The role of hydrogenotrophic methanogens in an acidogenic reactor

Huang, Wenhai

2016

Huang, W. (2016). The role of hydrogenotrophic methanogens in an acidogenic reactor.
Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/144042>

<https://doi.org/10.32657/10356/144042>

This work is licensed under a Creative Commons Attribution-NonCommercial 4.0
International License (CC BY-NC 4.0).

Downloaded on 26 Apr 2023 22:49:07 SGT

NANYANG
TECHNOLOGICAL
UNIVERSITY

**THE ROLE OF HYDROGENOTROPHIC
METHANOGENS IN AN ACIDOGENIC
REACTOR**

HUANG WENHAI
SCHOOL OF CIVIL AND ENVIRONMENTAL ENGINEERING
2016

THE ROLE OF HYDROGENOTROPHIC METHANOGENS IN AN ACIDOGENIC REACTOR

HUANG WENHAI

School of Civil and Environmental Engineering

A thesis submitted to Nanyang Technological University
in partial fulfillment of the requirement for the degree of
Doctor of Philosophy

2016

ACKNOWLEDGEMENT

My deepest gratitude would go first and foremost to my Ph.D. supervisor, Professor Ng Wun Jern. His constant trust and guidance allows me the opportunity and freedom to explore my own research interest. Even during my failures in the research work, Prof. Ng never grudges his encouragement. Second, I would like to express my heartfelt gratitude to my co-supervisor Professor Cohen Yehuda, who is always there to assist and guide me whenever I have difficulties.

I am also greatly indebted to Dr Wang Zhenyu who is both a mentor and helpful friend to me in research and in life. I would also like to extend my gratitude to Asst. Professor Zhou Yan who always shares her experience and knowledge without reservation.

Special thanks to my wonderful colleagues and friends in Advanced Environmental Biotechnology Center (AEBC), and also friends in other centers of Nanyang Environment and Water Research Institute (NEWRI) for their assistance in helping me during difficult times and effort in establishing an enjoyable atmosphere within NEWRI.

Last but not least, I would also like to thank my family, for their consistent support and confidence in me all through these years.

TABLE OF CONTENTS

ACKNOWLEDGEMENT	i
TABLE OF CONTENTS.....	ii
ABSTRACT	vi
LIST OF TABLES	viii
LIST OF FIGURES.....	ix
PUBLICATION LIST	xii
LIST OF SYMBOLS.....	xiii
CHAPTER 1 INTRODUCTION.....	1
1.1 Background.....	1
1.2 Problem statement	3
1.3 Objectives.....	3
CHAPTER 2 LITERATURE REVIEW.....	5
2.1 High-strength organic wastewater	5
2.2 Anaerobic degradation.....	6
2.2.1 Development of anaerobic degradation	6
2.2.2 Principle of anaerobic degradation	8
2.3 Two-phase anaerobic degradation.....	10
2.4 Acidogenic phase	11
2.4.1 Acidogenesis	13
2.4.2 Acetogenesis	13
2.5 Effect of hydrogen on acidogenic phase.....	15
2.6 Effect of pH on acidogenic phase	18

2.7 Microbial studies on acidogenic phase	21
2.8 Knowledge gap and concerns about methanogens in acidogenic reactors	23
CHAPTER 3 TWO-PHASE ANAEROBIC DEGRADATION OF HIGH-STRENGTH ORGANIC WASTEWATER.....	26
3.1 Introduction.....	26
3.2 Material and methods	27
3.2.1 AnSBR setup and substrate	27
3.2.2 Start-up procedures	28
3.2.3 Operation of the two-phase anSBR system	30
3.2.4 Analytical methods	31
3.2.4.1 Composition of VFAs	31
3.2.4.2 Composition of biogas	32
3.2.4.3 DNA extraction	32
3.2.4.4 16S ribosomal RNA gene real-time quantitative PCR (qPCR)	33
3.3 Results and discussion.....	35
3.3.1 Performance of two-phase anSBR.....	35
3.3.2 Microbial community dynamics in two-phase anSBR – based on qPCR results.....	40
3.4 Conclusions	43
CHAPTER 4 ROLE OF HYDROGENOTROPHIC METHANOGEN IN ACIDOGENIC REACTOR.....	44
4.1 Introduction.....	44
4.2 Material and methods	45
4.2.1 AnSBR setup and operation.....	45
4.2.2 Batch test.....	46

4.2.3 pH change test on laboratory-scale reactor R _A	47
4.2.4 Analytical methods	47
4.2.4.1 Chemical analysis.....	47
4.2.4.2 Biological analysis.....	48
4.3 Results.....	49
4.3.1 Batch tests with biomass from the R _A	49
4.3.1.1 Performance of batch test.....	49
4.3.1.2 Real-time qPCR analysis of microbial community	51
4.3.2 Effect of pH change on R _A	52
4.3.2.1 Performance of R _A before pH change	52
4.3.2.2 Performance of R _A after pH change	52
4.3.2.3 Microbial community dynamics in R _A	54
4.4 Discussions.....	54
4.4.1 Effects of pH on performance and microbes in serum bottle batch study.....	54
4.4.2 Effects of pH on performance and microbes in R _A	56
4.4.3 Effect of hydrogen on acidogenesis.....	57
4.4.4 Role of methanogens in acidogenic reactor	58
4.5 Conclusions.....	59
CHAPTER 5 IMPROVED PERFORMANCE OF ACID-REACTOR.....	61
5.1 Introduction.....	61
5.2 Material and methods	62
5.2.1 AnSBR setup and operation.....	62
5.2.1.1 Operation before connecting R _A and R _H	62
5.2.1.2 Operation after connecting R _A and R _H	65
5.2.2 Analytical methods	65
5.2.2.1 Chemical analysis.....	65

