopolymeric bases and if they contained average quality score below 25 over a 50 bp sliding window. Sequences were then aligned using the respective SILVA bacterial and archaeal database and again trimmed so that all sequences started from the same aligned position with at least half of the expected read length. The “pre-cluster” function was subsequently used to merge sequences with 1 bp difference. Chimeras were removed UCHIME using database-independent approach. OTUs were clustered at 97% sequence similarity with average neighbouring clustering algorithm. Normalization of sample size was conducted by “sub.sample” function in Mothur by resampling the same number of reads for each sample, based on the smallest sample size. Taxonomic classification at the phylum and genus level were done with naïve Bayesian classifier method using RDP training set 9 alignment database and taxonomy, bootstrapped 1,000 times with 50% cutoff. Relative abundance was calculated as the percentage of the number of sequences in the OTU by total number of respective archaeal or bacterial sequences in a sample. Heatmap was also constructed with heatmap.2 function from R using the bacterial data output from Mothur, whereby the OTUs containing less than 30 reads were removed for visualization purpose (**Appendix B**). The OTUs with their genus identification were aligned on the y-axis and were hierarchically clustered using Bray-Curtis algorithm. This heatmap was used to identify important classified bacterial genus for the microbial community analysis.

Table 4.2. List of primers targeting the 16S rRNA genes, used in the 454 pyrosequencing.

Target group	Function	Primer	
		identification	Sequence
Universal <i>Bacteria</i>	Forward primer	BAC357F	CCTACGGGAGGCAGCAG
	Reverse primer	BAC926R	CCGTCAATTYMTTTRAGT
Universal <i>Archaea</i>	Forward primer	ARC517F	GCYTAAAGSRNCCGTAGC
	Reverse primer	ARC909R	TTTCAGYCTTGCGRCCGTAC

4.3. Results and discussions

4.3.1. Reactor performances

Operation of a semi-continuous system to depict the first reactor of two-stage system was examined at different pHs. The reactor controlled at pH 4 was started slightly later at the 5th day and reactors at pH 10 and 11 were both started at the 8th day. Soluble COD concentrations in the control and pH 5 to 9 immediately increased from 170 mg SCOD.L⁻¹ at 0 days to the maximum at a range of 4,600 - 7,000 mg SCOD.L⁻¹ after 8 days (**Figure 4.1**). The soluble COD concentrations then increased even higher between 14 and 33 days as different batch of feed sludge contained much higher organic content. Ultimately, the soluble COD and VFA concentrations dropped as feed sludge was maintained from 37 days at a similar concentration to the initial feed sludge concentration, and the highest soluble COD was observed at pH 11 with around 12,000 mg SCOD.L⁻¹ (**Figure 4.1**). The highest VFA concentration was observed at pH 8 to 10 with 5,800 - 8,400 mg COD.L⁻¹, while the control contained 2,300 - 3,300 mg COD.L⁻¹ of VFA. The highest VFA concentration obtained in acidic condition (pH 5) was between 4,000 and 5,500 mg COD.L⁻¹, and it was around 3,800 mg COD.L⁻¹ at pH 7. Consistent with previous findings, alkaline conditions were better for VFA accumulation than acidic and neutral conditions (Chen et al. 2007; Jie et al. 2014). Stable VFA production was also achieved in this semi-continuous configuration to provide steady supply of suitable substrates for methanogenesis.

Figure 4.1. Sludge solubilization and acidification of the reactors at different pHs. The concentrations of soluble COD in reactors operated without pH adjustment (control) and acidic pH (A); and in reactors operated at neutral and alkaline pH (B). The concentrations of total VFA in the control and reactors at acidic pH (C), and in reactors operated at neutral and alkaline pH (D).

4.3.2. Effect of pH on the solubilization of organic matter

Higher sludge solubilization was achieved with higher pH, whereby pH 11 achieved the highest solubilization (25.9%) (**Figure 4.2**). The highest solubilization in acidic condition (pH 5) was at 8.2% and neutral condition achieved 6.8% solubilization. The organic compositions of the sludge soluble fractions tested in this study were methane (derived from acetic acid utilization via methanogenesis), VFA, carbohydrates and proteins. Other components not accounted for were categorised as others. Zhang et al. (2009) found elevated concentrations of ethanol and lactic acids during WAS acidification process in both increasing acidic and alkaline conditions, which may contribute to this other component. VFA formed almost the entire solubilization product in the control and pH 5 to 7 (up to

7.4%), indicating effective biological acidogenesis reactions. Meanwhile, VFA component maximized at around 12% from pH 8 to 10 even with increasing sludge solubilization, as proteins, carbohydrates and other components gradually increased. This shows that higher solubilization caused by sodium hydroxide did not necessarily translate to higher VFA production at pH 9 and above. Indeed, lower VFA production was observed at pH 11, where VFA formed 8.9% solubilization and carbohydrates, proteins and other components formed 2.1%, 6.5% and 8.3%, respectively. Poor acidogenesis was also observed at pH 4, where VFA production formed only 1.9% solubilization out of 5.2% sludge solubilization.

Figure 4.2. The average extent of sludge solubilization with the organic compositions (a), and the average VFA concentrations with its speciations (b) in the reactors after 37 days.

Acetic and propionic acids were always the predominant VFA species in all conditions, except for the control, pH 6 and pH 7 where acetic acid was converted into methane (**Figure 4.2B**). Acetic acid was preferred for subsequent methanogenesis reaction (Li et al. 2011). Butyric and isovaleric acids subsequently followed as the next most predominant VFA species in the reactors. These results were in agreement with the findings in previous batch study, that VFA composition was not affected by pH conditions, except at pH 6 and 7, where methanogenesis took place (Zhang et al. 2009).

Methane production in the control, pH 6 and 7 bioreactors contributed to 0.5% - 0.6% solubilization (**Figure 4.2A**). It was also detected at pH 8 but at lower percentage (0.09% solubilization). Besides sludge solubilization and acidogenesis being optimized in alkaline condition, pH could also serve to control VFA accumulation by inhibiting methanogenesis reaction.

VFA yields during the mixed sludge acidifications were calculated to be 410 ± 70 mg COD g VSS⁻¹ at pH 8 and 150 ± 40 mg COD g VSS⁻¹ with no pH control. These values were higher compared to previous batch WAS acidifications at 35°C between pH 4 to 11, where the highest VFA yield were obtained at pH 9 (298 mg COD g VSS⁻¹) and the reactor with no pH control gave 60 mg COD g VSS⁻¹ (Zhang et al. 2009). The type of substrate being treated could influence the sludge acidification. It had been discussed previously that primary sludge, which formed part of the mixed feed sludge in this study, was more easily degraded than WAS (Zhang et al. 2009). This possibly explained the higher VFA yield obtained in this study compared to the mesophilic batch acidification treating WAS alone.

In terms of total activity, it was found that amylase and protease activities were reduced below pH 6 and above pH 8 (**Figure 4.3**). Total enzymatic activities at pH 5 and below, as well as, pH 10 and above were reduced by more than 50%. In contrast, sludge solubilization was increased with higher pH or lower pH beyond the control (pH 6) (**Figure 4.2**), indicating the leading role of abiotic effect by acid or alkaline dosage to hydrolysis. The results also demonstrated the detrimental

impact of alkaline condition to the biotic contribution to hydrolysis. It could be inferred that this impact of high pH to bioprocess might also lead to the poor acidification efficiency at pH 9 and above. For both amylase and protease activities, the majority was found in the pellet and tightly bound EPS fractions, which was in agreement with previous reports (Higuchi et al. 2005; Yu et al. 2008).

Figure 4.3. Amylase (A) and protease activities (B) of EPS fractions from sludges at different pHs.

Soluble fractions of the control and pH 6 reactors gave the lowest RI signals relative to all other reactors (**Figure 4.4A** and **Figure 4.4B**), which was consistent with the extent of solubilization results in **Figure 4.2A**. Different molecular weight (MW) distributions were observed in the reactors at different pH. All samples showed a peak with MW size between 0.1 and 4 kDa. Meanwhile, another peak was detected from the pH 9, 10 and 11 soluble fractions which had a size larger than 500 kDa (**Figure 4.4B**).

Figure 4.4. Size exclusion chromatograms of the soluble fractions from the control and acidic reactors with refractive index (A), 254 nm (B) detectors; and chromatograms of the soluble fractions from the neutral and alkaline reactors at 77th days with refractive index (C), 254 nm (C) detectors. The dotted lines represent the retention time of the calibration standards and the arrows depicted the interpolated molecular weight (MW) based on **Eq. 4.1**.

Passing the soluble fractions through a UV detector at 254 nm did not yield obvious signals in all samples for the range between the 0.1 and 4 kDa (**Figure 4.4C** and **Figure 4.4D**), indicating that the substance detected on RI was not aromatic in nature and might represent VFA, as shown in **Figure 4.2A**. A large MW peak (> 500 kDa) was, however, observed at pH 9, 10 and 11 (**Figure 4.4D**). This large MW peak could have been derived from release of proteinaceous cell fragments or EPS into the soluble fractions (Eskicioglu et al. 2006), which was mediated by the sodium hydroxide dosed. In alkaline condition, proteins were denatured and EPS were solubilized to release the organic contents tightly bound within the sludge matrix.

Damage to the microbial cell in the sludge also occurred as alkali induced solubilization of membrane proteins and saponification of the membrane lipids on cell membranes (Mendonca et al. 1994). Previous investigation assessed the biodegradability of soluble fractions at different MW which were derived from WAS pretreated with microwave and thermal hydrolysis (96°C) (Eskicioglu et al. 2006). That study found that the soluble fractions containing MW larger than 300 kDa gave lower biodegradation rate than other soluble fractions containing smaller MW molecules. This could be due to the structural complexity and lower surface area to volume ratio of substances with higher MW. The presence of such high MW substances would be disadvantageous for subsequent use of this acidification mixed liquor for further bioprocess, e.g. AD. Hence, mixed sludge acidification is recommended at pH 8 at mesophilic (35°C) condition with 3 days SRT.

4.3.3. Effect of pH on microbial communities

4.3.3.1 Microbial quantitative analysis by qPCR

As the feed sludge also contains Bacteria and Archaea, changes to the microbial compositions of the feed sludge would be reflected in the respective reactors. **Figure 4.5** shows that there was an increase in the number of DNA copies in the feed sludge at 77 compared to 47 days. Consequently, a similar increase in DNA copies was also observed in all reactors. In general, both the archaeal and bacterial DNA copies were reduced compared to the feed sludge, except for the *Archaea* in the control, at pH 6 to 8 and pH 9 at 47 days, and the *Bacteria* in the control, at pH 6 to 9 at 47 days and pH 7 at 77 days. *Archaea* showed the highest abundance at pH 7, followed by the control, pH 6 and pH 8. On the contrary, the sludges at pH 4, 10 and 11 experienced almost one log of reductions in archaeal DNA copy abundance compared to the feed sludge (**Figure 4.5A**). As *Archaea* are typically found to be methanogens in anaerobic reactors, the results indicated that pH around neutral were more favourable for the growth of methanogens than acidic or alkaline conditions, hence the highest methanogenic activity recorded around neutral pH in this study (**Figure 4.2A**). It was also noted that despite the similar methane volume produced at pH 6 and 7, the archaeal abundance was slightly

higher at pH 7 than 6. This observation might be attributed to the different methanogenic communities present in the two conditions, which would be described later. Similarly, there were also quantitative reductions to the feed sludge in the bacterial qPCR results. Almost one log reduction was observed at pH 4, 10 and 11 at 77 days and it was halved at pH 5, 8 and 9 (**Figure 4.5B**). Since qPCR targeted universal *Bacteria*, it was unclear if the reduction of *Bacteria* abundances was attributed to the loss of microorganisms derived from the feed sludge, or the active acidogenic microorganisms. Nevertheless, qPCR confirmed the influence of acidic and alkaline conditions to the reduction of both archaeal and bacterial population abundances.

Figure 4.5. Microbial quantification by qPCR of the feed sludge and reactors at 47 and 77 days, targeting the *Archaea* (A) and *Bacteria* (B). The dotted lines represent the respective bacterial and archaeal quantification of the anaerobic seed sludge.

4.3.3.2 Microbial community analysis by 454 pyrosequencing

454 pyrosequencing targeting *Archaea* generated a total of 210,000 sequencing reads in this study. Sequence processing, clustering into OTUs at 97% similarity and normalization by Mothur reduced the number of reads to 1,544 reads in each sample. Taxonomic classification using RDP database resulted in 99% of the OTUs being classified to genus level and the rest as unclassified. All the archaeal genera identified were known methanogens (**Figure 4.6A**). *Methanolinea* and *Methanospirillum* were found to be predominant methanogens in the samples collected from all reactors including the feed and seed sludges (**Figure 4.6A**). The relative abundance of the hydrogenotrophic *Methanolinea* was between 41% and 93%, except at pH 7 and 8 with 28% - 48% relative abundance. Meanwhile, hydrogenotrophic *Methanospirillum*'s relative abundance was between 5% and 35%. Hydrogenotrophic methanogenesis seemed to be the important pathway for methanogenesis with short SRT during sludge acidification process and this had also been observed in other anaerobic sludge digestion processes (Kim et al. 2013). In addition, *Methanosarcina* was enriched in the control (naturally buffered to between pH 5.8 and 6.2), pH 6 and 7 reactors, but negligible in the other reactors. These enrichments were consistently observed in both the 47 and 77 days samples. *Methanosarcina* can grow between pH 5 and 8 (De Vrieze et al. 2012) and this coincided with the pH values where it was enriched. Hence, *Methanosarcina* was likely associated with methanogenesis between pH 6 and 7. In contrast, *Methanocorpusculum* was exclusively enriched at pH 7 and 8, as hydrogenotrophic *Methanocorpusculum* can grow between pH 6.5 and 7.5 (Anderson et al. 2009). *Methanocorpusculum* appeared to be cultivated better than *Methanosarcina* at pH 7 and 8, and would also likely to be associated with methanogenesis under these conditions. Characterization of the methanogenic communities by 454 pyrosequencing and their abundances by qPCR demonstrated that methanogens could be sustained and acclimated at pH 6, 7 and 8 with short SRT (3 days), which could be correlated with the methanogenic activities under these conditions.

Figure 4.6. The average relative abundances of archaeal OTUs with 97% similarity to genus level (A); and the average relative abundance of bacterial OTUs with 97% similarity to phylum level (B) after 47 and 77 days of reactor operation.

454 pyrosequencing targeting *Bacteria* generated a total of 300,000 sequencing reads in this study. Sequence processing, clustering to 97% similarity and normalization by Mothur reduced the number of reads to 955 reads in each sample. Microbial ecology in the anaerobic process is still yet to be understood as 29% of the bacterial OTUs could not be classified to genus level. Similar to the archaeal communities, the bacterial communities found in 47 days were related to those in 77 days.

Figure 4.7 shows that *Parabacteroides*, *Paludibacter* and *Caldisericum* were detected in the seed sludge, feed sludge, control and reactors in acidic, neutral and alkaline conditions. These genera had been commonly discovered in anaerobic digesters previously and were found to be involved in VFA production (Krieg et al. 2010). This implied that the feed sludge had developed acidogens which subsequently influenced the bacterial composition of the reactors in this study. However, these genera predominating in the feed sludge were largely replaced by other genera in all reactors. This proved that the reductions of bacterial DNA copies observed in qPCR at pH 4, 10 and 11 (**Figure 4.5B**) were attributed to the death of microorganisms derived from the feed sludge, while specialized microorganisms were enriched. The semi-continuous sludge acidification process with 3 days SRT between the control and pH 4 to 11 at 35°C in this study led to the acclimation of heterogeneous bacterial community structures capable of producing VFA. Importantly, the specific genera enriched in the reactors in this study were optimal in their respective operating pH conditions (**Figure 4.7** and **Table 4.3**). Hence, operating pH could be considered as one of the selective pressure for the enrichment of these different genera and that there was a wide diversity of microorganisms with acidogenic capabilities from pH 4 to 11.

Under acidic condition, *Hallella* was detected in the control, pH 4, 5 and 6 reactors, and *Phascolarctobacterium* in the control, pH 5 and 6, but not under neutral and alkaline conditions (**Figure 4.7**). *Prevotella* and *Clostridium_sensu_stricto* were enriched at pH 5 alone, while *Oscillibacter* only at pH 4. *Oscillibacter valericigenes*

Figure 4.7. Relative abundances of bacterial genera in the feed sludge, seed sludge and reactors after 47 and 77 days of reactor operation.

is known to be a mesophilic, anaerobic fermentative bacterium which produced valeric acid as the main fermentation product from carbohydrates with optimum pH of 6 - 6.5 (Iino et al. 2007). *Hallella* and *Prevotella* are anaerobic *Bacteria* that ferment carbohydrates and some proteins to succinic and acetic acids, which grew at pH 5.5 - 5.7 and 4.6 - 5, respectively (Krieg et al. 2010). The succinic acid produced could in turn be consumed by *Phascolarctobacterium* to produce propionic acid (Vos et al. 2009). Meanwhile, *Clostridium_sensu_stricto* species were grouped as *Clostridium* cluster 1, based on their 16S rRNA fingerprints but displayed a variety of phenotypic characteristics (Vos et al. 2009). Despite the acclimation of acidogens under acidic condition, the enzymatic activity at pH 4 and 5 (**Figure 4.3**) and the sludge solubilization mediated by acid dosage here were lower than in alkaline conditions. Consequently, less solubilized organic matters were available to be acidified (**Figure 4.2A**). In addition, *Hallella* and *Oscillibacter* were the genera enriched at pH 4, but their optimal growth conditions were not at pH 4 (Iino et al. 2007; Krieg et al. 2010). The enriched microbial communities at pH 4 might not be functioning optimally and this could also explain its low acidogenic efficiency, as the VFA component contributed only half of the sludge solubilization (**Figure 4.2A**).

Under alkaline condition, **Figure 4.7** shows that *Tissierella* could be detected at pH 8, 9 and 10. In addition, *Petrimonas*, *Proteiniphilum*, *Levilinea* and *Proteiniborus* could be found at pH 8 and 9. *Tissierella* grew optimally at mesophilic condition and produced acetic, butyric, isovaleric acids at optimum pH of 8.3 (Vos et al. 2009), which was similar condition to the pH range it was enriched in this study. *Levilinea* is an anaerobic microorganism isolated from an anaerobic reactor, which ferments sugars and amino acids into hydrogen, acetic and lactic acids between pH 6 - 7.2 (Yamada et al. 2006). *Petrimonas*, *Proteiniphilum* and *Proteiniborus* are fermentative *Bacteria*, producing mainly acetic acid, hydrogen and carbon dioxide (Vos et al. 2009; Krieg et al. 2010), with the latter two mainly using proteins as fermentative substrate, with tolerable pH ranges of 6 - 10 (Niu et al. 2008). *Petrimonas* grew optimally at pH 7.2 (Krieg et al. 2010). *Sedimentibacter* was only

Table 4.3. The pH value and sodium chloride concentration cultivation ranges of the identified genera in the reactors.

Phylum	Genus	Cultivation pH range	Cultivation NaCl range
<i>Bacteroidetes</i>	<i>Bacteroides</i>	7 - 8.5	N.R.
		5.0 - 7.6	
	<i>Paludibacter</i>	(optimum 6.6)	0 - 0.5%
		5.0 - 8.5	
	<i>Parabacteroides</i>	(optimum 7 - 7.5)	0 - 2%
			0 - 4%
	<i>Petrimonas</i>	around 7.2	(optimum 0%)
		6.0 - 9.7	
<i>Caldiserica</i>	<i>Proteiniphilum</i>	(optimum 7.5 - 8)	0 - 5%
	<i>Hallella</i>	5.5 - 5.7	N.R.
	<i>Prevotella</i>	4.6 - 5	N.R.
<i>Chloroflexi</i>		5.5 - 7.5	
<i>Firmicutes</i>	<i>Caldisericum</i>	(optimum 6.5)	0.5 - 1%
	<i>Levilinea</i>	6 - 7.2	0 - 0.25%
	<i>Phascolarctobacterium</i>	N.R.	N.R.
	<i>Clostridium_sensu_stricto</i>	varied	varied
		6.4 - 10	
	<i>Proteiniborus</i>	(optimum 8.5 - 8.8)	0 - 2%
	<i>Sedimentibacter</i>	7 - 8.2	N.R.
		6.5 - 8.5	
	<i>Tissierella</i>	(optimum 8.3)	N.R.
		6.5 - 9	
	<i>Guggenheimella</i>	(optimum 7.5 - 8)	N.R.
		6 - 10.5	0 - 6%
	<i>Anaerobranca</i>	(optimum 8.5 - 9.5)	(optimum 1 - 2.5%)
		6.5 - 10.5	
	<i>Alkalibaculum</i>	(optimum 8 - 8.5)	N.R.
	<i>Clostridium_XI</i>	varied	varied
		5.5 - 8.5	
	<i>Oscillibacter</i>	(optimum 6 - 6.5)	0 - 4%

enriched at pH 8. It was known to ferment proteins through Stickland-type reactions within pH 7 - 8.2 to produce VFA (Vos et al. 2009), and hence, was likely involved in the acidogenesis of proteinaceous matter at pH 8. This was consistent with a previous study which utilized stoichiometric analysis to suggest the Stickland-type pathway as the main pathway for VFA production during WAS acidification in alkaline condition (Liu et al. 2012). These results demonstrated that *Tissierella*, *Petrimonas*, *Proteiniphilum*, *Levilinea*, *Proteiniborus* and

Sedimentibacter which were enriched at pH 8 were able to grow in mild alkaline condition. Their biotic contribution, as proven by the hydrolytic enzymatic activities in **Figure 4.3**, when combined with the abiotic solubilization mediated by sodium hydroxide dosage at pH 8, were able to yield comparable VFA accumulation to that at pH 10 during the mesophilic mixed sludge acidification with 3 days SRT and 35°C in this study. Hence, acidification at pH 8 is recommended.

At higher pH values, *Guggenheimella* was enriched at pH 10, which utilized mainly proteins to produce VFA (**Figure 4.7**) (Vos et al. 2009). The enrichment of *Anaerobranca*, *Alkalibaculum* and *Clostridium_XI* were also observed at pH 10 and 11. Both *Anaerobranca* and *Alkalibaculum* were known to produce VFA. *Anaerobranca* was considered an extremophile, able to survive in alkaliphilic environment (pH 6 - 10.5) and sodium chloride concentration up to 6% (w/v) (**Table 4.3**) (Vos et al. 2009). Sodium plays a significant role in substrate uptake and ATP generation for alkaliphiles, including *Anaerobranca* (Kevbrin et al. 2005). High sodium in the reactors at pH 10 and 11 would have resulted from the high dosage of sodium hydroxide used for pH adjustment and this might drive the enrichment of *Anaerobranca* (Zhang et al. 2010). All the reactors operated at pH 9 and above also managed to enrich carbohydrate- and protein-utilizing acidogens, but the additional bioenergetics requirement to survive under the stressed condition of high pH and salinity might lead to the reduced acidogenic activities, as observed in this study (Kevbrin et al. 2005). Mixed sludge acidification at pH 8 provided a balance of abiotic solubilization by sodium hydroxide and preservation of microbial activity to give optimal VFA accumulation in this study, resulting in suitable acidified products for subsequent bioprocess application.

4.4. Conclusion

The operating pH in the first reactor of a two-stage anaerobic digestion system was optimized to enhance the sludge hydrolysis and acidogenesis step. Sludge acidogenesis was preferred at pH 8 despite higher solubilization at higher pH, where proteins, carbohydrates and other organic matter formed the remaining organic compositions. Different microbial populations were enriched as the reactors were operated at different pH, according to populations' respective optimal growth conditions. The hydrolytic enzymatic activity results revealed a reduction in biotic hydrolytic activities outside pH 6, 7 and 8 even when suitable acidogens were enriched, indicating the impairment of biological activities. The solubilization products after acidogenesis above pH 8 also contained aromatic substances with high MW with possibly low biodegradability and would be unfavourable for subsequent bioprocess. *Tissierella*, *Petrimonas*, *Proteiniphilum*, *Levilinea*, *Proteiniborus* and *Sedimentibacter* were identified at pH 8 and contributed to the highest acidogenesis efficiency in this study. The overall sludge digestion performance incorporating this enhanced sludge solubilization and acidogenesis process in a two-stage anaerobic system should be examined.

Chapter 5

Enhanced Sludge Digestion Performance and Microbial Community Analysis in Alkaline Two-Stage Anaerobic System

5.1. Introduction

The results reported in **Chapter 4** indicated that operating pH of 8 was optimal for enhanced sludge solubilization and acidogenesis at 35°C and 3 days solids retention time (SRT). pH 8 gave optimal sludge hydrolysis and acidogenesis, while allowing methanogenesis which provides alkalinity (via carbon dioxide generation) and consumes potentially inhibitory acetic acid and hydrogen in the stage-1 reactor. This chapter will apply these conditions as a two-stage anaerobic digestion system, which was anticipated to simultaneously combine chemical and biological modes of sludge digestion enhancement. Aside from the enhanced hydrolysis, this method is expected to reduce chemical requirements of the alkaline pretreatment (normally at pH 10 or above) (Lin et al. 1997; Zhang et al. 2010; Li et al. 2012), and the subsequent pH correction before stage-2. In this study, the stage-2 would be operated with a longer SRT of 17 days. The extended SRT in stage-2 was thought necessary to ensure more complete sludge digestion and methane production. Thermophilic condition (55°C) had also been proven to improve sludge degradability as a sludge pretreatment step, with the method known as temperature phased anaerobic digestion (TPAD) (Ge et al. 2011). Both mesophilic and thermophilic conditions would be tested on the alkaline enhanced stage-1 to compare the sludge digestion performances under these two conditions.

The results obtained in **Chapter 4** had also identified unique microbial communities associated with the enhanced sludge solubilization and acidification at pH 8. It is therefore of interest to examine the influence of these unique microorganisms developed in the first stage on the overall two-stage system and its performance. Pervin et al. (2013) established that a temperature shift from

35°C to 55°C caused a microbial community shift from mesophilic to thermophilic microbial communities, which lead to a substantial improvement in the sludge hydrolytic efficiency. However, the effect of stage-1's temperature shift under mild alkaline condition (pH 8) from mesophilic (35°C) to thermophilic (55°C) temperatures on the microbial communities is unknown.

This study aims to: 1) evaluate the extent of sludge solubilization achieved in the alkaline enhanced stage-1 under mesophilic and thermophilic conditions, 2) determine if sludge digestion efficiency could be enhanced in the alkaline two-stage system with the stage-1 under mesophilic (35°C) and thermophilic (55°C) conditions compared to the conventional single-stage AD system, and 3) characterize the microbial communities in the alkaline two-stage systems and correlate microbial community shifts in relation to process performance.

5.2. Materials and Methods

5.2.1. Reactor operation

Two continuous stirred tank reactor (CSTR) systems, which had been described in **Chapter 3**, namely the single-stage and two-stage anaerobic systems, were operated in parallel (**Figure 3.1**). The single-stage AD system had a working volume of 50 L, while the two-stage AD system comprised a 7.5 L stage-1 and 42.5 L stage-2 reactor. Both systems were operated at 20 days overall SRT, with the stage-1 of the two-stage system at 3 days SRT and stage-2 at 17 days SRT. pH was controlled automatically with 5M sodium hydroxide and hydrochloric acid as required. The single-stage system was maintained at 35°C, without pH control and remained between pH 6.9 and 7.1 throughout the study. The pH and temperature of the two-stage system were altered according to the operating periods in **Table 5.1**.

All reactors were seeded with anaerobic sludge collected from an anaerobic digester in Ulu Pandan municipal water reclamation plant, Singapore. The feed sludge was a mixture of primary and secondary sludges from the same plant, collected weekly, and stored at 4°C before use. The characteristics of the feed

sludge were $32,300 \pm 3,400$ mg TCOD L⁻¹; $1,800 \pm 600$ mg SCOD L⁻¹; 25.3 ± 1.8 g TS L⁻¹; 20.2 ± 1.3 g VS L⁻¹; and pH 5.7 to 6.1.

Table 5.1. Operating parameters of the two-stage systems (average $\pm$ standard deviation).

Reactor operating period	Stage-1 reactor		Stage-2 reactor	
	pH	Temperature	pH	Temperature
Period 1 (0 to 89 days)	8 ± 0.02	35°C	6.8 to 7.6 (without control)	35°C
Period 2 (90 to 165 days)	8 ± 0.02	55°C	7.6 to 7.8 (without control)	35°C
Period 3 (166 to 252 days)	8 ± 0.02	55°C	6.9 to 7.1	35°C

5.2.2. Analytical methods

50 mL samples were collected twice weekly from each reactor's sampling valve. All the analyses involved in this study were described in the analytical methods of **Chapter 3**. Dissolved total ammonia nitrogen from the soluble fraction was measured with the Nessler method kit (Hach, USA). Daily biogas volume produced was measured with a thermal-based gas flowmeter (McMillan, USA) fitted on the reactor in period 1 and with a wet gas meter (Ritter, Germany) in period 2 and 3. The extent of sludge solubilization was calculated following Ge et al. (2011) and the total COD removal and VS reduction were calculated based on mass balance. Both of the equations had been described in **Chapter 3**. Single-factor ANOVA and Tukey's test were calculated in Microsoft Excel (Analysis ToolPak), as statistical analysis of the TCOD removal, VS reduction and methane yield for the two reactor configurations and operating conditions ($p < 0.05$ was considered as statistically significant).

5.2.3. DNA extraction, amplicon 454 pyrosequencing and the bioinformatics analysis

The materials and methods for genomic DNA extraction, amplicon 454 pyrosequencing, sequence processing and bioinformatics analysis were described in **Chapter 4**. The sequences were archived in NCBI Sequence Read Archive (SRA) with the accession SRP054901 (<http://www.ncbi.nlm.nih.gov/genbank/>). Heatmap

was constructed using R with heatmap.2 function, after preprocessing with Mothur. For visualization purposes, OTUs with less than 20 sequences per OTU were removed. The samples (at x-axis) and OTUs with genus classification (at y-axis) were hierarchically clustered using Bray-Curtis algorithm. Good's coverages in all samples were from 97.7% to 99.8% for *Archaea* and ranged from 73.4% to 94.0% for *Bacteria*.

5.2.4. Economic assessment

Economic assessment is conducted based on the anaerobic digester in Ulu Pandan Water Reclamation Plant, Singapore. The influent sludge flow is $200 \text{ m}^3 \text{ d}^{-1}$ with sludge contents of $16.3 \text{ kg TSS m}^{-3}$ and $12.9 \text{ kg VSS m}^{-3}$ (Xie et al. 2007). Hence, volume of the stage-1 reactor is calculated to be 600 m^3 . The economic assessment accounts for the additional cost for the construction of stage-1 digester and additional operational cost (sodium hydroxide dosing), but more methane is generated and less sludge disposed. Capital costs for civil and electromechanical works are calculated as reported by Bolzonella et al. (2005) and calculated to be SGD 353,400. The cost of sodium hydroxide is SGD 410 per ton, sludge disposal (25% on TSS) is SGD 70 per ton and methane is SGD 0.19 per m^3 .

5.3. Results and discussion

5.3.1. Sludge solubilization in the alkaline enhanced stage-1 of the alkaline two-stage system

The extent of sludge solubilization in the alkaline enhanced stage-1 was calculated according to **Eq. (3.1)** and shown in **Figure 5.1**. Stage-1 operation in mesophilic condition during period 1 achieved 11% - 23% sludge solubilization. The application of mild alkaline condition in mesophilic stage 1 allowed this study's reactor to have higher sludge solubilization compared to a first reactor (pH 5.5) of a two-phase anaerobic system fed with the same feed sludge source, calculated to give around 7% solubilization at 35°C with 3 days SRT (**Chapter 3**). Besides sludge solubilization, acidogenesis is also important in the alkaline enhanced stage-1 to

provide necessary substrates (i.e. VFA) for subsequent methanogenesis in stage-2. During the mesophilic stage-1 start-up operation (0 to 59 days), TVFA accounted for an average of $10.8\% \pm 2.2\%$ sludge solubilization and methane production at average of $3.4\% \pm 0.9\%$ (**Figure 5.1A**). After the methanogens had acclimated (66 to 84 days), the average extent of solubilization was $12.8\% \pm 1.7\%$, TVFA's contribution was reduced to $4.1\% \pm 0.9\%$ solubilization while methane increased to $5.8\% \pm 1.3\%$. However, there was sCOD unaccounted for besides TVFA and methane, and this was identified as "others" at an average of $2.5\% \pm 1.4\%$. It is possible that such materials included protein, carbohydrate, ethanol, lactic acids or inert materials (Batstone et al. 2002; Zhang et al. 2009). **Figure 5.1B** shows that VFA composition in the mesophilic stage-1 was dominated by acetic acid, which was preferred for methanogenesis, followed by propionic acid. The results demonstrated that the simultaneous combination of alkaline mediated hydrolysis and biological activities indicated by VFA production and methanogenesis led to an enhanced sludge solubilization and acidogenesis in the mesophilic alkaline stage-1 in this study.

Higher solubilization was obtained in period 2 and 3 (thermophilic, 55°C) with 22% - 31% of sludge solubilization, compared to in period 1 (mesophilic, 35°C) (**Figure 5.1A**). This was consistent with previous findings comparing TPAD and mesophilic two-phase systems without alkaline condition (Ge et al. 2010; Kim et al. 2013). During the thermophilic alkaline enhanced stage-1 start-up operation (96 to 171 days), TVFA contributed an average of $14.9\% \pm 2.7\%$ sludge solubilization and methane production at average of $2.6\% \pm 0.9\%$. After the methanogens had acclimated (180 to 250 days), average extent of solubilization was $28.1\% \pm 2.0\%$ and there was no obvious difference between the TVFA contribution in solubilization which was at $16.1\% \pm 3.3\%$ as higher methane production contributed $5.4\% \pm 0.4\%$. The VFA composition in thermophilic stage-1 was also mainly acetic and propionic acids (**Figure 5.1B**). Combination of alkaline mediated hydrolysis by sodium hydroxide and the evidence of biological activities also enhanced sludge solubilization and its acidogenesis in the thermophilic alkaline

stage-1 in this study, but was found higher than that at mesophilic temperature. Specialized microbial communities associated with enhanced sludge hydrolysis, acidogenesis and methanogenesis enriched in the mesophilic and thermophilic alkaline enhanced stage-1 would be discussed later.

Figure 5.1. The extent of sludge solubilization with the organic compositions (a), and the VFA concentrations with its speciations (b) in the alkaline enhanced stage-1 at mesophilic (35°C) condition in period 1 and thermophilic (55°C) condition in period 2 and 3.