5.2.2.2 Molecular biology analysis	66
5.3 Results	67
5.3.1 Performance of anSBR before Connection.....	67
5.3.2 Performance of anSBR after Connection	71
5.3.3 Microbial community dynamics in the R _A and R _H	72
5.4 Discussions.....	74
5.4.1 Conversion of hydrogen to methane	74
5.4.1.1 Methane production from acetotrophic methanogens in R _A	75
5.4.1.2 Methane production from hydrogen produced in R _A ..	76
5.4.2 Microbial community changes in the reactors	77
5.4.3 Improved acidogenesis by external hydrogenotrophic methanogen.....	78
5.5 Conclusions	79
CHAPTER 6 CONCLUSIONS AND RECOMMENDATIONS	81
6.1 Major findings	81
6.2 Recommendations.....	82
REFERENCE	85

ABSTRACT

Two-phase anaerobic degradation, compared to single-stage anaerobic degradation, has the potential for higher stability, efficiency and biogas production. The acidogenic phase, as the first phase, not only provides the necessary substrate for the next phase, i.e. the methanogenic phase, it also helps to maintain stability of the whole process. Methanogens, especially hydrogenotrophic methanogens, not only keep a proper hydrogen partial pressure but also behave as syntrophic partner to acetogens and acetate-oxidizing bacteria in various biochemical reactions in the acidogenic phase.

A laboratory-scale two-phase anaerobic sequencing batch reactor system was studied to investigate the effects of pH shock on performance and microbial community structures in the reactors and batch studies. Results showed that pH shock caused increase in hydrogen content, low production of VFA and decrease of the hydrogenotrophic methanogen population. However, recovery of pH did not result in immediate recovery of acidogenic reactor performance. The recovery of acidogenic reactor performance only occurred with recovery of the hydrogenotrophic methanogen population and decrease of hydrogen. These observations indicated hydrogenotrophic methanogen helped to maintain a low hydrogen presence in the acidogenic reactor which would then better ensure healthy VFA production.

To investigate if external hydrogenotrophic methanogens could also help ease the hydrogen stress on VFA production in the acidogenic reactor at low pH, a laboratory-scale reactor designed to enrich hydrogenotrophic methanogens (R_H) was incorporated into the two-phase anSBR system. pH of the acidogenic reactor was maintained at 4.5 to inhibit the resident hydrogenotrophic methanogen in the acidogenic reactor and encourage hydrogen production. Results showed that R_H could effectively consume the hydrogen and improve

VFA production in acidogenic reactor which enhanced overall performance of the two-phase anSBR.

The findings indicated hydrogenotrophic methanogens play an important role in the acidogenic reactor. Maintaining proper methanogenic activity internally or externally for the acidogenic phase is essential to better ensuring an efficient performance of the overall two-phase anaerobic system.

LIST OF TABLES

Table 2.1 Comparison of aerobic and anaerobic treatment (Chan et al., 2009)	6
Table 3.1 Synthetic feed composition	29
Table 3.2 Two-phase anSBR operating parameters.....	30
Table 3.3 Operation schedule of R_A^a	31
Table 3.4 Primer and probe sets for qPCR (Yu et al., 2005; Bialek et al., 2011)	34
Table 3.5 Theoretical equivalent COD of respective VFA.....	35
Table 4.1 Operating parameters of R_A	46
Table 4.2 Effects of pH on acidogenesis	57
Table 5.1 Synthetic feed composition	64
Table 5.2 Operating parameters of R_H augmented two-phase anSBR	65

LIST OF FIGURES

Figure 2.1 Major degradative steps during anaerobic decomposition (Lema <i>et al.</i> , 1988; ElFadel <i>et al.</i> , 1997; Demirel and Scherer, 2008).....	9
Figure 2.2 Major intermediary products, products and microorganisms involved in acidogenic phase. APB: acid-producing bacteria (acidogens); ATB: acetogenic bacteria (acetogens); HATB: homoacetogens; AOB: acetate oxidizing bacteria; HUM: hydrogen-utilizing methanogens; AUM: acetate utilizing methanogens; ΔG : indicating syntrophic relationship between ATB and HUM (Mosey, 1983).	12
Figure 2.3 Relative rates of acid formation from glucose regulated by trace concentrations of hydrogen in the gas (Mosey, 1983).	17
Figure 2.4 Relationship between hydrogen content in biogas and ratios of metabolites (Won and Lau, 2011).....	18
Figure 2.5 The overall acidogenic activity as a function of pH (Yu and Fang, 2002, 2003).	20
Figure 3.1 Schematic diagram of the laboratory-scale two-phase anSBR.....	28
Figure 3.2 COD and VFA of R _A during Period I-IV. (a): COD of influent into R _A (Infl A), effluent from R _A (Effl A), theoretical COD of VFA in influent into R _A (VFA Infl A), effluent from R _A (VFA Effl A) and HAc in effluent from R _A (HAc Effl A); (b): Theoretical COD of effluent HPr (HPr Effl A) and HBu (HBu Effl A) from R _A	37
Figure 3.3 Biogas of R _A during Period I-IV: CH ₄ and CO ₂ content of biogas from R _A	38
Figure 3.4 Removal rate and AOF in R _A	38
Figure 3.5 COD and VFA of R _M during Period I-IV: COD of influent into R _M (Infl M), effluent from R _M (Effl M), theoretical COD of VFA in influent into R _M (VFA Infl M), effluent from R _M (VFA Effl M) and HAc in effluent from R _M (HAc Effl M).	39
Figure 3.6 Biogas of R _M during Period I-IV: CH ₄ and CO ₂ content of biogas from R _M	39