Increase in the ammonium ion had typically been associated with cell disruption and release of proteinaceous cellular content followed by biological deamination (Kayhanian 1999). **Figure 5.2** shows that The ammonium ion concentration in the alkaline enhanced stage-1 increased from 540 in period 1 to 790 mg N-NH₄⁺ L⁻¹ in period 2 and 680 mg N-NH₄⁺ L⁻¹ in period 3, corresponding to the higher sludge solubilization in the thermophilic condition (**Figure 5.1**). It was higher than that measured in feed sludge (105 - 200 mg N-NH₄⁺ L⁻¹), and was only slightly lower than in the single-stage and stage-2 (590 - 790 mg N-NH₄⁺ L⁻¹ and 790 - 860 mg N-NH₄⁺ L⁻¹, respectively). This further demonstrated the destruction of proteinaceous material and its deamination in both the mesophilic and thermophilic alkaline enhanced stage-1. Both the presence of ammonia and VFA conversion into methane and carbon dioxide in stage-1 could be useful in maintaining the pH value and reducing alkali consumption (Batstone et al. 2002). However, higher temperature and pH increased the speciation of inhibitory free ammonia (NH₃). Based on estimation, free ammonia concentrations were calculated to be 53 mg N-NH₃ L⁻¹ in period 1, 220 mg N-NH₃ L⁻¹ in period 2 and 190 mg N-NH₃ L⁻¹ in period 3.

Figure 5.2. Ammonium concentrations in the feed sludge, single-stage and two-stage systems.

Previous studies reported tolerable free ammonia concentration to be below 150 mg N-NH₃ L⁻¹ in anaerobic system (Kayhanian 1999). Methanogenesis in the thermophilic stage-1 would have been adversely affected, but methane production in thermophilic stage-1 (4.3 ± 0.3 L d⁻¹) still persisted and was only slightly lower than during mesophilic stage-1 operation (5.0 ± 0.9 L d⁻¹) (**Figure 5.3D**). Methanogens tolerant to inhibitory ammonia concentration might be developed in the thermophilic alkaline enhanced stage-1 and this would be evaluated later.

5.3.2. The overall sludge digestion and methane production

Parallel operation of the alkaline two-stage and the conventional single-stage AD systems showed that the former consistently achieved higher COD removal, VS reduction and methane production than the single-stage system at 20 days SRT (**Figure 5.3**). The average TCOD removal, VS reduction, methane production and methane yield in the alkaline two-stage system with mesophilic stage-1 (period 1) were $44 \pm 6.3\%$, $39.8 \pm 5.9\%$, 12.8 ± 1.1 L d⁻¹ and 0.160 ± 0.027 L g TCOD_{added}⁻¹, respectively; whereas these were $37 \pm 5.1\%$, $30.9 \pm 6.5\%$, 10.2 ± 0.9 L d⁻¹, and 0.126 ± 0.018 L g TCOD_{added}⁻¹, respectively, in the single-stage system (period 1). This resulted in the mesophilic-mesophilic alkaline two-stage system operated in period 1 generating 18.9%, 28.9% and 27.2% higher TCOD removal, VS reductions and methane yield over the conventional single-stage system in period 1. On the other hand, operation of a mesophilic-mesophilic two-phase anaerobic system acidic first reactor fed with the same feed sludge source reported in **Chapter 3** achieved only 31.6% VS reduction. Ge, et al. (2011) also operated a mesophilic-mesophilic two-phase system (2 + 14 days SRT), with phase-1 at pH 6.6, fed with sludge of similar characteristics as presented in this study, and obtained 34% VS reduction. This indicated the greater sludge destruction capability associated with the alkaline two-stage anaerobic system.

Moving the alkaline enhanced stage-1 from mesophilic (35°C) to thermophilic (55°C) conditions during period 2 did further enhance sludge solubilization efficiency in stage-1. Consequently, it gave slightly higher overall process performance compared to the alkaline two-stage system with mesophilic stage-1.

Average TCOD removal of $46.4 \pm 4.2\%$, VS reduction of $41.2 \pm 4\%$, methane production of $15.2 \pm 0.8 \text{ L d}^{-1}$ and methane yield of $0.177 \pm 0.017 \text{ L g TCOD}_{\text{added}}^{-1}$ were obtained in the alkaline two-stage system with thermophilic stage-1. However, these differences were found to be not statistically significant ($p < 0.05$).

Figure 5.3. Organic loading rate, OLR (A), TCOD removal (B), VS reduction (C) and methane production (D) of the single-stage and alkaline two-stage AD systems.

This implied that the stage-2 also played an important role in the overall sludge digestion of the two-stage system. It is possible that the extended operation of

stage-2 at 17 days SRT masked the accelerated hydrolytic efficiency achieved in thermophilic alkaline enhanced stage-1, and hence, no substantial difference in the overall alkaline two-stage systems' COD removal, VS reduction and methane yield when stage-1 was operated in mesophilic and thermophilic temperatures. Since hydrolysis is known to be the rate-limiting factor in sludge digestion, higher sludge solubilization associated with thermophilic alkaline enhanced stage-1 operation might allow for shorter SRT requirement in stage-2, but further study is required. Considering the additional energy required maintaining a thermophilic (55°C) condition, the small process improvement in this study would not merit departure from the mesophilic alkaline enhanced stage-1 in the alkaline two-stage anaerobic system at SRT of 3 + 17 days (**Appendix D**).

The highest pH reached in stage-2 during period 1 and 2 were 7.6 and 7.8, respectively. To ensure sludge digestion and methane production would not be adversely affected by pH, stage-2 pH was controlled at pH 7.1 by dosing 5M hydrochloric acid from 167 days in period 3. The adjustment did not produce significantly different organic reduction ($47 \pm 3.1\%$ average TCOD removal) and methane yield (0.177 ± 0.015 L g TCOD_{added}⁻¹) compared to period 1 and 2 ($p < 0.05$). Most alkaline pretreatment strategies which employed alkaline addition to pH 10 or above required acid dosage to neutralize the pretreated sludge, before anaerobic digestion (Zhang et al. 2010; Kim et al. 2013). The use of mild alkaline (pH 8) condition in this study demonstrated that the mild alkaline condition coupled with mesophilic conditions in the alkaline enhanced stage-1, and self-buffered pH and mesophilic conditions in stage-2 would be both energetically and chemically sustainable.

Table 5.2. Economic estimate based on laboratory results between conventional single-stage and alkaline two-stage anaerobic digestion systems.

Additional expense for NaOH dosage (per year)	\$	54,200
Additional income from enhanced sludge reduction (per year)	\$	17,700
Additional income from enhanced methane production (per year)	\$	8,800
Net savings (per year)	\$	(27,700)

Table 5.2 presents the economic estimate between operating conventional single-stage and alkaline two-stage anaerobic digestion system. Despite the improvement in higher sludge reduction and methane production, the cost of chemical dosing was higher and the operation of alkaline two-stage anaerobic system operation did not result in positive net savings. However, a strict pH control in the stage-1 reactor may not be necessary. It can be seen that the extent of solubilization of semi-continuous reactor controlled every 12 hours at pH 8 in **Chapter 4** (around 15%) was similar to the average extent of solubilization of mesophilic stage-1 in this study, which was automatically controlled in real-time (11 – 23%). Lime may also be used as a cheaper alternative to sodium hydroxide. In addition, Shi et al. (2015) found that higher influent solids concentration resulted in higher sludge solubilization efficiency under the same alkali to sludge TS ratio, with the highest sludge solubilization at 90 g TS L⁻¹. Therefore, there is further room to optimize pH control of the stage-1 which may reduce chemical consumption and the amount of treated sludge to be disposed; resulting in positive net savings for the alkaline two-stage anaerobic system.

In many places, digested sewage sludge are dewatered and utilized as nutrient source (nitrogen and phosphate) in agricultural application (Tchobanoglous et al. 2003). Enhanced sludge digestion through the alkaline two-stage anaerobic system would decrease the amount of nutrients available. This is because enhanced digestion involves higher degree of cellular break-up which releases the

intracellular content containing nitrogen and phosphorus. This was evident in **Figure 5.2**, which showed more ammonium being released to the soluble fraction of the stage-2 than single-stage reactor. The released nutrients are then removed from the digestate during sludge dewatering. However, there are alternative methods to recover this nutrients as fertilizers by forming struvite or magnesium ammonium phosphate hexahydrate ($\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$), which had been reported previously in the literature (Ueno and Fujii 2001; Tong and Chen 2009).

5.3.3. Microbial community characterization

A total of 319,211 archaeal 16S rRNA sequences and 227,539 bacterial 16S rRNA sequences were obtained using 454 pyrosequencing. These sequences were processed using Mothur and R to reveal the temporal community shifts and the respective microbial compositions in the single-stage and alkaline two-stage systems.

5.3.3.1. Microbial shifts and identification of Archaea

Methanogenic taxa could only be found under the domain *Archaea* (Garcia et al. 2000), and were the only consortium found in the archaeal-specific 16S rRNA pyrosequencing. **Figure 5.4** presents the clustering of reactor samples according to their similarity in microbial community structures. It could be seen that the archaeal communities in the single-stage reactor in all periods and stage-2 in period 2 and 3 were closely related. The second archaeal cluster consisted of archaeal communities in stage-1 and stage-2 at 45 and 70 days during period 1, and the third cluster consisted of archaeal communities in the feed sludge, stage-1 at 0 and 12 days and stage-1 in period 2 and 3 (**Figure 5.4**). These results proved that the operation of alkaline enhanced stage-1 in mild alkaline condition with mesophilic and thermophilic temperatures led to enrichment of different specialized methanogens. However, all samples were mainly predominated by *Methanolinea* (OTUA00001) and *Methanospirillum* (OTUA00002 and OTUA00004), both of which were hydrogenotrophic methanogens (Garcia et al. 2000). In fact, these two genera were also detected abundantly in the feed sludge. Known acetate-utilizing methanogens, *Methanosaeta* and *Methanosarcina*, were not

found in any sample, except *Methanosarcina* (OTUA00005) in the mesophilic alkaline enhanced stage-1. This finding is consistent with previous data that hydrogenotrophic *Methanomicrobiales* predominated in AD processes treating sewage sludge (Jaenicke et al. 2011; Kim et al. 2013). It was suggested that acetoclastic methanogenesis contributed to around two-thirds of methane production in an anaerobic process (Speece 1996; Garcia et al. 2000). Hydrogenotrophic methanogens were in fact, more robust and hence, more abundant than acetoclastic methanogens in the presence of high ammonia and acetic acid (Karakashev et al. 2005; Lü et al. 2013). The predominance of hydrogenotrophic methanogens in this study might be selected by ammonia, released during the digestion of proteinaceous materials in the sludge.

The second cluster revealed that controlling the pH at 8 and SRT of 3 days in the mesophilic alkaline enhanced stage-1 during period 1 drove the adaptation of *Methanosarcina* (OTUA00005) and *Methanobrevibacter* (OTUA00008 and OTUA00012) (**Figure 5.4**). The subsequent stage-2 also contained *Methanosarcina* and *Methanobrevibacter*, albeit at lower relative abundances. Temporal analysis of the methanogenic community indicated the contribution of *Methanosarcina* and *Methanobrevibacter* to methanogenesis in the mesophilic alkaline enhanced stage-1. The relative abundances of *Methanosarcina* and *Methanobrevibacter* OTUs (OTUA00005, OTUA00008 and OTUA00012) rose from the 12th day (0%, 1.3%, and 0.15%, respectively) to the 45th day (19.3%, 9.2% and 8.2%, respectively). The increase of the relative abundances corresponded to the increase in methane production from 2.2 L d⁻¹ at 12 days to 2.8 L d⁻¹ at 45 days, and later, 4.2 L d⁻¹ at 70 days (**Figure 5.3D**). *Methanosarcina* was known to be stress-tolerant and was normally enriched in the presence of high ammonia (> 1,500 mg N-NH₄⁺ L⁻¹) and VFA concentrations (> 8.5 mM acetate) (Karakashev et al. 2005; Lü et al. 2013). VFA accumulation observed in stage-1 occurred due to faster rate of VFA production by acidogens than VFA utilization by methanogens, as enhanced sludge solubilization occurred (Zheng et al. 2013). The activity of methanogens in the alkaline enhanced stage-1 is beneficial since methanogens consumed accumulated

VFA or hydrogen under the enhanced sludge solubilization condition, which could be potentially inhibitory to further acidogenic and methanogenic reactions (Chen et al. 2008; Stams and Plugge 2009).

Figure 5.4. Heatmap depicting the average counts (log₂-transformed) of archaeal OTU (97% similarity) in each sample after “sub.sample” and OTUs < 20 counts removed. The samples (x-axis) and OTUs (y-axis) were both hierarchically clustered using Bray-Curtis similarity index. The OTUs were classified at genus level using RDP database with 50% threshold. Period 1 was represented by 12, 45 and 70 days; period 2 by 116 and 158 days; and period 3 by 217 days. The symbols besides the OTUs indicated its predominance throughout an operating period in: (●) SS, single stage reactor; (x) TS1, stage-1 of alkaline two-stage system; and (□) TS2, stage-2 of alkaline two-stage system. Feed sludge was abbreviated as FS.

Figure 5.4 shows that the thermophilic alkaline enhanced stage-1, which had formed another separate cluster, enriched a thermophilic hydrogenotrophic methanogen, *Methanothermobacter* (OTUA00013) (Garcia et al. 2000). An adaptation process was also observed where the increase in relative abundances of *Methanothermobacter* (OTUA00013) from 116 days (0.8%) to 158 days (2.9%) and 217 days (9.2%) (**Appendix E1**) correlated with the increase in methane production with 1.1 L d⁻¹ at 116 days, 2.8 L d⁻¹ at 158 days and 4.3 L d⁻¹ at 217 days (**Figure 5.3D**). It could also be inferred that *Methanothermobacter* was involved in the methanogenesis in the presence of inhibitory free ammonia concentration (190 to 220 mg N-NH₃ L⁻¹) during the thermophilic alkaline enhanced stage-1 operation. Previous study reported that *Methanothermobacter* replaced *Methanosarcina* as dominant methanogen in a thermophilic anaerobic process treating chicken manure containing up to 2,000 mg N-NH₃ L⁻¹ (Niu et al. 2013). This demonstrated the adaptation of ammonia-tolerant methanogen for the operation of thermophilic alkaline enhanced stage-1 at 55°C and pH 8.

5.3.3.2. Microbial shifts and identification of Bacteria

Microorganisms under the domain *Bacteria* were able to carry out various metabolic functions, including hydrolysis, acidogenesis and acetogenesis reactions in AD process (Nelson et al. 2011). **Figure 5.5** shows that the microbial communities in the single-stage and mesophilic-mesophilic alkaline two-stage systems were clustered separately. In addition, even further microbial differentiation occurred when the mesophilic alkaline enhanced stage-1 was adjusted to thermophilic condition. Similar to the archaeal communities, the results showed that the microbial community structures in the single-stage and alkaline two-stage anaerobic systems were non-random, and that specialized microbial populations developed as different reactor configurations and operating conditions were applied.

Bacteria in the single-stage reactor remained clustered together throughout this study, as no process modification was applied (**Figure 5.5**). The single-stage reactor was mainly predominated by the unclassified *Flavobacteriales* (OTUB00001),

Levilinea (OTUB00003), *Paludibacter* (OTUB00007) and unclassified *Bacteroidetes* (OTUB00009). Bacterial communities in the seed sludge of the two-stage system were also closely related to this single-stage system community. Functionally, these microorganisms are associated with hydrolysis and acidogenesis of carbohydrates, proteins and lipids, and ensured the production of VFA for methanogenesis (Ariesyady et al. 2007; Krieg et al. 2010; Regueiro et al. 2012). Acetogenic microorganisms, such as *Smithella* (OTUB00012) and *Synergistaceae* (OTUB00033) were also detected in the single-stage and all stage-2s. These microorganisms performed the oxidation of thermodynamically challenging substrates (e.g. propionic acid and some species of amino acids) into acetate or carbon dioxide in the presence of its hydrogen-utilizing microorganisms, which in this case, was hydrogenotrophic methanogens (Stams and Plugge 2009).

Figure 5.5 shows that the maintenance of pH 8 and 3 days SRT led to a microbial community shift from the single-stage bacterial consortium to a consortium acclimated for the operating conditions of the mesophilic alkaline enhanced stage-1 reactor. The shift was mainly attributed to the enrichment of *Flavobacteriaceae* (OTUB00002), *Proteiniborus* (OTUB00013), unclassified *Firmicutes* (OTUB00016), *Saccharofermentans* (OTUB00020), *Levilinea* (OTUB00025) and *Tissierella* (OTUB00063), while those *Bacteria* predominant at 0 day were reduced. These enriched OTUs were found to be up to 26%, 8%, 5%, 6%, 5% and 5% in relative abundances, respectively, throughout period 1 (**Appendix E3**). Their predominance was detected from the 12th day sample, and occurred along with the increase in sludge solubilization to 23%, in which 18% and 3% were in the form of VFA and methane, respectively (**Figure 5.1**). It was noted that *Levilinea* and *Flavobacteriaceae* were also observed in the stage-2 and single-stage reactors, but their high relative abundances in the mesophilic alkaline enhanced stage-1 suggested their activities in this reactor, too. *Proteiniborus*, *Saccharofermentans* and *Tissierella* were classified under the order *Clostridiales*. *Proteiniborus* had been found to perform protein hydrolysis and acidogenesis with optimum pH of 8.5 - 8.8

Figure 5.5. Heatmap depicting the average counts ($\log_2$ -transformed) of bacterial OTU (97% similarity) in each sample after “sub.sample” and OTUs < 20 counts removed. The samples (x-axis) and OTUs (y-axis) were both hierarchically clustered using Bray-Curtis similarity index. The OTUs were classified at genus level using RDP database with 50% threshold. Period 1 was represented by 12, 45 and 70 days; period 2 by 116 and 158 days; and period 3 by 217 days. The symbols besides the OTUs indicated its predominance throughout an operating period in: (●) SS, single stage reactor; (x) TS1, stage-1 of alkaline two-stage system; and (□) TS2, stage-2 of alkaline two-stage system. Feed sludge was abbreviated as FS.

to produce acetate, alcohol and hydrogen (Niu et al. 2008); *Saccharofermentans* fermented hexoses, polysaccharides and alcohols to produce acetate, lactate and fumarate at pH 5.0 - 7.5 (Chen et al. 2010); while *Tissierella* had been isolated from human faeces and anaerobic digesters, and produced acetic acid from creatine at optimum pH of 8.3 (Vos et al. 2009). Together with *Flavobacteriaceae* and *Levilinea*, these microorganisms could grow at pH 8 and were presumably associated with the enhanced hydrolysis of sludge organic components and their acidogenesis in the mesophilic alkaline enhanced stage-1. Previous microbial community analysis in a mesophilic (35°C) acidic first reactor (pH 5.2 – 5.5) of a two-phase system also yielded similar bacterial communities at order level to those in this study's mesophilic alkaline enhanced stage-1 (Shimada et al. 2011). Shimada et al. (2011) observed the predominance of *Clostridia*, while *Flavobacteriales* and *Clostridiales* predominated in the mesophilic acidic first reactor (pH 5.5) of a two-phase system investigated in **Chapter 3**. It has to be noted that the *Clostridia* and *Flavobacteriales* enriched in this two-stage system might be different at genus-level, representing possibly different metabolic capabilities. Nevertheless, different activities were obtained with 32% overall VS reduction efficiency in the latter study, but 39.8% overall VS reduction with the alkaline two-stage system in this study. This demonstrated that the combination of chemical (via sodium hydroxide mediated hydrolysis) and biological effects (via acclimation of specialized microorganisms) in the alkaline two-stage anaerobic system was able to enhance sludge digestion efficiency compared to a single-stage and two-phase system.

Application of thermophilic condition in the alkaline enhanced stage-1 induced strong selective pressure to the bacterial community as the lowest bacterial richness was observed (**Figure 5.5**). Here, unclassified *Clostridiales* of different OTU to that in the mesophilic alkaline enhanced stage-1 (OTUB00005, OTUB00023 and OTUB00044) and *Haloplasma* (OTUB00006) formed the predominant *Bacteria*. They were up to 47%, 18%, 13% and 38% in relative abundance, respectively; whereas none was detected in the feed sludge and single-stage reactor (**Appendix**

E4). *Haloplasma* had been detected previously in an anaerobic leach bed reactor treating rye silage and straw materials, operated at 65°C, pH 8 and 21 days SRT (Rademacher et al. 2012), which was similar to the operating temperature and pH of the thermophilic alkaline enhanced stage-1 in this study. Interestingly, *Haloplasma's* phylum *Tenericutes* was not described in the AD community meta-analysis by Nelson et al. (2011) and presents a rare microbial clade that are yet to be explored in AD process. Comparison against previous studies also found that the operation of thermophilic stage-1 at pH 8 in this study enriched a different microbial population to those in the thermophilic first reactor of the TPAD system without mild alkaline condition (Pervin et al. 2013). Despite the low bacterial richness in thermophilic alkaline enhanced stage-1 (**Figure 5.5**), solubilization and acidogenesis were higher than its mesophilic counterpart (**Figure 5.1A**). Sludge solubilization in the thermophilic alkaline enhanced stage-1 at 219 days was 26% (18% VFA and 4% methane), while sludge solubilization in the mesophilic stage-1 at 70 days was 11% (4% VFA and 5% methane). Thermophilic *Bacteria* were known to have higher metabolic activity, including acidogenesis and extracellular enzyme productions for hydrolysis, compared to its mesophilic counterpart (Zbransk et al. 2000), and would explain the improved performance. Hence, the enhanced sludge solubilization and acidogenesis associated with operating stage-1 at mesophilic mild alkaline condition could be further improved by adapting these specialized thermophilic bacteria at 55°C and pH 8 in stage-1.

Nevertheless, the higher solubilization observed in the alkaline enhanced stage-1 at thermophilic conditions (**Figure 5.1A**) was likely compensated in the subsequent stage-2, as the slight improvement to the overall alkaline two-stage system organic reduction and methane production were not statistically significant in this study. The highest extent of solubilization in mesophilic and thermophilic alkaline enhanced stage-1 were 23% and 31%, respectively. On the other hand, overall TCOD reduction of the two-stage system reached an average of 44% with the mesophilic stage-1 and 46.4% with the thermophilic stage-1, confirming the role of stage-2 for further sludge solubilization and digestion. This was also supported by

the detection of hydrolytic and acidogenic *Bacteria* in the stage-2s, which originated from their respective mesophilic and thermophilic alkaline enhanced stage-1s (**Figure 5.5**). The adjustment of operating pH of the stage-2 from pH 7.8 (period 2) to 7.1 (period 3) did not cause microbial community shift (from the stage-2 sample at 158th days in period 2 to 217th days in period 3) (**Figure 5.5**), and consequently, also no significant difference to the TCOD removal and methane yield. While many have argued the optimum pH for AD is between 6.5 and 7.2 (Appels et al. 2008; Zhang et al. 2010), this study has shown the microorganisms in stage-2 tolerated pH up to 7.8, which was induced by the sodium hydroxide dosage in the alkaline enhanced stage-1. Microorganisms tolerant to mild alkaline condition enriched in the mesophilic and thermophilic alkaline enhanced stage-1 would have been transferred to the respective stage-2; and were evident in the microbial community analysis in **Figure 5.5**. Hence, the stage-2 of the two-stage system in this study could be operated without prior pH adjustment to neutral.

Some similarities were observed comparing the bacterial communities in the continuous mesophilic stage-1 reactor (3 days SRT) in this Chapter and those in the semi-continuous mesophilic reactor controlled at pH 8 and 3 days SRT in **Chapter 4**. *Proteiniborus*, *Levilinea*, *Tissierella* were enriched in both reactors, but, there were also other different taxons that were enriched in individual reactor. Furthermore, the continuous mesophilic stage-1 reactor enriched *Methanosarcina* and *Methanobrevibacter*, which likely contributed to methanogenesis in this case; while the semi-continuous mesophilic reactor controlled at pH 8 enriched another genus, *Methanocorpusculum*. The operating pH of the semi-continuous system was adjusted every 12 hours, resulting in variations of pH 7.1 – 8.2, whereas the continuous system maintained strict pH control of 7.98 – 8.02. Interestingly, *Methanocorpusculum* was not enriched in the continuous mesophilic stage-2 operated at 17 days SRT (pH 6.8 – 7.8), and this may be due to the difference in SRT. The results suggested that different reactor configuration may result in different microbial community structure.

Nevertheless, temporal microbial community analysis in this study found that microbial communities from the same reactor with controlled operating parameters were clustered together over the span of the experimental period (**Figure 5.5**). This finding suggested that deterministic factors, in this case the reactor operating parameters and environmental conditions, determined microbial community dynamics and supported the niche based theory in microbial ecology. Recent studies using high throughput sequencing technology have also supported niche based theory to explain microbial dynamics in anaerobic processes (Vanwonterghem et al. 2014; Luo et al. 2015). Under a properly controlled condition, microbial community dynamics would then be expected to be predictable. Hence, molecular tools might be used as early warning signal to process inhibition or failure. In the alkaline enhanced stage-1, acclimation of *Bacteria* was noted to be faster at 12 days or less, compared to the methanogens' adaptation, occurring between 12 days and 45 days (**Figure 5.4** and **Figure 5.5**). Enhanced hydrolysis and acidogenesis were thus achieved faster than methanogenesis. As a consequence, process imbalance in the alkaline enhanced stage-1 and then subsequently in stage-2 might occur during the adaptation process. High VFA production associated with enhanced sludge solubilization could accumulate beyond inhibitory concentrations, leading to process failure. Therefore, monitoring of key methanogens, such as *Methanosarcina*, *Methanobrevibacter* and *Methanothermobacter* could serve as a predictive or troubleshooting tool in the future to ensure a successful reactor operation.

5.5. Conclusion

This study showed that the mesophilic (35°C) alkaline two-stage anaerobic system incorporating the combination of alkaline mediated hydrolysis and biological activities in the stage-1 achieved higher TCOD removal, VS reduction and methane yield compared to the conventional single-stage system. Thermophilic condition (55°C) further enhanced sludge solubilization and acidification in the alkaline

enhanced stage-1 and could potentially reduce system SRT. Microbial community analysis revealed the enrichment of specialized microorganisms in the alkaline two-stage system, which was capable of tolerating mild alkaline condition and was associated with enhanced hydrolysis and acidogenesis of the feed sludge's organic components. Therefore, enhanced sludge digestion could proceed without the need for pH neutralization and so eliminated the additional chemical requirement. Temporal microbial community analysis also indicated that the microbial community structure is influenced by deterministic, rather than stochastic factors associated with the reactor operating conditions, which may influence the microbial community dynamics. This implied that microbial community dynamics were predictable and under properly controlled operating conditions, microbiological molecular tools might be applied as monitoring tools to predict process inhibition, failure or to be used for process troubleshooting.

Chapter 6

Anaerobic Co-digestion of Sewage Sludge with Waste Cooking Oil in Alkaline Two-Stage Anaerobic System

6.1. Introduction

The anaerobic sludge digestion process does not only reduce volatile solids (VS) and improve sludge quality, but also produces a renewable source of bioenergy, e.g. methane (Tchobanoglous et al. 2003; Appels et al. 2008). Fat, oil and grease (FOG) are a type of organic waste commonly found in urban areas or industries, which contained high energy content but is not easily treated by anaerobic digestion (AD). FOG can be collected from the grit collection chamber in a wastewater treatment plant (WWTP) or kitchen grease traps or interceptors (Long et al. 2012). Hydrolysis of lipid found in the FOG into long chain fatty acids (LCFA) and glycerol proceeds easily, but LCFA may accumulate to inhibitory concentrations, resulting in the failure of the anaerobic process (Hwu et al. 1998). Furthermore, FOG tends to lack necessary nutrients for microbial growth during AD process. Anaerobic co-digestion of sewage sludge with FOG becomes an attractive option to improve a digester's methane yield as sufficient nutrients are available in the sewage sludge and potential LCFA inhibition can be mitigated. Furthermore, it has been estimated that the biogas produced in the anaerobic sludge co-digestion with FOG could support over 50% of the energy requirement of a WWTP compared to 20% - 40% with conventional anaerobic sludge mono-digestion (Long et al. 2012).

However, there are three main problems associated with treatment of lipid rich substrates by AD processes: (1) mass transport limitation as lipid or LCFA adsorb onto the cellular surfaces (Pereira et al. 2004), (2) sludge floatation leading to washout (Hwu et al. 1998), and (3) specific LCFA inhibition to anaerobic

microorganisms (Hwu and Lettinga 1997; Shin et al. 2003). Firstly, lipids and LCFA can adsorb to sludge and cellular surfaces. Hence, the substrate and products going in and out of the sludge are hindered (Pereira et al. 2002). The second reason applies to anaerobic processes involving sludge blanket, such as the upflow anaerobic sludge blanket (UASB) process (Hwu et al. 1998). In such process, sludge blanket may be unable to settle effectively because LCFA adsorbs to the sludge and it has a relatively low density (Hwu et al. 1998). Consequently, the sludge floats and gets washed out of the system, leading to process failure. Thirdly, LCFA has been found to severely inhibit syntrophic acetogens and methanogens (Rinzema et al. 1994; Hwu et al. 1998). The inhibition mechanisms have been proposed to be related to LCFA adsorption onto cell surfaces, which irreversibly impairs the transport and protective functions of the cell (Rinzema et al. 1994). Galbraith and Miller (1973) also suggested a surfactant effect by LCFA in the sludge matrix causing damage the cell membrane. However, recent studies suggest a rethink to the inhibition mechanism as more studies reveal that inhibition is not permanent, but involves a reversible mass transfer limitation on cell surfaces, a reversible metabolic inhibition mechanism, or their combination (Pereira et al. 2004). The inhibition mechanism and severity may also be influenced by the type of LCFA as had been demonstrated previously (Kim et al. 2004; Pereira et al. 2004).

The application of alkaline two-stage anaerobic configuration described in **Chapter 5** is expected to address these challenges and further improve methane yield. Higher LCFA loading was reported to be tolerated by the traditional (acidic/neutral pH) two-phase than single-stage configuration, as the first reactor can act as a buffer to protect the sensitive acetogens and methanogens (Kim et al. 2004). The alkaline condition in the alkaline enhanced stage-1 functions as an FOG pretreatment by saponification. Saponification involves the hydrolysis of glycerides in the FOG into glycerol and fatty acid salts with higher solubility (Carrere et al. 2012). This could in turn be easily utilized by microorganisms compared to the insoluble lipids, which cause adsorption and mass transport limitation on cell surfaces (Battimelli et al. 2009). On the other hand, excessive saponification could

cause foaming problem and cellular membrane destruction during mixing. Hence, mild alkaline condition as maintained in the alkaline enhanced stage-1 of the alkaline two-stage system is necessary. Moreover, the use of thermophilic rather than mesophilic temperature may also enhance methane yield by increasing microbial activities, the diffusion coefficients and lipid solubility in aqueous media (Chipasa and Mędrzycka 2006; Kabouris et al. 2009).

The sensitive LCFA degrading syntrophs and methanogens had been identified as key populations driving LCFA oxidation and methanogenesis in anaerobic processes (Sousa et al. 2009; Stams et al. 2012). So far, LCFA degrading bacterium have been identified belonging to the genera *Syntrophus* (*Proteobacteria* phylum), *Syntrophomonas* and *Thermosyntropha* (*Firmicutes* phylum), working in syntrophy with hydrogenotrophic methanogens, such as *Methanobacterium*, *Methanoautotrophicum* and *Methanospirillum* (Sousa et al. 2009). However, the microbial community involved in the anaerobic co-digestion of sludge and FOG is expected to be more complicated but is not well understood. Previous studies established that a microbial community diverges when fed with different type of substrates, such as FOG (Ziganshin et al. 2013; De Francisci et al. 2015). A more dramatic shift in the bacterial community was observed than in the archaeal community when a digester was switched from performing municipal waste mono-digestion to co-digestion with FOG (Martín-González et al. 2011; Lauwers et al. 2013). However, most of these studies were performed in a single-stage configuration and rarely in two-stage configuration (Kim et al. 2004).

Waste cooking oil (WCO) was chosen in this study as the FOG substrate. This study aims to extend the alkaline two-stage anaerobic digestion system to treat a mixture of sewage sludge and WCO. Increasing load of WCO in the feed will be tested to define the limit of the mixing ratio of WCO and sewage sludge in two-stage alkaline anaerobic co-digestion system. Microbial community shifts is elucidated to determine the key populations and investigate the relations between microbial communities, physiochemical profiles and reactor performances.

6.2. Materials and methods

6.2.1. Reactor start-up and operation

Semi-continuous reactors were operated in 2 L glass bottles with 1.8 L working volume, maintained at 3 + 17 days SRT for the alkaline two-stage system, by daily draw-fill method. The alkaline two-stage anaerobic systems were operated at 3 + 17 days SRT. A single-stage anaerobic reactor (20 days SRT and 35°C) fed with only sewage sludge served as control. A summary of the operating parameters of the different anaerobic systems were presented in **Table 6.1**. Mixed liquor mixing and temperature were controlled using temperature controlled magnetic stirrers, while pH was checked and adjusted once daily by dosing 5 M sodium hydroxide, when required. The reactors were initially seeded with anaerobic sludge treating sewage sludge from Ulu Pandan water reclamation plant, Singapore, and purged with nitrogen to eliminate oxygen in the headspace. The feed sludge is municipal mixed sewage sludge, composed of primary and secondary sludge from the same plant. WCO was prepared by boiling new vegetable cooking oil at 180°C for 8 hours and left to cool to room temperature. The feed sludge characteristics were 25.4 ± 3.0 g TS.L⁻¹; 20.3 ± 2.5 g VS.L⁻¹; $37,200 \pm 2,800$ mg TCOD.L⁻¹; $2,000 \pm 400$ mg SCOD.L⁻¹, pH 5.8 - 6.0 and characteristics of the waste cooking oil were 902.5 ± 4.6 g TS L⁻¹; 902.5 ± 4.6 g VS L⁻¹; $2,880,600 \pm 23,900$ mg TCOD L⁻¹; $2,344,400 \pm 48,400$ mg SCOD L⁻¹. WCO characterization revealed that TS = VS, and 81% of the SCOD (determined as centrifugation at 13,000 xg for 5 min and filtration through 0.45 µm) was equivalent to TCOD (SCOD/TCOD = 0.81).

Table 6.1. The operating parameters of the alkaline two-stage system in the anaerobic co-digestion of sludge with waste cooking oil (WCO).