Figure 3.7 Quantification of 16S rRNA gene concentration of bacteria and methanogenic communities in: (a) R _A and (b) R _M of Period I-IV.	42
Figure 3.8 RDM of: (a) R _A and (b) R _M of Period I-IV.	43
Figure 4.1 COD, VFA, biogas and pH information during batch test: (a) COD of TVFAs in effluent (TVFA Effl), COD of effluent (COD Effl), COD of HBU and HAc in effluent (HBU Effl, HAc Effl); (b) CH ₄ and H ₂ content of biogas from serum bottles and pH of respective effluent. CO ₂ data are not shown, since the content of CO ₂ = (100% - [CH ₄] - [H ₂]). The dash line marked the day when NaOH was added to increase the pH in serum bottles (Day 11).	50
Figure 4.2 Quantification of 16S rRNA gene concentration of bacteria and methanogenic communities in serum bottle I-III at the start of the batch test (No. 0) and at the end of the batch test (No. I-III).	51
Figure 4.3 Performance and microbial community structure of R _A . (a) COD, VFA variation: COD of influent into R _A (Infl A) and effluent from R _A (Effl A); COD of TVFA in influent into R _A (VFA Infl A) and effluent from R _A (VFA Effl A); COD of HAc in effluent (HAc Effl A); (b) Biogas: CH ₄ , CO ₂ and H ₂ content of biogas from R _A ; (c) Quantification of 16S rRNA gene concentration of methanogenic communities and bacteria in the R _A	53
Figure 5.1 Schematic diagram of the R _H augmented two-phase anSBR: a) before the Connection; b) after the Connection.	63
Figure 5.2 COD and VFA of R _A : COD of influent into R _A (Infl A), effluent from R _A (Effl A), theoretical COD of HAc in influent into R _A (HAc Infl A), effluent from R _A (VFA Effl A) and HAc in effluent from R _A (HAc Effl A).	68
Figure 5.3 COD and VFA of effluent from R _M and R _H : effluent from R _M and R _H (Effl M and H), theoretical COD of effluent VFA from R _M and R _H (VFA Effl M and H) and HAc in effluent from R _M and R _H (HAc Effl M and H).	69
Figure 5.4 Theoretical COD of effluent HPr from R _A and R _M	69
Figure 5.5 Biogas of R _A and R _H : H ₂ content in R _A and CH ₄ content in R _H	70
Figure 5.6 Biogas of R _A and R _H : H ₂ production rate in R _A and CH ₄ production rate in R _H	70

Figure 5.7 Biogas of R_M : CH_4 and CO_2 content in R_M	71
Figure 5.8 Biogas of R_M : CH_4 and CO_2 production rate in R_M	71
Figure 5.9 Quantification of 16S rRNA gene concentration of bacteria and methanogenic communities in R_A	73
Figure 5.10 Quantification of 16S rRNA gene concentration of bacteria and methanogenic communities in R_H	74
Figure 6.1 Major processes occurring in acidogenic reactor.	82

PUBLICATION LIST

PATENT

- Wang Z., Zhou Y., Huang W., Ng, W. J., 2014. A process for mitigating sulfate impact on and enhancing methane production in anaerobic systems (Application No. PCT/SG2014/000033)
- Wang Z., Zhou Y., Huang W., Ng, W. J., 2013. Method and apparatus for carbon dioxide capture and energy recovery enhancement (Application No. PCT/SG2013/000153)

JOURNAL PAPER

- Huang W., Wang Z., Guo Q., Wang H., Zhou Y., Ng W. J., 2016. Pilot-scale landfill with leachate recirculation for enhanced stabilization. *Biochemical Engineering Journal*. 105, 437–445.
- Huang W., Wang Z., Zhou Y., Ng W. J., 2014. The Role of Hydrogenotrophic Methanogens and Hydrogen Regulated Performance in an Acidogenic Reactor. *Chemosphere*. 140, 40-46.

LIST OF SYMBOLS

anSBR	Anaerobic Sequencing Batch Reactor
AOF	Acidified Organic Fraction
ATP	Adenosine Triphosphate
BOD	Biochemical Oxygen Demand
CH ₄	Methane
CO ₂	Carbon Dioxide
COD	Chemical Oxygen Demand
CSTR	Continuous Stirred-Tank Reactor
H ₂	Hydrogen
HAc	Acetic Acid
HBu	Butyric Acid
HCa	Capronic Acid
HHe	Heptanoic Acid
HPr	Propionic Acid
HRT	Hydraulic Retention Time
HVa	Valeric Acid
MLSS	Mixed Liquor Suspended Solids
MLVSS	Mixed Liquor Volatile Suspended Solids
NAD ⁺	Nicotinamide Adenine Dinucleotide
NADH	Reduced Nicotinamide Adenine Dinucleotide
OLR	Organic Loading Rate
PBS	Phosphate Buffer Solution
PCR	Polymerase Chain Reaction
PLC	Programmable Logic Controller
R _A	Acidogenic Reactor
RDM	Relative Dominance of Methanogen
R _H	Hydrogenotrophic Reactor
R _M	Methanogenic Reactor

R_{AH}	Gaseous Phase of Combination of R_A and R_H
SBR	Sequencing Batch Reactor
SCOD	Soluble Chemical Oxygen Demand
SRT	Solid Retention Time
TCD	Thermal Conductivity Detector
VFA	Volatile Fatty Acid

CHAPTER 1 INTRODUCTION

1.1 Background

High-strength organic wastewaters, such as food processing wastewater and landfill leachate, due to their biodegradability, are often treated aerobically or anaerobically (Lema *et al.*, 1988; Burgoon *et al.*, 1999; Rajbhandari and Annachhatre, 2004; Chrobak and Ryder, 2005; Scott *et al.*, 2005). Compared with aerobic treatment, though anaerobic treatment may not result in as high a treated effluent quality, energy requirement is much lower and there is also the benefit of producing combustible methane – an energy source. As energy cost rises, anaerobic treatment of wastewater becomes increasingly favored, especially as pretreatment for high-strength organic wastewaters.

However, there also are limitations in the anaerobic process. To achieve stable performance, close monitoring and maintenance of the anaerobic process is required due to its inherent complexity. Any disturbance, such as organic and/or hydraulic over loadings and fluctuations in pH, can easily cause process failure. Intermediary products (e.g., VFAs) from acidogenesis tend to be produced faster than their utilization by methanogens, leading to accumulation of organic acids. If a proper balance is not kept between acidogens and methanogens, as organic acids accumulate and pH drops, methanogenesis will be inhibited.

To help address this problem, the idea of two-phase (i.e., acidogenic phase and methanogenic phase) anaerobic process was proposed by Pohland and Ghosh (Pohland and Ghosh, 1971). By phase separation, optimum growth conditions could be developed for both acidogens and methanogens, the substrate turnover rate would be increased, which consequently would improve treatment efficiency, overall process stability and methane production (Cohen *et al.*, 1980, 1982).

As the first phase, the acidogenic phase would not only produce the necessary substrate for the next phase, it is also essential for the overall stability of the

whole process (Ince, 1998). However, various microorganisms, such as acidogens, acetogens, homoacetogens and methanogens, and the complex conversions between acetate, other VFAs, hydrogen and carbon dioxide are involved in this phase (Mosey, 1983). All of these microorganisms, and their biological reactions and products can affect the acidogenic phase.

Hydrogen plays an essential role in keeping the redox balance within the acidogenic phase and so affects the distribution of acid products arising from fermentation of organic matters. Reports in the literature on acidogenesis suggest conflicting conclusions regarding the relationship between hydrogen content/production and VFA product distribution (Mosey, 1983; Mu *et al.*, 2006; Li *et al.*, 2010; Wu *et al.*, 2010; Won and Lau, 2011). Similarly, pH as an important factor that influences the acidogenesis process was reported to be closely related to degradation of substrate and distribution of organic acid products (Ren *et al.*, 1997; Yu and Fang, 2002, 2003). However, reports on its effect on acidogenesis also show conflicting results (Yu and Fang, 2002, 2003; Li *et al.*, 2010; Wu *et al.*, 2010). These process-oriented studies regarding hydrogen and/or pH may provide useful guidelines for improving performance of the acidogenic process, but not necessarily better understanding of the intrinsic bioprocesses within the acidogenic process without adequate data concerning the microorganisms in these acidogenic reactors.

With development of culture-independent molecular techniques based on detection of specific regions of 16s-rRNA genes, quantitative analysis of microbial communities has also been enabled recently (Yu *et al.*, 2005; Yu *et al.*, 2006; Kim *et al.*, 2011). These new techniques provided an improved method to seek understanding of the relationship between microbial structures, the dynamics and process performance.

1.2 Problem statement

As the “key” microorganisms in the acidogenic phase, acidogens have been extensively investigated. Intuitively it would seem reasonable only few studies have investigated methanogens in the acidogenic phase (Mizuno et al., 1998; Shimada et al., 2011). However, methanogens, such as the hydrogenotrophic methanogens, can play an important role in the acidogenic phase (Shimada et al., 2011) . They not only keep a proper partial pressure of hydrogen in the acidogenic phase, but also behave as syntrophic partner for acetogens and acetate-oxidizing bacteria in various biological reactions (Madigan and Brock, 2009). It should be noted, due to their heterogeneous nature, acidogens are perceived as being more robust and adaptable than methanogens; methanogens in the acidogenic phase where conditions favor the acidogens, are therefore likely to be more vulnerable and thus potentially more sensitive to environmental changes in the reactor. Any change, like loading fluctuations, may therefore have a larger impact on resident methanogens than acidogens in acidogenic reactor. A change in the microbial structure in terms of the methanogenic community may consequently affect acidogenic process performance (Mosey, 1983; Yu and Fang, 2002, 2003). Therefore, research on methanogens therein is also essential for better understanding of the performance of acidogenic reactors.

1.3 Objectives

The objectives of this project are to understand the role of methanogens in the acidogenic phase, and investigate the microbial community dynamics in the reactors during operation of a two-phase anaerobic system. The following were investigated:

1. Performance change and microbial community dynamics in two-phase anaerobic reactors during phase separation;
2. Effects of pH shock on performance and microbial community structure of reactors;

3. Defining key microbes and major pathways in the acidogenic reactor under and after pH stress;
4. Possible methods for improving acidogenic reactor performance under pH stress.

While existing reports on effects of hydrogen and pH showed conflicting results on acidogenesis in acidogenic phase, this project, instead of conducting more investigation on performance in response to pH change, tended to explore the performance of an acidogenic reactor in response to methanogen population changes. The results would indicate the essential role of methanogens in acidogenic reactor when performance of the reactors shifted not with pH change but with increase and decrease of residential methanogen population. Further, the effects of external methanogens on an inhibited (by low pH) acidogenic reactor were also investigated to further confirm the role of methanogens for acidogenic reactor. Therefore, keeping a proper methanogenic activity internally or externally for the acidogenic reactor is essential to guarantee an efficient performance of overall two-phase anaerobic system.