Period	Feed ratio of feed sludge : WCO (g VS)	Meso stage-1		Thermo stage-1		Meso-meso/Thermo-meso Stage-2	
		pH	Temp.	pH	Temp.	pH	Temp.
1	75-80 : 20-25	6.6 - 8.2	35°C	7 - 8.2	55°C	6.9 - 7.1	35°C
2	65-70 : 30-35	6.6 - 8.2	35°C	7 - 8.2	55°C	6.9 - 7.1	35°C
3	55-65 : 35-35	6.6 - 8.2	35°C	7 - 8.2	55°C	6.9 - 7.1	35°C
4	45-52 : 48-55	6.6 - 8.2	35°C	7 - 8.2	55°C	6.9 - 7.1	35°C

6.2.2. Analytical methods

All the chemical analyses involved in this study were described in the analytical methods of **Chapter 3**. Daily biogas volume produced was measured with a wet gas meter (Ritter, Germany). The extent of sludge solubilization was calculated following Ge et al. (2011) and the total COD and VS reductions were calculated based on mass balance, both described in the analytical methods of **Chapter 3**.

6.2.3. DNA extraction, amplicon 454 pyrosequencing and the bioinformatics analysis

The microbial community characterization was performed on the samples after 63 days (period 1), 140 days (period 2), 175 days (period 3), 210 days (period 4 before process failure) and 252 days (period 4 after process failure). The materials and methods for genomic DNA extraction, 454 pyrosequencing, sequence processing and bioinformatics analysis are described in **Chapter 4**. The sequences were archived in NCBI Sequence Read Archive (SRA) with the accession SRS895459 and SRP058605 (<http://www.ncbi.nlm.nih.gov/genbank/>). Heatmap was constructed using R with heatmap.2 function, after preprocessing with Mothur. For visualization purposes, OTUs with less than 50 sequences per OTU were removed. The samples (at x-axis) and OTUs with genus classification (at y-axis) were hierarchically clustered using Bray-Curtis algorithm. Canonical correspondence analysis was also constructed using R with cca function.

6.3. Results and discussion

6.3.1. Reactor performance

Anaerobic co-digestion of sewage sludge with WCO was investigated at increasing ratio of WCO to system OLR based on VS mass, which consequently increased the organic loadings. The study conducted during experimental period 1 (0 to 77 days) was with WCO to OLR VS ratio of 20% - 25% (system OLR of 2.02 ± 0.18 g TCOD L⁻¹ d⁻¹), period 2 (76 to 140 days) with 30% - 35% ratio (2.59 ± 0.31 g TCOD L⁻¹ d⁻¹), period 3 (141 to 182 days) with 35% - 45% ratio (2.81 ± 0.41 g TCOD L⁻¹ d⁻¹) and period 4 (183 to 266 days) with 48% - 55% ratio (2.99 ± 0.33 g TCOD L⁻¹ d⁻¹) (Table 6.2).

Table 6.2. The reactor operating parameters and performances in the control and alkaline two-stage anaerobic systems (average $\pm$ standard deviation).

	Period	Single-stage sludge	Meso-meso two-stage sludge + WCO	Thermo-meso two-stage sludge + WCO
OLR (mg TCOD L ⁻¹ d ⁻¹)	1	1.52 ± 0.14	2.02 ± 0.18	
	2	1.56 ± 0.08	2.59 ± 0.31	
	3	1.38 ± 0.07	2.81 ± 0.41	
	4	1.16 ± 0.15	2.99 ± 0.33	
Total COD removal (%)	1	38.7 ± 4.6	56.6 ± 2.8	55.6 ± 2.6
	2	34.2 ± 4.6	60.6 ± 6.1	59.0 ± 7.1
	3	23.9 ± 5.8	61.4 ± 6.9	58.6 ± 6.7
	4	33.7 ± 9.0	43.5 ± 3.3	20.7 ± 11.0
VS reduction (%)	1	34.1 ± 2.7	54.0 ± 2.2	53.9 ± 1.9
	2	29.8 ± 4.8	57.8 ± 4.9	57.1 ± 3.2
	3	20.1 ± 8.2	56.6 ± 3.9	55.0 ± 4.6
	4	29.3 ± 10.0	53.9 ± 6.1	55.3 ± 10.5
Methane yield (L g TCOD _{added} ⁻¹)	1	0.17 ± 0.02	0.33 ± 0.01	0.33 ± 0.02
	2	0.17 ± 0.02	0.36 ± 0.03	0.36 ± 0.04
	3	0.17 ± 0.04	0.32 ± 0.04	0.35 ± 0.02
	4	0.19 ± 0.03	0.001 ± 0.001	0.006 ± 0.002
Methane yield (L g VS _{added} ⁻¹)	1	0.27 ± 0.02	0.54 ± 0.02	0.55 ± 0.04
	2	0.26 ± 0.03	0.63 ± 0.03	0.63 ± 0.05
	3	0.29 ± 0.06	0.72 ± 0.02	0.69 ± 0.04
	4	0.33 ± 0.05	0.002 ± 0.001	0.011 ± 0.002

The enhancement of methane yield by anaerobic co-digestion of sludge with WCO by the alkaline two-stage system was demonstrated, as it reached $0.55 \text{ L g VS}_{\text{added}}^{-1}$ in period 1 (**Table 6.2**). This was almost double compared to the yield in the control, a single-stage system without co-digestion with $0.27 \pm 0.02 \text{ L g VS}_{\text{added}}^{-1}$. VS reduction and TCOD removal were also higher in co-digestion system than the control, indicating the degradation of WCO into biogas (54% vs. 34.1% VS reduction and 56.6% vs. 34.1% TCOD removal in period 1). The methane yield of the alkaline two-stage anaerobic co-digestion systems at $0.33 \text{ L g COD}_{\text{added}}^{-1}$ was also comparably higher than the highest value obtained in **Chapter 5**, performing anaerobic sludge mono-digestion with thermophilic alkaline enhanced stage-1 at $0.177 \text{ L g COD}_{\text{added}}^{-1}$. The additional methane recovered can be converted into heat and electricity to support the energy requirement of the WWTP (Long et al. 2012).

Gradual increase of WCO load from period 1 to 3 resulted in an increase of methane yield in both two-stage systems to $0.63 \text{ L g VS}_{\text{added}}^{-1}$ in period 2; and $0.72 \text{ L g VS}_{\text{added}}^{-1}$ for mesophilic-mesophilic and $0.69 \text{ L g VS}_{\text{added}}^{-1}$ for thermophilic-mesophilic alkaline two-stage systems in period 3 (**Table 6.2**). Methane yield of $0.72 \text{ L g VS}_{\text{added}}^{-1}$ was the highest methane yield obtained in this study by applying mesophilic-mesophilic two-stage system with WCO ratio of 35 - 45% (VS) to system under OLR of $2.81 \pm 0.41 \text{ g TCOD L}^{-1} \text{ d}^{-1}$ in period 3. **Figure 6.1** shows that the effluent VS of both two-stage systems at the WCO ratio of 35 – 45% (VS) were similar to the control. Thus, the additional input of WCO to enhance biogas production did not affect the final amount of sludge to be disposed. Characteristics of the FOG substrate can influence the resulting methane yield that can be obtained. However, this maximum methane yield obtained in this study was within the higher range compared to previous results (**Table 6.3**). FOG obtained from restaurant grease waste tend to also consist of wastewater and food waste particles giving lower substrate VS concentration ($200 - 300 \text{ g L}^{-1}$) (Wan et al. 2011; Wang et al. 2013), compared to WWTP grease trap residue or WCO used in this study ($400 - 900 \text{ g L}^{-1}$) (Kabouris et al. 2009; Noutsopoulos et al. 2013). The closest comparable substrate was performed by Lauwers et al. (2013) co-digesting waste

Figure 6.1. Organic loading rate, OLR (A), effluent VS concentration (B), TCOD concentration (C), non-soluble COD concentration (D), soluble COD and TVFA concentrations (E) of the single-stage control and alkaline two-stage AD systems.

activated sludge (WAS) with vegetal oil, which obtained lower maximum methane yield ($0.44 \text{ L g VS}_{\text{added}}^{-1}$) compared to the alkaline two-stage system configuration in this study. The alkaline enhanced system in this study also gave higher maximum methane yield compared to anaerobic co-digestion with WWTP grease trap residue using two-phase configuration with mesophilic and thermophilic temperatures (at $0.54 - 0.66 \text{ L g VS}_{\text{added}}^{-1}$) (Kabouris et al. 2009).

Table 6.3. Comparisons of maximum methane yield in different anaerobic co-digestion systems treating sewage sludge with FOG.

Substrate	Sludge : FOG ratio (VS)	Configuration	OLR ($\text{g VS L}^{-1} \text{ d}^{-1}$)	Methane yield ($\text{L g VS}_{\text{added}}^{-1}$)	References
Mixed sludge + restaurant grease waste	77:23	CSTR single-stage 20 days SRT, 36°C	1.58	0.63	(Razaviaran i et al. 2013)
WAS + municipal grease waste	64:36	Semi-continuous single-stage 15 days SRT, 37°C	2.34	0.6	(Wan et al. 2011)
WAS + municipal grease waste	34.5:65.5	CSTR single-stage 20 days SRT, 37°C	2.16	0.75	(Wang et al. 2013)
Mixed sludge + WWTP grease trap residue	40:60	Semi-continuous single-stage 15 days SRT, 35°C	3.5	0.49	(Noutsopoulos et al. 2013)
Mixed sludge + WWTP grease trap residue	52:48	CSTR single-stage 12 days SRT, 35°C	4.35	0.51	(Kabouris et al. 2009)
		CSTR single-stage 12 days SRT, 52°C	4.35	0.61	
Mixed sludge + WWTP grease trap residue	52:48	CSTR two-phase 1+12 days SRT, $24^{\circ}\text{C} + 35^{\circ}\text{C}$	4.35	0.54	(Kabouris et al. 2009)
		CSTR two-phase 1+12 days SRT, $24^{\circ}\text{C} + 52^{\circ}\text{C}$	4.35	0.66	
WAS + vegetal oil	60:40	CSTR single-stage 20 days SRT, 37°C	2.58	0.44	(Lauwers et al. 2013)
Mixed sludge + waste cooking oil	55-65:35-45	Semi-continuous alkaline two-stage 3 + 17 days SRT, $35^{\circ}\text{C} + 35^{\circ}\text{C}$	1.48	0.72	Present study
	55-65:35-45	Semi-continuous alkaline two-stage 3 + 17 days SRT, $55^{\circ}\text{C} + 35^{\circ}\text{C}$	1.48	0.69	

Further increase to 48% - 55% WCO ratio resulted in process failure in period 4. Negligible methane was produced as the effluent VS, total COD, non-soluble COD, soluble COD and TVFA concentrations increased (**Table 6.2** and **Figure 6.1**). TVFA accumulation and negligible methane production indicated that the methanogenesis step of converting VFA into methane was impaired. Moreover, the accumulation of non-soluble COD indicated that the solubilization of sludge's particulates or lipids from the WCO had also been interrupted. However, VFA could still be produced and detected in all the reactors' effluents, demonstrating that acidogenesis had not been completely inhibited. The microbial community dynamics will be evaluated later to help understand the role of microbial communities in the anaerobic co-digestion process with alkaline two-stage systems which undergo optimal or inhibitory WCO loading.

Figure 6.2. The average extent of sludge solubilization with the organic compositions in the mesophilic (A) and thermophilic (B) alkaline enhanced stage-1 in anaerobic co-digestion of sludge with waste cooking oil.

Increasing digester's operating temperature had been previously found to increase digestion performance in sludge mono-digestion (**Chapter 5**) (Han et al. 1997; Ge et al. 2011) and in co-digestion of sludge with FOG (Kabouris et al. 2009). In contrast, there is no obvious improvement to sludge solubilization in the alkaline enhanced stage-1 performing co-digestion under thermophilic than mesophilic conditions (**Figure 6.2**). pH adjustment was performed once daily to the alkaline enhanced stage-1 and it was recorded that the sodium hydroxide dosage was higher in mesophilic (0.051, 0.043, 0.044 and 0.051 g NaOH g TS⁻¹ in period 1, 2, 3 and 4, respectively) than in thermophilic operation (0.048, 0.038, 0.029 and 0.025 g NaOH g TS⁻¹ in period 1, 2, 3 and 4, respectively). Sodium hydroxide functioned to destroy sludge EPS, cell walls and saponified lipid substrates to improve solubilization performance. This difference in sodium hydroxide dosage might have compensated the expected solubilization improvement which should have been observed in the thermophilic alkaline enhanced stage-1. Consequently, the methane yield, VS reduction and TCOD removal observed between the two alkaline two-stage systems were not significantly different (**Table 6.2**). Anaerobic co-digestion of sewage sludge with WCO in continuous configuration with continuous pH adjustment is expected to produce a better comparative assessment in the future. Nevertheless, the implementation of mesophilic-mesophilic alkaline two-stage anaerobic co-digestion system reported in this study managed to achieve one of the highest methane yields compared to previous studies performing anaerobic co-digestion of sewage sludge with FOG (**Table 6.3**).

The alkaline two-stage anaerobic system can also be applied to treat sludges from other municipal water treatment plants. This is because the characteristics of municipal sludges are not dramatically different. In general, sludge from industrial wastewater treatment bioprocesses can also be treated by the alkaline two-stage anaerobic systems as the wastewater is also treated by a bioprocess. However, preliminary biotoxicity testing is advised to ensure that the industrial wastewater can be treated by AD process (Stuckey and McCarty 1984).

6.3.2. Microbial community analysis

6.3.2.1. Bacterial community structures

A total of 149,494 sequences were obtained from the bacterial amplicon pyrosequencing, which was trimmed to 1,012 sequences per sample, after sequence processing by Mothur. **Figure 6.3** shows that bacterial profile in the anaerobic seed sludge was similar to those in the control single-stage reactor samples. However, there was a microbial community shift from the anaerobic seed sludge community to bacterial communities of the different reactors of the alkaline two-stage systems, although only the mesophilic-mesophilic and thermophilic-mesophilic stage-2s were closely related to each other. The mesophilic and thermophilic stage-1s formed their own clusters as the WCO mixing ratio was increased from 20% - 25% at 63 days to 48 - 55% at 252 days. However, a separate cluster was observed in the mesophilic-mesophilic and thermophilic-mesophilic stage-2s at 252 days, when process failure occurred. These results indicated the bacterial adaptation from a community performing sludge mono-digestion to a community suitable for co-digestion of sludge with WCO, and that inhibitory level of WCO induced a community shift in the stage-2s.

Compared to the single-stage control reactor, the mesophilic alkaline enhanced stage-1 was enriched with *Ruminococcaceae*, *Fusobacterium*, *Sporanaerobacter* and *Bacteroides* (**Figure 6.3**). *Ruminococcaceae* (*Firmicutes* phylum) mainly ferments carbohydrates and had been associated with degradation of particulate organics substrates in human gut (Walker et al. 2008). *Fusobacterium* (*Fusobacteria*), *Sporanaerobacter* (*Firmicutes*) and *Bacteroides* (*Bacteroidetes*) were capable of proteins and carbohydrates fermentation (Hernandez-Eugenio et al. 2002; Krieg et al. 2010), and would be involved in the degradation of the sludge substrate. De Francisci et al. (2015) correlated *Bacteroides*' functional genes with high efficiency of polysaccharides degradation, and possibly assisted in the hydrolysis of polysaccharide-related compositions of the sewage sludge in this study. Some species of *Fusobacterium* and *Bacteroides* also demonstrated lipase

Figure 6.3. Heatmap depicting the average counts (log₂-transformed) of bacterial OTU (97% similarity) in each sample after “sub.sample” and OTUs < 50 counts removed. The samples (x-axis) and OTUs (y-axis) were both hierarchically clustered using Bray-Curtis similarity index. The OTUs were classified at genus level using RDP database with 50% threshold.

activity, which can facilitate hydrolysis of WCO into glycerol and LCFA (Rudek and Haque 1976; Jensen et al. 2008). Further increase of WCO loading in period 4 (48 – 55%) at 210 and 252 days led to additional enrichment of *Dysgonomonas*

(*Bacteroidetes* phylum), *Escherichia-Shigella* (*Proteobacteria*), *Megamonas* (*Firmicutes*) and *Veillonellaceae* (*Firmicutes*), which were also capable of producing lipolytic activities (Shoshichi 1974; Vos et al. 2009; Krieg et al. 2010). *Megamonas* is also able to ferment glycerol, a product of lipid hydrolysis, into VFA (De Francisci et al. 2015). This indicated that increasing the FOG load also led to a microbial community shift in the mesophilic alkaline enhanced stage-1 to a community suitable for lipolytic activity.

In contrast, the bacterial community structure of the thermophilic alkaline enhanced stage-1 was similar throughout period 1 to 4. A different bacterial community was enriched in the thermophilic than mesophilic condition, mainly dominated by *Anaerobaculum*, *Thermoanaerovibrio*, *Clostridium_XII* and *Tepidimicrobium*. *Clostridium_XII* consisted of multiple species which displayed a variety of phenotypic characteristics (Vos et al. 2009). *Tepidimicrobium* is a proteolytic bacterium and grows at 50°C (Slobodkin et al. 2006), similar to the operating temperature of thermophilic alkaline enhanced stage-1. *Anaerobaculum* ferments proteins and carbohydrates at optimum temperature of 55 – 60°C (Vos et al. 2009). It was reported that *Anaerobaculum*'s growth in oil reservoir was stimulated by the addition of palm oil analogue (Li et al. 2012), and indicated *Anaerobaculum*'s postulated function in lipid degradation. In addition, *Thermoanaerovibrio* grows at optimum temperature of 50 – 65°C and ferments amino acids in syntrophy with hydrogenotrophic methanogens (Vos et al. 2009). These enriched microorganisms were expected to play important role in enhanced hydrolysis and acidogenesis of proteins, carbohydrates and lipids in the thermophilic stage-1.