CHAPTER 2 LITERATURE REVIEW

2.1 High-strength organic wastewater

High-strength organic wastewaters, such as food processing wastewater and landfill leachate, are characterized by high COD, BOD, nutrients, and organic and inorganic contents. Such wastewaters, if discharged without proper treatment, can threaten the ecology of receiving water bodies. Due to the biodegradability of high-strength organic wastewaters, aerobic and anaerobic treatment are often preferred for pollution control of these wastewaters (Lema *et al.*, 1988; Burgoon *et al.*, 1999; Rajbhandari and Annachhatre, 2004; Chrobak and Ryder, 2005; Scott *et al.*, 2005). With appropriate process design and control, almost all wastewaters with a BOD/COD ratio greater than 0.5 can be treated biologically (Tchobanoglous *et al.*, 2003). In the aerobic process, organic pollutants are converted to biomass and carbon dioxide by microorganisms (aerobes) using free or dissolved oxygen; while in the anaerobic process, the organic matter is degraded into methane, carbon dioxide and water in the absence of oxygen. The former process is more commonly used for treatment of wastewaters with lower organic strength (biodegradable COD concentration less than 1000 mg/L) to achieve a high degree of treated effluent quality; while the latter process is more suitable for the pretreatment of high strength organic wastewaters (biodegradable COD concentrations over 4000 mg/L) (Chan *et al.*, 2009). Moreover, in anaerobic treatment of organic wastewaters, while achieving the objective of pollution control, resources such as combustible methane can also be recovered. General merits of aerobic and anaerobic treatment are highlighted in Table 2.1.

As listed in Table 2.1, though anaerobic treated effluent quality is typically lower than the aerobically treated, energy requirement in the latter is also much higher. As energy cost rises, anaerobic treatment (or pretreatment) of wastewater is increasingly favored nowadays.

Table 2.1 Comparison of aerobic and anaerobic treatment (Chan *et al.*, 2009)

Feature	Aerobic	Anaerobic
Organic removal efficiency	High	High
Effluent quality	Excellent	Moderate to poor
Organic loading rate	Moderate	High
Sludge production	High	Low
Nutrient requirement	High	Low
Alkalinity requirement	Low	High for certain industrial waste
Energy requirement	High	Low to moderate
Temperature sensitivity	Low	High
Startup time	2–4 weeks	2–4 months
Odor	Less opportunity for odors	Potential odor problems
Bioenergy and nutrient recovery	No	Yes
Mode of treatment	Total (depending on feedstock characteristics)	Essentially pretreatment

2.2 Anaerobic degradation

2.2.1 Development of anaerobic degradation

The chronological development of anaerobic biotechnology could date back to in 1776, when Volta firstly recognized that anaerobic process could convert organic matters to methane gas. In 19th century, a septic tank was built in the city of Exeter, England, in 1895 by Donald Cameron to collect methane for heating and lighting (McCarty, 2001). With further development of anaerobic technology, the focus then shifted from wastewater treatment to sludge treatment. Sludge digestion became increasingly popular in larger cities, and the importance of methane gas generation was widely recognized. Methane gas was used for heating digester; it was collected and delivered to municipal gas systems, and it was used for power generation for operating biological wastewater treatment systems. Today, anaerobic digestion is widely adopted for

the stabilization of municipal sludge and animal manure, and recovery of useful renewable energy—methane and biosolids.

However, due to a failure to understand the fundamental of the process, application of anaerobic biotechnology was limited until 1950. Stander (1950) was the first to recognize the importance of solids retention time (SRT) for successful anaerobic treatment of different wastewaters. SRT is an important parameter for selecting a suitable bioreactor because anaerobes grow slowly during metabolic generation of methane, hydrogen and other products. It is essential to select a bioreactor configuration that decouples the hydraulic retention time (HRT) from SRT. Such decoupling can maintain a significantly high SRT/HRT ratio and prevents washout of slow-growing anaerobes. Other considerations include feedstock types (solid, liquid, or gaseous), product inhibition, bioenergy recovery, and mass transfer limitations. This has been the basis for the development of the so-called high-rate anaerobic reactor in which SRT and hydraulic retention time (HRT) were uncoupled.

The development of high-rate anaerobic reactor led to a wider application of anaerobic biotechnology, particularly for industrial wastewater treatment and biogas recovery. Applications of high-rate anaerobic reactors in industrial wastewater treatment worldwide covered industries including Breweries, beverages, landfill leachate, paper, pulp and food (Chan *et al.*, 2009).

Anaerobic degradation has nowadays become one of the most promising technologies able to synchronize human activities with natural cycles. In Europe, for example, anaerobic degradation has become quite widespread and has been able to produce 8.3 Mtoe (megatonnes of oil equivalent) of energy, which represents approximately 0.4 % of the 1,703 Mtoe of primary energy consumption of the 27 countries of the European Union in 2009 (EurObserv'er, 2010).

2.2.2 Principle of anaerobic degradation

Anaerobic processes are defined as biological processes in which organic matters are metabolized in an environment free of dissolved oxygen or its precursors (e.g., H_2O_2). Anaerobic process is classified as either anaerobic fermentation or anaerobic respiration depending on the type of electron acceptors.