Similar to the alkaline two-stage anaerobic sludge mono-digestion systems in **Chapter 5**, hydrolysis and acidogenesis still occurred in the stage-2. The maximum solubilization observed in the alkaline enhanced stage-1 during co-digestion was 21.2% (**Figure 6.2**) while TCOD removal in the stage-2s ranged from 55.6% to 61.4% (**Table 6.2**). This demonstrated that further solubilization occurred in the

stage-2 during co-digestion. Bacterial communities in both stage-2s were found closely related from period 1 to 3 and before inhibition at period 4 (**Figure 6.3**). These bacterial communities were different to those in their respective stage-1s, and were mainly dominated by *Syntrophomonas*, *Candidatus_Cloacamonas* and unclassified genera of *Bacteroidetes* and *Flavobacteriaceae*. Hydrolysis and acidogenesis of sewage sludge and possibly lipids from the WCO could have been performed by the *Bacteroidetes* and *Flavobacteriaceae*. The *Flavobacteriaceae* detected in the stage-2s was derived from the anaerobic seed sludge inoculum and was also present in the control (single-stage sludge mono-digestion system), hence, indicating its role in sewage sludge digestion. Regueiro et al. (2012) found that higher percentage of *Bacteroidetes* was associated with higher hydrolytic activities found in anaerobic digesters treating sewage sludge, brewery and sugar industries wastewater, hence, indicating its contribution to protein and carbohydrate hydrolysis and acidogenesis in this study. *Candidatus_Cloacamonas* had been previously detected in an anaerobic sludge mono-digestion and a co-digestion reactor treating sewage sludge with restaurant grease waste (Pelletier et al. 2008; Razaviarani and Buchanan 2014) and *in silico* analysis indicated that it was a syntrophic bacterium capable of degrading amino acids and propionic acid (Pelletier et al. 2008). Hence, degradation of propionic acid (also a product of glycerol acidogenesis) was likely performed by *Candidatus_Cloacamonas* and also by *Smithella* in the stage-2s (Liu et al. 1999). *Syntrophomonas* is one of the few microorganisms which perform beta-oxidation of saturated and unsaturated LCFA (Sousa et al. 2009). *Syntrophomonas* can also degrade butyric acids generated from protein/carbohydrate acidogenesis or LCFA acetogenic reactions (Wu et al. 2007). As LCFA formed the intermediate of lipid degradation during the co-digestion of WCO with sewage sludge, *Syntrophomonas* is critical to the digestion performance and stability of the anaerobic co-digestion process.

Operating concerns regarding LCFA accumulation during anaerobic co-digestion of sludge with FOG or fatty wastewaters had been widely documented (Hwu et al. 1998; Palatsi et al. 2010; Silvestre et al. 2011; Long et al. 2012). In this study, LCFA

degrading microorganism was identified to be *Syntrophomonas* but was only detected in stage-2s, but not in any stage-1 (**Figure 6.3**). On the other hand, the operation of an acidogenic phase (35°C, 0.76 days SRT, pH 6.7 - 7.3) treating fat-containing wastewater achieved 35% LCFA removal in the acidogenic phase, but its bacterial community structure was not characterized (Kim et al. 2004). *Syntrophomonas* grows optimally at 37°C – 40°C with pH 7.0 – 7.6 (Wu et al. 2007). It was possible that operating the stage-1 at pH 8 was not preferred for the growth of *Syntrophomonas* and hence, its absence in the alkaline enhanced stage-1. Secondly, high hydrogen partial pressure could inhibit the syntrophic LCFA acetogenesis reactions. Biogas analysis revealed that hydrogen concentration in the mesophilic and thermophilic stage-1 increased between period 1 and 4, from 0.17 - 0.72 mol% to 0.32 - 3.02 mol%, respectively; while the hydrogen concentrations in the stage-2s were below 0.01 mol%. Hydrogen could be removed by hydrogenotrophic methanogenesis in anaerobic processes and methane productions were indeed observed in the stage-1s, albeit at low level (**Figure 6.2**). **Chapter 4** established that methanogenesis in stage-1 was retarded at pH 8. Hydrogenotrophic methanogenesis in the alkaline enhanced stage-1s of alkaline two-stage co-digestion systems might not be able to keep up with hydrogen production, and hence, the hydrogen accumulated to inhibitory levels to LCFA acetogenesis.

As the WCO ratio increased to the inhibitory 48% - 55% (period 4), it is interesting to note that there was no significant microbial community shift (**Figure 6.3**) and severe reduction in the extent of solubilization (**Figure 6.2**) in both mesophilic and thermophilic stage-1s. On the other hand, microbial community shift occurred in the stage-2s at 252 days when inhibitory WCO ratio was applied in period 4. The shift was mainly attributed to the reduction of populations enriched in the stage-2 (i.e. *Syntrophomonas*, *Candidatus_Cloacamonas* and unclassified genera of *Bacteroidetes* and *Flavobacteriaceae*) and replaced with *Petrimonas*, unclassified *Anaerolineaceae* and *Bacteria* derived from their respective stage-1s (**Figure 6.3**). This implied that the microbial communities and their activities in stage-1 were not

significantly impacted by increasing WCO loading to 48% - 55%. Since the metabolic functions in stage-1 involved sludge hydrolysis/acidogenesis and lipid hydrolysis, microorganisms involved in these metabolic steps seemed to be more tolerant to LCFA inhibition compared to the LCFA degrading, propionic acid syntrophic acetogens and methanogens in the stage-2. Similarly, Hanaki et al. (1981) also found that glucose acidogenesis and lipid hydrolysis could still occur even when LCFA had induced inhibition on the LCFA acetogenesis and subsequent methanogenesis. Canonical correspondence analysis would be described later to correlate the microbial community structures in the stage-2s with process parameters.

6.3.2.2. Archaeal community structures

A total of 184,459 sequences were obtained from the archaeal amplicon pyrosequencing, which was trimmed to 1,710 per sample, after sequence processing by Mothur. **Figure 6.4** shows that the archaeal communities were identified to be known methanogens. Archaeal communities took longer than 63 days to compose the respective clusters based on reactor configurations. Similar to the bacterial communities, clustering of archaeal communities could be generally observed according to the reactor sample but from period 2 (138 days) and above, except for the mesophilic-mesophilic and thermophilic-mesophilic stage-2s which were closely related. This lag phase could be due to the slower growth rate of methanogens compared to acidogens (Miron et al. 2000; Batstone and Jensen 2011), and had also been observed in **Chapter 5**. This clustering was then maintained as higher ratio of WCO was introduced up to 35% - 45% at 175 days in period 3 and early period of period 4 at 210 days before VFA accumulation occurred. Another distinct cluster was again observed as the mesophilic-mesophilic and thermophilic-mesophilic stage-2s clustered together at 252 days (period 4), when the process failed.

Figure 6.4. Heatmap depicting the average counts ($\log_2$ -transformed) of archaeal OTU (97% similarity) in each sample after “sub.sample” and OTUs < 50 counts removed. The samples (x-axis) and OTUs (y-axis) were both hierarchically clustered using Bray-Curtis similarity index. The OTUs were classified at genus level using RDP database with 50% threshold.

As expected, reactor operating conditions (pH and temperature) of the alkaline two-stage systems resulted in distinct archaeal community structures after 63 days. Taxonomic analysis of the archaeal amplicons revealed that all of the samples were predominated by the hydrogenotrophic *Methanolinea* and *Methanospirillum*, except in the stage-2s (**Figure 6.4**). Instead, the stage-2s were dominated by *Methanospirillum* and *Methanoculleus*. **Chapter 4** found that the alkaline enhanced stage-1 in sludge mono-digestion was enriched with

Methanosarcina and *Methanobrevibacter* under mesophilic condition, *Methanothermobacter* under thermophilic condition, and *Methanolinea* and *Methanospirillum* in the stage-2. Operation of the alkaline enhanced stage-1 in anaerobic co-digestion also revealed the specific enrichment of the thermophilic *Methanothermobacter* in the thermophilic stage-1, but *Methanobrevibacter*, *Methanobacterium* and *Methanosphaera* were enriched in the mesophilic stage-1.

This archaeal diversity in this study was similar to that observed in a survey of 21 full-scale anaerobic digesters, which found *Methanobacterium*, *Methanothermobacter* and *Methanoculleus* in many anaerobic co-digestion systems (Sundberg et al. 2013). *Methanospirillum* had been identified previously to be syntrophic partner of *Syntrophomonas* and *Syntrophus* in LCFA degradation (Sousa et al. 2009). In cultivation, *Methanoculleus* had been found to work in syntrophy with acetic acid oxidizing *Bacteria* (Schnürer et al. 1999). *Methanoculleus* had also been isolated in an oil field in China and an anaerobic digester treating wastewater of a palm oil plant, which implied its syntrophic relationship with an LCFA degrading bacterium, too (Zellner et al. 1998; Cheng et al. 2008). All the methanogens identified in this study were hydrogenotrophic methanogens, except for *Methanosarcina* which was able to utilize either acetic acid or hydrogen (Garcia et al. 2000). Lipid (triglyceride) hydrolysis results in the production of LCFA and glycerol (Long et al. 2012). Degradation of this LCFA and propionic/butyric acids (product of glycerol acidogenesis) then required the syntrophic association between acetogenic *Bacteria* and their hydrogenotrophic methanogens in AD (Stams et al. 2012). Hydrogenotrophic methanogenesis pathway is thus especially important in anaerobic co-digestion of sewage sludge and FOG.

Increasing the WCO ratio to the inhibitory 46% - 55% in period 4 did not result in obvious archaeal community shifts in the mesophilic and thermophilic stage-1s, but significant community shifts were observed in the meso-meso and thermo-meso stage-2s due to the reduction of *Methanoculleus* (**Figure 6.4**). Despite the

absence of cultivation data in syntrophy with LCFA degrading syntrophs, the reduction of *Methanoculleus* along with the reduction of *Syntrophomonas* at 252 days, strongly suggested the important role of *Methanoculleus* in LCFA degradation during co-digestion of sewage sludge with WCO.

It was indicated in **Chapter 5** that different reactor configurations (continuous and semi-continuous system) produced different microbial communities. Therefore, comparison is made between semi-continuous mesophilic mono-digestion reactor controlled at pH 8 in **Chapter 4** and the semi-continuous mesophilic co-digestion stage-1 in this Chapter. Previous studies had established that AD of different substrates enrich different microbial groups (Martín-González et al. 2011; Sundberg et al. 2013; De Francisci et al. 2015). This also applies to the alkaline two-stage system in this study. The archaeal communities in the mesophilic mono-digestion stage-1 reactor was enriched with *Methanosarcina* and *Methanobrevibacter*, whereas it was *Methanobrevibacter*, *Methanobacterium* and *Methanosphaera* in the mesophilic co-digestion stage-1 reactor. For the bacterial communities, the mono-digestion reactor was strongly associated with proteins and carbohydrates degradation, such as *Tissierella*, *Petrimonas*, *Proteiniphilum*, *Levilinea*, *Proteiniborus* and *Sedimentibacter*. In contrast, microbial communities in the co-digestion reactor enriched different groups of Bacteria and those which were associated with lipid degradation, such as *Ruminococcaceae*, *Fusobacterium*, *Sporanaerobacter* and *Bacteroides*. The results suggested that different groups of microorganisms were enriched when the alkaline two-stage system treated a different substrate composition.

6.3.2.3. Correlation between microbial communities and reactor parameters

Since no obvious bacterial and archaeal community shifts were observed in the stage-1s, the canonical correspondence analysis mainly focused on the correlation between bacterial, archaeal communities in the stage-2s and process parameters.

Figure 6.6. Canonical correspondence analysis (CCA) between the microbial community of single-stage control, thermophilic-mesophilic stage-2, relative abundance of the key genera and process parameters.

of the stage-2 at 252 days when the process failed, were associated with higher SCOD, TVFA and were dominated by *Bacteria* and methanogens enriched in their respective stage-1s (Figure 6.5 and Figure 6.6). This indicated that process failure was due to the interruption of acetogenic and methanogenic reactions that were expected to be performed in the stage-2s. Identification of the important role of propionic acid oxidizing *Candidatus_Cloacamonas*, LCFA degrading *Syntrophomonas* and the hydrogenotrophic partner, *Methanoculleus* in this study

gives way for future application of targeting these key microorganisms as process monitoring tools to prevent process failure or even to induce failing reactors with these consortia to ensure stable process operation.

6.4. Conclusion

Anaerobic co-digestion of sewage sludge with WCO in the alkaline two-stage anaerobic digestion systems under increasing WCO loading was investigated. Maximum methane yield of $0.72 \text{ L g VS}_{\text{added}}^{-1}$, 56.6% VS reduction and 61.1% TCOD removal were achieved with mesophilic-mesophilic two-stage system at 35% - 45% ratio (VS) of WCO to overall loading, which is one of the highest methane yields reported for the anaerobic co-digestion of sewage sludge with FOG. This study demonstrated the feasibility of dramatically enhancing methane production with lipid-rich waste, to support the WWTP's energy requirement. The meso-meso two-stage alkaline anaerobic co-digestion system achieved 148% higher methane yield than a conventional single-stage sludge mono-digestion system. Different fermentative *Bacteria* (*Firmicutes*, *Bacteroidetes*, *Fusobacteria* and *Proteobacteria*) were enriched in the mesophilic and thermophilic alkaline enhanced stage-1s and they were not significantly influenced by increasing WCO loading in this study. Matching the *Bacteria* identified with literature data revealed their possible contribution in enhanced sludge and WCO hydrolysis and acidogenesis in mild alkaline condition. Meanwhile, *Candidatus_Cloacamonas*, *Syntrophomonas* and *Methanoculleus* dominated in the stage-2s, performing the critical LCFA acetogenesis and methanogenesis. However, they were reduced at higher WCO loading ratios (48% - 55%). Syntrophic acetogens and methanogens were more susceptible to LCFA inhibition than fermentative *Bacteria* and could be targeted as process monitoring tools or bioaugmentation consortium to ensure a stable process operation in anaerobic co-digestion system.

Chapter 7

Conclusions and Recommendations

7.1. Conclusions

This study investigated the enhancement of anaerobic sludge digestion process by biological method (i.e. two-stage configuration) and its underlying microbial community structures. Sludge digestion enhancement was explored by implementing phase separation (**Chapter 3**), optimizing the operating pH (**Chapter 4**) and the operating temperature of stage-1 (**Chapter 5**). Microbiological molecular methods were applied to further understand the complex microbial community structures and dynamics in enhanced anaerobic sludge digestion. In addition, the alkaline two-stage system developed in this study was also applied for anaerobic co-digestion of sewage sludge with fat, oil and grease (FOG) to present an alternative solution to waste FOG management as a source of renewable energy (**Chapter 6**).

Sludge digestion enhancement strategy by operating a two-phase AD CSTR system demonstrated higher sludge digestion efficiency and methane production compared to the conventional single-stage AD CSTR system at SRT of 12 days (2 + 10 days) (**Chapter 3**). This improvement was attributed to the decrease in non-soluble COD reduction efficiency in the single-stage system at 12 days SRT, which indicates poor sludge hydrolysis efficiency. On the other hand, microbiological analysis identified distinct fermentative *Flavobacteriales* and *Clostridiales* enriched in the acidogenic reactor throughout all SRTs tested, which suggested their contribution to the enhanced hydrolytic efficiency and ultimately, the two-phase system's process stability at the lower SRT (12 days). The opportunity to reduce SRT is promising in many regions with limited land area, as reactor volumes can hence be reduced. Additionally, methanogenesis was found to proceed in the phase-1 reactor, which can be useful to regulate alkalinity and consume potentially inhibitory acetic acid and hydrogen. Thus, a two-stage AD system is preferred.

Amongst the different pH tested (pH 4 – 11), operation of the first reactor at pH 8 gave the optimal acidified sludge mixed liquor, while still preserving methanogenic activities (**Chapter 4**). Increased sludge solubilization does not necessarily translate to higher biodegradability. Hydrolysis at pH 9 and above was mainly led by abiotic factors and negatively impacted on biological activities; and produced large molecular weight (MW) aromatic substances which tend to indicate poor biodegradability. Moving the alkaline enhanced stage-1 (pH 8) from mesophilic (35°C) to thermophilic temperature (55°C) further increased the sludge solubilization and acidification (**Chapter 5**). Microbiological analysis revealed the enrichment of fermentative *Bacteria* in the mesophilic and thermophilic alkaline enhanced stage-1, all of which functioned and maintained enhanced sludge solubilization and acidogenesis under mild alkaline condition. Despite the improved sludge digestion efficiency and methane production, the economic assessment of the alkaline two-stage anaerobic system suggested negative net savings due to the high chemical cost, compared to the conventional single-stage anaerobic system. A strategy to resolve this issue was discussed and proposed.

The enhancement of energy recovery by increasing methane production was further evaluated in anaerobic co-digestion of sewage sludge at increasing loadings of waste cooking oil (WCO) in the mesophilic-mesophilic and thermophilic-mesophilic alkaline two-stage systems (**Chapter 6**). Both alkaline two-stage systems could tolerate up to 35% - 45% of WCO ratio (VS) to overall loading, but failed when a ratio of 48% - 55% was tested. Microbiological analysis revealed that both mesophilic and thermophilic alkaline enhanced stage-1s mainly performed sludge hydrolysis, acidogenesis and WCO hydrolysis; while the stage-2 also conducted all these in addition to VFA acetogenesis, LCFA, glycerol acetogenesis and methanogenesis. Propionic acid oxidizing *Candidatus_Cloacamonas*, LCFA degrading *Syntrophomonas* and the hydrogenotrophic methanogen, *Methanoculleus* were enriched in the stage-2s and were identified to be key populations that were strongly correlated to the stability of process performance. In this study, the mesophilic-mesophilic alkaline two-stage anaerobic co-digestion

system achieved 148% higher methane yield than in the single-stage mono-digestion system. This demonstrated the feasibility of co-digestion of sludge with WCO to enhance methane production and hence, renewable energy recovery to support wastewater treatment plants' energy demand.