In the conventional anaerobic process, generally four major steps take place sequentially. Hydrolysis firstly degrades complex macromolecular organics (i.e., proteins, lipids and carbohydrates) to simpler monomers such as amino acids and sugars; then by acidogenesis, fermentative bacteria (acidogens) convert these smaller organics into hydrogen, carbon dioxide and intermediary products, such as volatile fatty acids (VFAs) and ethanol; acetogenesis then takes place and converts these intermediary products into acetic acid; these steps are then followed by methanogenesis which utilizes these acetic acids, hydrogen and carbon dioxide to produce methane (shown in Figure 2.1).

Figure 2.1 Major degradative steps during anaerobic decomposition (Lema *et al.*, 1988; ElFadel *et al.*, 1997; Demirel and Scherer, 2008).

Two groups of microorganisms, in particular, are considered essential to the overall performance of the process. One is acidogens which are fast-growing bacteria that convert biodegradable organics into substrates for subsequent methanogenesis. Various bacteria in anaerobic systems are capable of producing organic acids. They can be obligate anaerobes, aero-tolerant anaerobes or facultative anaerobes. The second group is the methanogens which convert products from the acidogens to methane and so complete the anaerobic process. Unlike acidogens which are comprised of diverse groups of bacteria, methanogens are obligate anaerobes and classified into only five orders, i.e., Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales and Methanopyrales within the domain of Archaea. Most methanogens preferred a temperature between mesophilic 20 °C and thermophilic conditions 60 °C, but Methanopyrales grow only under extremely high temperature (higher than 80 °C), and Methanococcales are commonly not

found in anaerobic treatment bioreactors. The other three orders of methanogens would be more typically present in anaerobic treatment processes. Among these three orders of methanogens, Methanobacteriales and Methanomicrobiales are hydrogenotrophic methanogens, which utilize only hydrogen and carbon dioxide or formate to produce methane; while Methanosarcinales are acetotrophic methanogens in which the family *Methanosaetaceae* utilize only acetate, and *Methanosarcinaceae* utilize acetate as well as various other methyl compounds and hydrogen (Madigan and Brock, 2009).

Due to differing growth characteristics of acidogens and methanogens, one single set of operating condition is not possible to maximize the activities of both acidogens and methanogens. Acidogens are favored by shorter SRTs and lower pH (4.5-6.5), while methanogens prefer longer SRTs and neutral pH (6.5-7.5). Moreover, for a properly functioning anaerobic system, balance among the various microorganisms is required. Since the growth rate of acidogens is much higher than methanogens, intermediary products (e.g., VFAs) from acidogenesis tend to be produced faster than their utilization by methanogens, leading to possible accumulation of organic acids. If a proper balance is not kept between acidogens and methanogens, as organic acids accumulate and pH drops, methanogenesis will be inhibited making the whole anaerobic system acidic and eventually fail.

2.3 Two-phase anaerobic degradation

Due to the above reasons, Pohland and Ghosh (1971) first proposed the idea of separating the two main groups of microorganisms physically into serial phases (reactors) to maximize the growth rate of each. Several techniques have been developed to achieve phase separation – e.g., membrane separation, kinetic control, and pH control (Pohland and Mancy, 1969; Cohen *et al.*, 1979; Chung *et al.*, 1998). Among these techniques, a combination of kinetic control and pH

control had proven to be the most successful and has been used in research and application of two-phase anaerobic degradation (Tchobanoglous *et al.*, 2003). In the first phase (acidogenic phase), a pH of 4.5-6.5 and a short SRT is conducive to high production of VFAs. In the second phase (methanogenic phase), a neutral pH and a longer SRT is introduced to enhance methanogen activity and thus maximizes removal efficiency of pollutants and biogas production.

By phase separation, optimum growth conditions could be developed for the acidogens and methanogens, leading to production of more suitable substrate for methanogens from the acidogenic phase; inhibitory effects to methanogens caused by accumulation of VFAs and pH fluctuation can be circumvented; and compared with single stage anaerobic degradation, since acidogenesis and methanogenesis are physically separated, the substrate turnover rate is also increased, consequently improving treatment efficiency and methane production (Cohen *et al.*, 1980, 1982).

2.4 Acidogenic phase

As the first phase of the two-phase anaerobic degradation process, the acidogenic phase not only produces the necessary substrate for the next phase, it is also anticipated to help maintain stability of the whole process. Unlike the methanogens in the methanogenic phase, the nature of bacteria in the acidogenic phase is more heterogeneous. This property of acidogenic bacteria enables the acidogenic phase to act as a metabolic buffer for the methanogenic phase during organic and/or hydraulic over loadings and fluctuations. Moreover, materials that are toxic to methanogens may also be removed in the acidogenic phase (Ince, 1998).

Generally, the acidogenic phase consists of two major steps – acidogenesis and acetogenesis. These two steps convert organic matters from small organic

molecules into substrates, such as acetate and hydrogen for the methanogenic phase (Figure 2.2).

Figure 2.2 Major intermediary products, products and microorganisms involved in acidogenic phase. APB: acid-producing bacteria (acidogens); ATB: acetogenic bacteria (acetogens); HATB: homoacetogens; AOB: acetate oxidizing bacteria; HUM: hydrogen-utilizing methanogens; AUM: acetate utilizing methanogens; ΔG : indicating syntrophic relationship between ATB and HUM (Mosey, 1983).

2.4.1 Acidogenesis

Taking degradation of carbohydrates as example, carbohydrates are hydrolyzed to glucose. Glucose then undergoes glycolysis and is converted to pyruvate. Free energy is released in this process and used to form high-energy compound adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide (NADH). Since the NADH is the reduced form of NAD^+ , it has the potential to release one hydrogen atom and form hydrogen gas. This is one of the sources of hydrogen. Pyruvate is not stable and tends to be converted to other fatty acids, such as acetate, propionate and butyrate, with the assistance of various coenzymes (HSCoA and NADH). Hydrogen is also produced in these processes.

Following equations can represent the above reactions:

Of the three reactions listed above, the first is preferred because by production of acetate, more energy is conserved for the growth of acidogenic bacteria and it also provides the acetotrophic methanogens with the prime substrates for methane production (Mosey, 1983; Madigan and Brock, 2009). The other reactions, i.e., the formation of propionate and butyrate, are acidogens' response to accumulation of hydrogen. Diversion of fermentation of glucose towards butyrate reduces both the hydrogen output and acid load on the system. Production of propionate actually reverses hydrogen production. These reactions can help the acidogens maintain the redox balance in the system.

2.4.2 Acetogenesis

Following acidogenesis, acetogens further convert the propionate and butyrate into acetate in the following reactions:

As is indicated by the above equations, conversion of propionate and butyrate to acetate is endergonic under standard conditions (thermodynamically unfavored), thus additional energy source is needed to facilitate the reactions. In anaerobic degradation, the energy is provided by hydrogen-utilizing methanogens (hydrogenotrophic methanogens) which utilize the hydrogen and carbon dioxide to produce methane.

While the utilization of carbon dioxide and hydrogen provide the necessary energy for acetogens to produce acetate, the hydrogen produced by acetogens can also be used as electron donor for methanogenesis by hydrogenotrophic methanogens, making a syntrophic relationship between acetogens and hydrogenotrophic methanogens.

Besides the above reaction, acetate can also be produced in another acetogenesis process.

Unlike the syntrophic degradation of fatty acids with production of hydrogen and/or carbon dioxide, this acetogenesis process produces acetate as sole product. This is also why the acetogen involved in this reaction is called homoacetogen.

Another bacteria involved in the acidogenic phase are the acetate-oxidizing bacteria which reverse the above reaction. Since oxidization of acetate is endergonic, acetate-oxidizing bacteria also live syntrophically with hydrogenotrophic methanogens. Some of the acetate-oxidizing bacteria are also homoacetogens (Hattori, 2008).

Accordingly, producing acetate from organic matters in the acidogenic phase is a complicated process. It involves varieties of microorganisms such as acidogens, acetogens, homoacetogens and even methanogens (mainly hydrogenotrophic methanogens) and also conversions between acetate, other VFAs, hydrogen and carbon dioxide. All of these microorganisms, biological reactions and products can affect the acidogenic phase. Therefore, an understanding of how they affect the acidogenic phase is essential to improve the efficiency and the overall performance of two-phase anaerobic system.

2.5 Effect of hydrogen on acidogenic phase

Among the products of the acidogenic phase, hydrogen is one that can have a major effect on acid fermentation. Many fermentative bacteria in the acidogenic phase produce hydrogen, which provides a specific mechanism to dispose of excess electrons through the activity of hydrogen producing enzymes in bacteria. As distinguished from methane production, hydrogen is one of the intermediates formed during anaerobic fermentation, which means, hydrogen is not always released to the outer surface during the reaction. It can be available for other reactions where necessary. Many of the major processes involved in the phase produce or consume hydrogen (Figure 2.2). It can affect the cleavage of glucose in anaerobic degradation by altering the proportions of fermented products, such as acetate, propionate, and butyrate. Mosey (1983) established a theoretical relationship between relative rates of acid formation (ratio of the formation rate of organic acid to the unregulated rate of uptake of glucose) and concentration of hydrogen in the biogas (Figure 2.3). It showed increased concentration of

hydrogen slows down the glycolysis of glucose but speeds up the conversion from pyruvate to butyrate and propionate in acidogenesis reactions by lowering the ratio of $[NAD^+]/[NADH]$. It may also inhibit the acetogenesis of butyrate and propionate to acetate by acetogenic bacteria for thermodynamic reasons (see eqn. 4 and 5).

However, a recent study on an acidogenic-phase anaerobic sequencing batch reactor (anSBR) treating a sucrose-rich synthetic wastewater for hydrogen production found opposite relationships between acid metabolites and hydrogen content in biogas, showing the ratio of HPr/HAc increased as hydrogen content in biogas decreased [Figure 2.4, (Won and Lau, 2011)]. Other but conflicting results on the relationship between hydrogen content/production and production of butyrate as a metabolite also exist. Mu *et al.* (2006) found that during startup of an acidogenic granule-based reactor, as partial pressure of hydrogen increased in the biogas, a fermentative type shift from acetate to butyrate occurred. Wu *et al.* (2010), however, investigated an acid-phase anSBR treating liquid swine manure with glucose supplement and found that optimal acetate production with highest HAc/HBu ratio (mass to mass: 2.42 ± 0.13) was found at pH 5.0 when hydrogen content and production was also the highest (i.e., 36.9%). The authors attributed these results to the higher production of hydrogen from fermentation of glucose to acetate than butyrate, according to eqn. 1 and 3. Interestingly, with a different seed sludge treating liquid swine manure with supplement using a similar anSBR, the same research group derived completely different conclusion (Li *et al.*, 2010). It was found that 5.0 was still the optimal pH for hydrogen production, but HAc/HBu (mass to mass: 0.10) was lowest. These contradictory research results on relationship between hydrogen content/production and acid fermentation have complicated understanding of the effects of hydrogen on fermentation of organic matters in acid-phase anaerobic reactors. It should be noted, however, though these studies generally relate the hydrogen content or concentration in the biogas to the acidogenic metabolites,

hydrogen content in biogas was not the direct cause of any interruption of acidogenesis. It was the dissolved hydrogen in the solution that contributed to such changes in acidogenesis. The reason why gas content was used in these studies was that according to Henry's law, the solubility of gas is closely related to the gas content in gaseous phase.