The use of high throughput sequencing method to analyze the 16S rRNA gene amplicons, i.e. 454 pyrosequencing, in **Chapter 4**, **Chapter 5** and **Chapter 6** presents more comprehensive microbial profiles, cheaper and faster than could be obtained by traditional methods involving Sanger sequencing, e.g. denaturing gradient gel electrophoresis (DGGE) (**Chapter 3**). The data obtained from 454 pyrosequencing is more thorough and extensive and advocate more applications of high throughput sequencing methods in the future. In general, *Bacteroidetes*, *Firmicutes* and *Chloroflexi* formed the main bacterial phyla involved in the enhanced sludge hydrolysis and acidogenesis in this study, followed by *Tenericutes* as detected in the thermophilic alkaline enhanced stage-1 and *Proteobacteria* and *Fusobacteria* during WCO co-digestion. Syntrophic acetogens, i.e. propionic acid oxidizing *Smithella*, *Candidatus_Cloacamonas* and LCFA degrading *Syntrophomonas*, could only be grown in the stage-2, having pH 7 requirement for cultivation. Hydrogenotrophic methanogens were identified to be the predominant methanogens in anaerobic sludge digestion processes in this study, in contrast with previous estimations. It could have been due to hydrogenotrophic methanogens' higher tolerance to higher ammonia concentration compared to acetoclastic methanogens, which was released during the degradation of proteinaceous material in the sludge. *Methanolinea* and *Methanospirillum* were detected to be the predominant methanogens during sludge mono-digestion process and *Methanoculleus* to be strongly associated with WCO co-digestion process. Interestingly, *Methanosarcinaceae* and *Methanobacteriaceae* were enriched in pH ranges that were known to be unsuitable for methanogenesis at pH 5 and 8; and indicated the potential applications of specialized methanogens in those unconventional pH ranges.

In this study, microbial community structures were found to be influenced by the operating and environmental conditions, such as SRT, pH, temperature, substrate characteristics and concentrations of VFA or ammonia. Temporal microbiological analysis conducted in **Chapter 4**, **Chapter 5** and **Chapter 6** revealed that key microbial populations were shared between a common reactor configuration (SRT, pH and temperature), resulting in clustered community structures with similar process parameters. This finding supported the niche based theory of microbial ecology, that microbial communities are assembled by deterministic rather than stochastic factor, under properly controlled conditions of the anaerobic processes. Therefore, microbial monitoring by molecular tools can be applied in properly defined anaerobic processes as predictive tools to process performance. For instance, *Candidatus_Cloacamonas*, *Syntrophomonas* and *Methanoculleus* may be monitored and predict potential organic overloading in anaerobic co-digestion of sewage sludge with WCO. Key populations identified can also be utilized for process improvement or recovery by bioaugmentation. In addition, microbial dynamics, interactions and the resulting process performance presented in this study can also be translated into process modelling and better understanding of the AD process.

7.2 Recommendations for future research

Based on this study, the following items are suggested as future research:

- The alkaline enhanced stage-1 of the two-stage system described in **Chapter 5** and **Chapter 6** did not allow acetogenesis to occur, possibly due to the undesirable operating pH to cultivate the syntrophic acetogens. Process optimization is recommended to accommodate acetogenesis in the stage-1 and reduce VFA or LCFA load into the stage-2, therefore, accelerating the overall sludge digestion kinetics. Recycling stage-2 sludge into the stage-1 or further stage separation to make three-stage system can be considered to address this matter.

- The alkaline two-stage anaerobic system requires considerably high sodium hydroxide dosage and affected the economic viability of the process. Different operational control strategy (e.g. semi-continuous reactor operation and/or chemical dosing by intervals) needs to be considered to obtain optimal sludge solubilization and acidogenesis. In addition, economic assessment has to be performed for each optimization effort.
- When it was first introduced, 454 pyrosequencing was the preferred high throughput sequencing technology due to the longer read length and faster run time (MacLean et al. 2009). As longer read is obtained, more hypervariable regions of the 16S rDNA sequence can be covered for better phylogenetic analysis. However, 454 pyrosequencing lacks a reversible terminating nucleotide, meaning that more than one nucleotide can be incorporated in a homopolymer region and generate base insertion deletion error (Loman et al. 2012). Recently, Illumina is able to generate longer reads at around 400 bp, the length which 454 pyrosequencing had traditionally generated (Sinclair et al. 2015) and at lower cost than 454 pyrosequencing (0.5 USD/Mb vs 28 USD/Mb) (Loman et al. 2012). Furthermore, better read quality can be obtained because Illumina technology uses sequencing-by-synthesis which has a lower error rate and is not as susceptible to insertion deletion errors as 454 pyrosequencing (Loman et al. 2012). Therefore, Illumina sequencing technologies have to be considered for future study.
- The microbial community analysis in this study was based on 16S rRNA fingerprints to construct phylogenetic relationship and identify taxonomy. However, the functional significance of identified population may not be conclusively defined. Moreover, there are still many unclassified populations and their roles in AD process remained unknown. Isotope labelling methods, such as stable isotope probing (SIP), microautoradiography fluorescence in-situ hybridization (MAR-FISH) can be considered in the future. Furthermore, metagenomics, metatranscriptomics,

metaproteomics and metabolomics study are increasingly feasible since the advent of high throughput sequencing technology, and are valuable tools for microbiological studies in AD process.

- As the microbial community structures in AD process in this study are found to be strongly influenced by deterministic factor, practical tools may be developed to monitor, and even enhance the AD process. Monitoring of the key populations as predictive tools are currently performed with qPCR or ecological microarrays by routine quantification of 16S rDNA or functional gene during operation. However, the methods are costly and impractical to be performed as routine monitoring tools. Further research may be directed to develop an automated microfluidic-based platform which can perform routine quantification by qPCR or hybridization-based methods. Moreover, the identification of key microbial function groups opens the avenue for bioaugmentation to rapidly start-up, improve the performance or recover a failing digester.

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Supplementary information

Appendix A. List of standard plasmids for qPCR calibration.

Target group	Standard strain	Cloning primers	Plasmid size (bp)
<i>Bacteria</i>	<i>Escherichia coli</i> K12	Bac8F - Prk1492R	4,518
<i>Methanobacteriales</i>	<i>Methanobacterium formicicum</i> M.o.H. (DSM 863)	Arc63F - Prk1392R	4,330
<i>Methanomicrobiales</i>	<i>Methanobrevibacter arboriphilicus</i> DH1 (DSM 1536)	Arc63F - Prk1392R	4,326
	<i>Methanospirillum hungatei</i> JF1 (DSM 864)	Arc21F - Prk1492R	4,450
<i>Methanosarcinaceae</i>	<i>Methanomicrobium mobile</i> BP (DSM 1539)	Arc21F - Prk1492R	4,453
	<i>Methanosarcina acetivorans</i> C2A (DSM 2834)	Arc21F - Prk1492R	4,462
<i>Methanosaetaceae</i>	<i>Methanosarcina barkeri</i> MS (DSM 800)	Arc21F - Prk1492R	4,463
	<i>Methanosarcina mazei</i> Go1 (DSM 3647)	Arc21F - Prk1492R	4,459
	<i>Methanosaeta concilli</i> GP6 (DSM 3671)	Arc63F - Prk1392R	4,393

Appendix B. Heatmap depicting the average counts (log₂-transformed) of bacterial OTU (97% similarity) in each acidification reactor at different pHs, after “sub.sample” and OTUs < 30 counts removed. The OTUs (y-axis) was hierarchically clustered using Bray-Curtis similarity index. The OTUs were classified at genus level using RDP database with 50% threshold.

Appendix C1. The 454 pyrosequencing tags for universal *Archaea* and *Bacteria* primer amplicons, investigating microbial community in **Chapter 4**.

Sample ID	Sample	Archaeal amplicon tags	Bacterial amplicon tags
517y72	Seed	TATTACAG	AATATGGA
517y73	Seed	TATTAGCG	AATCACAA
517y74	Feed 47 days	TATTATGG	AATCAGCA
517y75	Feed 47 days	TATTCATG	AATCATGA
517y76	Control 47 days	TATTCGAG	AATCCATA
517y77	Control 47 days	TATTCTCG	AATCCGAA
517y78	pH 4 47 days	TATTGAGG	AATCCTCA
517y79	pH 4 47 days	TATTGCTG	AATCGAGA
517y80	pH 5 47 days	TATTGTAG	AATCGTAA
517y81	pH 5 47 days	TCAACCAG	AATCTACA
517y82	pH 6 47 days	TCAACGCG	AATCTCGA
517y83	pH 6 47 days	TCAACTGG	AATCTGTA
517y84	pH 7 47 days	TCAAGATG	AATGACCA
517y85	pH 7 47 days	TCAAGGAG	AATGAGGA
517y86	pH 8 47 days	TCAGCACG	AATGATTA
517y87	pH 8 47 days	TCAGCCGG	AATGCCAA
517y88	pH 9 47 days	TCAGCGTG	AATGCGCA
517y89	pH 9 47 days	TCAGGAAG	AATGCTGA
517y90	pH 10 47 days	TCAGGCCG	AATGGATA
517y91	pH 10 47 days	TCAGGTTG	AATGGTCA
517y92	pH 11 47 days	TCAGTCAG	AATGTAGA
517y93	pH 11 47 days	TCAGTGCG	AATGTCTA
517y112	Feed 77 days	TATTACAG	CAGCTACA
517y113	Feed 77 days	TATTAGCG	CAGCTCGA
517y114	Control 77 days	TATTATGG	CAGCTGTA
517y115	Control 77 days	TATTCATG	CAGGACCA
517y116	pH 4 77 days	TATTCGAG	CAGGAGGA
517y117	pH 4 77 days	TATTCTCG	CAGGATTA
517y118	pH 5 77 days	TATTGAGG	CAGGCCAA
517y119	pH 5 77 days	TATTGCTG	CAGGCGCA
517y120	pH 6 77 days	TATTGTAG	CAGGCTGA
517y121	pH 6 77 days	TCAACCAG	CAGGTAGA
517y122	pH 7 77 days	TCAACGCG	CAGGTCTA
517y123	pH 7 77 days	TCAACTGG, TCAGTGCG	CAGGTTAA
517y124	pH 8 77 days	TCAAGATG	CAGTAACA
517y125	pH 8 77 days	TCAAGGAG	CAGTACGA
517y126	pH 9 77 days	TCAGCACG	CAGTAGTA
517y127	pH 9 77 days	TCAGCCGG	CAGTCGGA
517y128	pH 10 77 days	TCAGCGTG	CAGTCTTA
517y129	pH 10 77 days	TCAGGAAG	CGTAACAA

517y130	pH 11 77 days	TCAGGCCG	CGTAAGCA
517y131	pH 11 77 days	TCAGGTTG	CGTAATGA

Appendix C2. The 454 pyrosequencing tags for universal *Archaea* and *Bacteria* primer amplicons, investigating microbial community in **Chapter 5**.

Sample ID	Sample	Archaeal amplicon tags	Bacterial amplicon tags
517y7	Single-stage 0 days	TCAGCACG	ATCGGCTA
517y8	Single-stage 0 days	TCAGCCGG	ATCGGTAA
517y10	Stage-1 0 days	TCAGGAAG	ATCGTCGA
517y12	Stage-1 0 days	TCAGGTTG	AAGGCATG
517y13	Stage-2 0 days	TCAGTCAG	ATCTACCA
517y14	Stage-2 0 days	TCAGTGCG	ATCTAGGA
517y15	Stage-2 0 days	TCAGTTGG	ATCTATTA
517y16	Feed 12 days	TCATAATG	ATCTCCAA
517y17	Feed 12 days	TATTACAG	ATCTCGCA
517y19	Single-stage 12 days	TATTATGG	AACCAGCT
517y20	Stage-1 12 days	TATTCATG	AACCAGGA
517y21	Stage-1 12 days	TATTCGAG	AACCAGGC
517y22	Stage-2 12 days	TATTCTCG	AACCAGGT
517y24	Feed 45 days	TATTGCTG	AACCAGTG
517y25	Feed 45 days	TATTGTAG	AACTCACG, AAGGCGTA
517y27	Single-stage 45 days	TCAACGCG	ACAACCGA
517y28	Single-stage 45 days	TCAACTGG	ACAACGTA
517y30	Stage-1 45 days	TCAAGGAG	AAGGCGTT, AACTCATA AAGGCGTG, AAGGCTAA, AACTCATC
517y31	Stage-1 45 days	TCAGCACG	AACTCATC
517y33	Stage-2 45 days	TCAGCGTG	AACCATCT
517y34	Stage-2 45 days	TCAGGAAG	ACAAGCCA
517y37	Feed 70 days	TCAGTCAG	AACCATGT
517y38	Feed 70 days	TCAGTGCG	AACCGAGA
517y39	Single-stage 70 days	TCAGCCGG	AACCGAGC
517y40	Single-stage 70 days	TCAGCGTG	AACCGAGG
517y42	Stage-1 70 days	TCAGGCCG	AACTCCAG
517y43	Stage-1 70 days	TCAGGTTG	AACTCCAT
517y44	Stage-1 70 days	TCAGTCAG	AAGGCGAA
517y46	Stage-2 70 days	TCAGTTGG	AACCGCAC
517y47	Stage-2 70 days	TCATAATG	AAGGCGAT, AACCGCAG
517y48	Feed 116 days	TATTACAG	CAGTCGGA
517y49	Feed 116 days	TATTAGCG	CAGTCTTA
517y50	Single-stage 116 days	TATTATGG	CGTAACAA
517y51	Single-stage 116 days	TATTCATG	CGTAAGCA

517y52	Stage-1 116 days	TATTCGAG	CGTAATGA
517y53	Stage-1 116 days	TATTCTCG	CGTACATA
517y54	Stage-2 116 days	TATTGAGG	CGTACGAA
517y55	Stage-2 116 days	TATTGCTG	CGTACTCA
517y56	Feed 158 days	TATTGTAG	ACCAACGA
517y57	Feed 158 days	TCAACCAG	CGTAGCTA
517y58	Single-stage 158 days	TCAACGCG	CGTAGTAA
517y59	Single-stage 158 days	TCAACTGG	CGTATACA
517y60	Stage-1 158 days	TCAAGATG	CGTATCGA
517y61	Stage-1 158 days	TCAAGGAG	CGTATGTA
517y62	Stage-2 158 days	TCAGCACG	CGTCACCA
517y63	Stage-2 158 days	TCAGCCGG	CGTCAGGA
517y64	Feed 217 days	TCAGCGTG	ACCAAGTA
517y65	Feed 217 days	TCAGGAAG	CGTCCGCA
517y66	Single-stage 217 days	TCAGGCCG	CGTCCTGA
517y67	Single-stage 217 days	TCAGGTTG	CGTCGATA
517y68	Stage-1 217 days	TCAGTCAG	CGTCGGAA
517y69	Stage-1 217 days	TCAGTGCG	CGTCGTCA
517y70	Stage-2 217 days	TCAGTTGG	CGTCTAGA
517y71	Stage-2 217 days	TCATAATG	CGTCTCTA

Appendix C3. The 454 pyrosequencing tags for universal *Archaea* and *Bacteria* primer amplicons, investigating microbial community in **Chapter 6**.

Sample ID	Sample	Archaeal amplicon tags	Bacterial amplicon tags
517y72	Seed	TATTACAG	AATATGGA
517y98	Feed 63 days	TATTCGAG	AATTCGGA
517y100	Control 63 days	TATTGAGG	AATTGCAA
517y102	Thermo stage-1 63 days	TATTGTAG	AATTGTGA
517y104	Thermo-meso stage-2 63 days	TCAACGCG	ACAACCGA
517y108	Meso stage-1 63 days	TCAGCACG	ACAATCAA
517y110	Meso-meso stage-2 63 days	TCAGCGTG	ACAATTGA
517y185	Control 138 days	TATTACAG	CAGTCTTA
517y187	Meso stage-1 138 days	TATTATGG	CGTAAGCA
517y188	Meso-meso stage-2 138 days	TATTCATG	CGTAATGA
517y189	Thermo stage-1 138 days	TATTCGAG	CGTACATA
517y190	Thermo-meso stage-2 138 days	TATTCTCG	CGTACGAA
517y191	Control 175 days	TATTGAGG	CGTACTCA
517y193	Meso stage-1 175 days	TATTGTAG	CGTAGCTA
517y194	Meso-meso stage-2 175 days	TCAACCAG	CGTAGTAA
517y195	Thermo stage-1 175 days	TCAACGCG	CGTATACA
517y196	Thermo-meso stage-2 175 days	TCAACTGG	CGTATCGA

517y197	Control 210 days	TCAAGATG	CGTATGTA
517y199	Meso stage-1 210 days	TCAGCACG	CGTCAGGA
517y201	Thermo stage-1 210 days	TCAGCGTG	CGTCCGCA
517y202	Thermo-meso stage-2 210 days	TCAGGAAG	CGTCCTGA
517y203	Control 252 days	TCAGGCCG	CGTCGATA
517y205	Meso stage-1 252 days	TCAGTCAG	CGTCGTCA
517y206	Meso-meso stage-2 252 days	TCAGTGCG	CGTCTAGA
517y207	Thermo stage-1 252 days	TCAGTTGG	CGTCTCTA
517y208	Thermo-meso stage-2 252 days	TCATAATG	AATATGGA

Appendix D. Energy balance calculation to compare mesophilic-mesophilic and thermophilic-mesophilic alkaline two-stage systems.

Heat energy balance was calculated as described by Lu et al. (2008). Heat energy input is the sum of heat energy required to raise the temperature of influent feed sludge and heat losses. Heat loss via the piping system was reported to be negligible and heat losses via the digester's surfaces accounted for 2% to 8% of energy required to heat the influent. Additionally, the effluent of alkaline enhanced stage-1 under thermophilic condition needed to be cooled down and part of the heat energy can be recovered to heat influent feed sludge. Heat output is the heat energy generated from the methane production. Hence, the heat energy balance is the total of heat output minus the total of heat input.

$$\text{Heat input} = \rho_{\text{sludge}} \times Q \times \gamma_{\text{sludge}} \times (T_{\text{digester}} - T_{\text{influent}}) \times (1 + \kappa)$$

Where ρ_{sludge} is specific density of sludge (1000 kg m^{-3}), Q is influent sludge flow ($0.0025 \text{ m}^3 \text{ d}^{-1}$), γ_{sludge} is specific heat capacity of sludge ($4.18 \text{ kJ kg}^{-1} \text{ }^{\circ}\text{C}^{-1}$), T_{digester} is temperature in digester (35°C or 55°C), T_{influent} is temperature of influent sludge (24°C), κ is percentage of heat losses (0.08).

$$\text{Heat recovered} = \gamma_{\text{sludge}} \times Q \times C_{\text{sludge}} \times (T_{\text{digester}} - T_{\text{influent}}) \times (1 + \kappa) \times (\varphi)$$

Where φ is percentage of heat recovered (0.85).

$$\text{Heat output} = \varepsilon \times V \times \text{COD}_{\text{in}} \times E$$

Where ε is the heat calorific value of methane ($35,800 \text{ kJ m}^{-3}$), V is methane yield of system ($0.160 \text{ m}^3 \text{ kg COD}_{\text{added}}^{-1}$ for mesophilic alkaline two-stage system and $0.177 \text{ m}^3 \text{ g COD}_{\text{added}}^{-1}$ for thermophilic alkaline two-stage system), COD_{in} is amount of COD added ($0.08369 \text{ kg COD d}^{-1}$ for mesophilic alkaline two-stage system and $0.08370 \text{ kg COD d}^{-1}$ for thermophilic alkaline two-stage system) concentration of COD at inlet, E is the heat energy conversion efficiency (0.55).

Table D1. Summary of the heat energy balance between the two systems.

	Mesophilic alkaline two-stage system	Thermophilic alkaline two-stage system
Heat energy input (kJ d⁻¹)	124	158
Heat energy output (kJ d⁻¹)	264	292
Heat energy balance (kJ d⁻¹)	+ 140	+ 134

Appendix E. Relative abundances of the microbial communities investigated in **Chapter 5**.

Table E1 – Relative abundance percentages of archaeal genera in the feed sludge, single-stage, stage-1 and stage-2 reactors. Minor taxons with less than 2% relative abundance in any sample were grouped as *Archaea* < 2%. Period 1 was represented by 12, 45 and 70 days samples; period 2 by 116 and 158 days samples; and period 3 by 217 days sample.

	Feed sludge			Single-stage				Stage-1				Stage-2			
Genus / Timepoint (days)	12	45	70	0	12	45	70	0	12	45	70	0	12	45	70
<i>Methanobrevibacter</i>	0.4	1.8	0.1	0.3	0.0	0.2	0.3	0.0	1.7	19.8	9.1	0.0	0.1	1.3	3.6
<i>Methanoculleus</i>	0.0	0.0	0.0	0.3	2.7	0.4	0.3	0.0	0.0	0.3	0.0	0.0	0.2	1.0	0.5
<i>Methanolinea</i>	74.6	55.4	77.4	82.1	83.4	53.3	79.7	88.5	75.5	40.3	34.3	92.3	97.9	49.2	57.1
<i>Methanosarcina</i>	0.2	0.2	1.9	0.3	0.0	0.0	0.1	0.0	0.0	19.6	21.5	0.2	0.0	0.1	9.8
<i>Methanospirillum</i>	24.2	40.9	19.7	16.4	12.6	45.3	19.5	11.2	22.3	17.9	34.2	7.1	1.7	47.0	27.9
<i>Methanothermobacter</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
<i>Archaea</i> < 2%	0.6	1.7	0.9	0.6	1.2	0.9	0.2	0.3	0.5	2.1	0.8	0.3	0.0	1.5	1.1

	Feed sludge			Single-stage			Stage-1			Stage-2	
Genus / Timepoint (days)	116	158	217	116	158	217	116	158	217	158	217
<i>Methanobrevibacter</i>	0.2	0.2	0.6	0.1	0.2	0.1	0.1	0.1	0.0	0.0	0.0
<i>Methanoculleus</i>	0.0	0.0	0.0	0.1	0.6	0.5	0.0	0.0	0.0	6.6	4.0
<i>Methanolinea</i>	85.2	58.2	76.5	75.5	77.9	84.4	75.0	69.4	68.5	58.0	76.2
<i>Methanosarcina</i>	0.2	5.6	0.8	0.0	0.1	0.0	0.0	0.0	0.1	0.0	0.0
<i>Methanospirillum</i>	14.1	35.3	21.7	23.9	20.6	14.5	23.5	26.6	21.2	34.5	17.4
<i>Methanothermobacter</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.8	3.0	9.5	0.1	1.9
<i>Archaea</i> < 2%	0.4	0.7	0.4	0.4	0.6	0.4	0.6	0.9	0.7	0.7	0.5

Table E2 – Relative abundance percentages of bacterial phyla in the feed sludge, single-stage, stage-1 and stage-2 reactors. Minor taxons with less than 2% relative abundance in any sample were grouped as *Bacteria* < 2%. Period 1 was represented by 12, 45 and 70 days samples; period 2 by 116 and 158 days samples; and period 3 by 217 days sample.

	Feed sludge			Single-stage				Stage-1				Stage-2			
Phylum / Timepoint (days)	12	45	70	0	12	45	70	0	12	45	70	0	12	45	70
<i>Actinobacteria</i>	1.8	6.1	2.7	3.3	2.5	5.9	2.2	2.0	1.5	3.6	1.3	2.6	3.2	7.8	2.5
<i>Bacteroidetes</i>	28.1	28.9	30.8	30.3	39.1	19.5	39.1	17.6	46.3	36.4	32.8	26.1	35.9	23.2	34.1
<i>Caldiserica</i>	2.1	1.4	1.4	2.4	2.2	1.1	3.4	11.7	0.9	0.4	1.1	8.2	3.6	0.7	1.1
<i>Chloroflexi</i>	4.1	5.7	5.0	15.5	6.3	38.2	8.1	6.4	5.8	3.4	7.5	11.3	6.2	11.1	8.6
<i>Firmicutes</i>	15.4	25.0	10.4	14.7	13.0	8.0	10.6	21.5	33.0	43.8	38.1	18.3	15.5	28.8	26.5
<i>Nitrospira</i>	1.7	1.1	1.8	0.1	0.0	0.1	0.7	1.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0
<i>Proteobacteria</i>	38.1	25.5	40.2	18.3	13.4	7.8	18.9	23.5	8.8	7.6	13.7	18.1	22.7	13.4	15.9
<i>Spirochaetes</i>	0.2	0.7	0.3	1.1	3.9	1.9	4.5	2.7	0.1	0.4	0.3	2.1	1.8	0.9	1.2
<i>Synergistetes</i>	1.3	0.8	0.4	3.2	3.1	2.5	2.9	4.1	1.2	2.2	1.1	5.2	4.9	7.5	5.6
<i>Tenericutes</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.9	0.0	0.0	0.0	0.1
<i>U_Bacteria</i>	4.0	3.2	3.6	9.8	15.5	14.4	9.0	7.3	1.8	1.6	2.5	6.4	4.6	5.9	3.6
<i>Bacteria</i> < 2%	3.3	1.7	3.4	1.3	0.8	0.8	0.6	2.2	0.6	0.6	0.8	1.7	1.5	0.7	0.8

	Feed sludge			Single-stage			Stage-1			Stage-2	
Phylum / Timepoint (days)	116	158	217	116	158	217	116	158	217	158	217
<i>Actinobacteria</i>	2.3	2.3	1.5	2.1	1.2	0.8	0.6	1.2	0.1	1.4	1.9
<i>Bacteroidetes</i>	29.8	33.4	43.8	31.0	37.8	33.3	1.5	2.9	0.4	44.3	38.4
<i>Caldiserica</i>	3.4	3.5	2.0	2.9	1.7	2.0	0.1	0.3	0.2	0.5	0.1
<i>Chloroflexi</i>	5.3	4.9	5.3	18.6	20.9	8.3	0.2	0.8	1.3	3.7	3.2

<i>Firmicutes</i>	14.8	15.8	8.0	14.8	13.4	12.3	53.6	64.3	55.5	27.9	23.3
<i>Nitrospira</i>	4.3	5.5	3.0	1.0	0.2	0.8	0.0	0.0	0.0	0.0	0.0
<i>Proteobacteria</i>	33.8	30.0	30.9	15.8	9.9	18.1	2.0	3.5	2.4	13.9	19.9
<i>Spirochaetes</i>	0.2	0.2	0.4	5.3	9.2	8.0	0.1	0.1	0.2	3.0	3.7
<i>Synergistetes</i>	1.3	0.9	0.4	2.2	1.2	1.3	0.1	0.3	0.0	2.8	5.4
<i>Tenericutes</i>	0.0	0.0	0.0	0.0	0.0	0.0	39.5	20.6	37.5	0.0	0.2
<i>U_Bacteria</i>	2.2	2.2	2.2	5.6	4.1	14.4	2.1	6.0	2.2	2.1	3.2
<i>Bacteria < 2%</i>	2.6	1.3	2.7	0.8	0.4	0.8	0.1	0.1	0.2	0.3	0.8

Table E3. – Relative abundance percentages of the representative OTUs described in **Figure 5.4** and **Figure 5.5** during period 1.

			Feed sludge			Single-stage				Stage-1				Stage-2			
Phylum	Genus	OTU / Timepoint (days)	12	45	70	0	12	45	70	0	12	45	70	0	12	45	70
<i>Euryarchaeota</i>	<i>Methanobrevibacter</i>	OTUA00008	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.2	9.2	6.2	0.0	0.1	0.5	2.3
	<i>Methanobrevibacter</i>	OTUA00012	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	8.2	2.0	0.0	0.0	0.6	0.8
	<i>Methanothermobacter</i>	OTUA00013	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	<i>Methanolinea</i>	OTUA00001	73.4	54.8	76.2	80.5	81.0	52.1	74.3	87.9	74.4	39.7	32.4	91.8	96.2	41.2	48.3
	<i>Methanosarcina</i>	OTUA00005	0.2	0.1	0.7	0.2	0.0	0.0	0.0	0.0	0.0	19.3	21.2	0.2	0.0	0.1	9.6
	<i>Methanospirillum</i>	OTUA00002	13.9	24.9	9.8	6.8	4.6	22.9	10.6	7.3	15.3	12.0	10.0	3.5	0.2	22.5	10.3
	<i>Methanospirillum</i>	OTUA00004	4.7	6.5	4.3	2.6	2.7	6.8	3.1	2.1	4.1	3.0	3.0	1.0	0.0	2.5	2.1
<i>Bacteroidetes</i>	<i>U_Flavobacteriaceae</i>	OTUB00002	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	22.7	25.6	7.1	0.0	4.6	1.3	2.9
	<i>U_Flavobacteriaceae</i>	OTUB00037	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.7	0.0	0.0	0.0	0.7
	<i>U_Flavobacteriales</i>	OTUB00001	0.3	0.0	0.1	13.8	20.3	9.0	9.9	3.6	0.0	0.1	0.0	6.6	8.5	5.1	7.4
	<i>Paludibacter</i>	OTUB00007	1.5	6.5	2.7	0.4	2.1	0.4	2.4	1.4	1.4	1.0	2.7	1.9	0.4	0.4	2.2
	<i>U_Bacteroidetes</i>	OTUB00009	0.9	0.5	0.7	2.9	3.1	2.0	6.0	5.2	0.1	0.1	0.8	4.5	4.2	0.8	0.6
<i>Chloroflexi</i>	<i>Levilinea</i>	OTUB00003	0.1	0.0	0.0	9.2	4.9	25.4	4.3	0.3	0.0	0.1	0.0	5.3	2.5	0.4	0.2
	<i>Levilinea</i>	OTUB00025	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	3.6	1.4	5.2	0.0	0.4	3.5	4.1
<i>Firmicutes</i>	<i>Proteiniborus</i>	OTUB00013	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	8.0	5.0	6.4	0.0	2.0	3.2	2.5
	<i>Tissierella</i>	OTUB00063	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.0	0.5	0.7	0.0	0.7	0.6	0.3
	<i>Saccharofermentans</i>	OTUB00020	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	6.3	1.5	0.0	0.0	1.9	1.1
	<i>U_Clostridiales</i>	OTUB00005	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	<i>U_Clostridiales</i>	OTUB00023	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	<i>U_Clostridiales</i>	OTUB00044	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	<i>U_Firmicutes</i>	OTUB00016	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.4	5.1	1.3	0.0	0.7	2.8	2.1
<i>Proteobacteria</i>	<i>Smithella</i>	OTUB00012	0.0	0.0	0.0	0.8	1.0	0.4	1.4	0.2	0.0	0.2	0.0	4.1	10.5	3.6	2.7
<i>Synergistetes</i>	<i>U_Synergistaceae</i>	OTUB00033	0.0	0.1	0.1	0.6	0.6	0.8	0.8	1.1	0.6	0.5	0.3	1.0	2.0	3.6	2.2
<i>Tenericutes</i>	<i>Haloplasma</i>	OTUB00006	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table B2 – Relative abundance percentages of the representative OTUs described in **Figure 5.4** and **Figure 5.5** during period 2 and 3.

			Feed sludge			Single-stage			Stage-1			Stage-2	
Phylum	Genus	OTU / Timepoint (days)	116	158	217	116	158	217	116	158	217	158	217
<i>Euryarchaeota</i>	<i>Methanobrevibacter</i>	OTUA00008	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	<i>Methanobrevibacter</i>	OTUA00012	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	<i>Methanothermobacter</i>	OTUA00013	0.0	0.0	0.0	0.0	0.0	0.0	0.7	2.9	9.2	0.1	1.9
	<i>Methanolinea</i>	OTUA00001	83.6	57.4	75.0	74.3	57.4	82.6	73.9	67.7	67.5	54.0	71.6
	<i>Methanosarcina</i>	OTUA00005	0.1	4.2	0.6	0.0	4.2	0.0	0.0	0.0	0.1	0.0	0.0
	<i>Methanospirillum</i>	OTUA00002	6.5	14.4	9.4	9.6	14.4	5.9	13.1	15.0	11.6	6.3	7.1
	<i>Methanospirillum</i>	OTUA00004	2.6	7.0	5.2	3.5	7.0	3.0	4.7	5.1	4.7	2.1	2.9
<i>Bacteroidetes</i>	<i>U_Flavobacteriaceae</i>	OTUB00002	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0
	<i>U_Flavobacteriaceae</i>	OTUB00037	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	16.1	6.1
	<i>U_Flavobacteriales</i>	OTUB00001	0.1	0.3	0.1	6.1	13.7	8.7	0.0	0.0	0.0	11.3	11.3
	<i>Paludibacter</i>	OTUB00007	1.8	6.1	7.3	2.4	4.2	2.5	0.4	1.7	0.1	0.1	0.1
	<i>U_Bacteroidetes</i>	OTUB00009	1.1	1.5	0.8	3.3	2.9	3.4	0.0	0.0	0.1	0.4	0.0
<i>Chloroflexi</i>	<i>Levilinea</i>	OTUB00003	0.0	0.0	0.0	13.0	17.2	5.1	0.0	0.0	0.0	0.0	0.0
	<i>Levilinea</i>	OTUB00025	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1
<i>Firmicutes</i>	<i>Proteiniborus</i>	OTUB00013	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0
	<i>Tissierella</i>	OTUB00063	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	<i>Saccharofermentans</i>	OTUB00020	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0
	<i>U_Clostridiales</i>	OTUB00005	0.0	0.0	0.0	0.0	0.0	0.0	47.0	17.9	28.9	4.1	1.9
	<i>U_Clostridiales</i>	OTUB00023	0.0	0.0	0.0	0.0	0.0	0.0	0.0	17.8	14.5	1.3	2.2
	<i>U_Clostridiales</i>	OTUB00044	0.0	0.0	0.0	0.0	0.0	0.0	0.8	13.0	4.2	1.2	1.1
	<i>U_Firmicutes</i>	OTUB00016	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.6
<i>Proteobacteria</i>	<i>Smithella</i>	OTUB00012	0.0	0.0	0.0	0.8	0.1	1.6	0.0	0.0	0.0	4.7	6.5
<i>Synergistetes</i>	<i>U_Synergistaceae</i>	OTUB00033	0.0	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.6	0.7
<i>Tenericutes</i>	<i>Haloplasma</i>	OTUB00006	0.0	0.0	0.0	0.0	0.0	0.0	37.7	18.1	35.4	0.0	0.2