Figure 2.3 Relative rates of acid formation from glucose regulated by trace concentrations of hydrogen in the gas (Mosey, 1983).

Figure 2.4 Relationship between hydrogen content in biogas and ratios of metabolites (Won and Lau, 2011).

2.6 Effect of pH on acidogenic phase

Other than hydrogen, another important factor that cannot be overemphasized with respect to the acidogenic phase is pH. pH can greatly influence the biochemical reactions of microbes which cover many aspects of microbial metabolism (e.g., eqn. 1-3 and 7); pH also affects the growth of microbes, distribution of microbial species and even the morphology and structures of microbes (Kisaalita *et al.*, 1987). Many researches on effects of pH on acidogenesis of different waste waters have been carried out. The metabolic pathways involving acetic and butyric acid production appeared to favor a pH ranging from 4.5 to 6.0 (Guo *et al.*, 2010). Yu and Fang studied the effects of pH on acidogenesis of synthetic gelatin-rich wastewater and dairy wastewater (Yu and Fang, 2002, 2003). The authors found that overall acidogenesis performance improved with increasing pH, in the pH range of 4.0 – 6.0 (Figure 2.5). Fraction of HAc in effluent organic products decreased for around 15% with pH decreasing from 6.0 to 4.0. Meanwhile VFA production was also halved

during the decrease of pH. However, data from other reports suggested otherwise (Li *et al.*, 2010; Wu *et al.*, 2010). No specific relationship between VFA production and pH was observed from pH 4.7 to 5.9 in the study of Li *et al.* (Li *et al.*, 2010). Similar results were found in the study of Wu *et al.* (Wu *et al.*, 2010). It was found that VFA concentration did not change much at pH from 4.4 to 5.0 but decreased greatly when pH further increased to 5.6. For HAc, however, its proportion in total soluble microbial products increased with pH increasing from 4.4 to 5.0, and then decreased as pH further increased to 5.6. The authors attributed this phenomenon to microbial community shift. It was suggested that in pH 4.4-5.0, the microbial community was dominated by hydrogen-producing bacteria, while in the pH range of 5.0-5.6, methanogenic consortium predominated the microbial community. The increase in the methanogenic consortium caused consumption of VFA, leading to lower concentration of VFA and HAc in the effluent.

pH is an important factor that influences the acidogenesis process and is closely related to degradation of substrate and distribution of organic acid products (Ren *et al.*, 1997; Yu and Fang, 2002, 2003). However, the conflicting results of these studies indicated that other than pH, there may still be other factors influencing the acidogenesis process, even when pH was the unique operating variable.

Figure 2.5 The overall acidogenic activity as a function of pH (Yu and Fang, 2002, 2003).

Although detailed operating parameters and performance data (such as VFAs and biogas) were obtained and analyzed in these studies on hydrogen and pH in the acidogenesis process, data concerning the microorganisms in these acid-phase reactors which would be essential to explain some of the phenomena has been lacking. Analysis of microbial activities has been derived from the performance of these bioreactors but not from direct evidence of observed microbial changes in the bioreactors. Indeed, studies on effects of different operating parameters (such as pH, temperature and HRT) on performance of

acidogenic reactors are important as these may provide general guidelines for improving their performance, but it is still the microorganisms inside the bioreactors that define the process performance. Operating parameters affect the microorganisms which in turn affect process performance. Therefore, an understanding of the microbial community structure and dynamics under different operating conditions is essential in aiding more effective control of the anaerobic process.

2.7 Microbial studies on acidogenic phase

Until very recently, in almost all engineered anaerobic systems, evaluation of activities of methanogens was largely process-oriented. Measurable metabolic parameters, such as methane production and substrate biodegradation, were determined as a function of the whole community (Yu *et al.*, 2005). Determining contribution of the individual methanogenic group to these parameters was nearly impossible, since culturing the archaea in vitro is difficult (Raskin *et al.*, 1994; Sekiguchi *et al.*, 2001). To overcome these difficulties, culture-independent molecular techniques based on detection of specific regions of 16S-rRNA genes were developed and have been successfully applied to community studies of microbes in anaerobic digesters. Substantial qualitative analyses of microbial communities in anaerobic treatment have provided valuable information for better understanding of anaerobic processes (Liu *et al.*, 2002a; Shin *et al.*, 2010a; Zhang *et al.*, 2011). Shin *et al.* (2010a) investigated two sets of two-phase anaerobic digesters treating food waste-recycling wastewater, and found that though generally stable operation was achieved with the overall COD removal efficiencies, performance of acidogenic reactors changed significantly which coincided with bacterial transition within the reactors.

Quantitative analysis of methanogenic communities has also been enabled with development of real-time PCR technique (Yu *et al.*, 2005; Yu *et al.*, 2006), providing more precise technology to seek understanding of relationship

between microbial structures as well as their dynamics and performance of reactors. Yu *et al.* (2005) developed six group-specific methanogenic primer and probe sets, which can separately detect four orders (Methanobacteriales, Methanococcales, Methanomicrobiales and Methanosarcinales) and two families (*Methanosarcinaceae* and *Methanosaetaceae*) of the order Methanosarcinales. This key development in real-time PCR techniques has aroused interest among researchers on quantitative analysis of methanogenic community in anaerobic digesters (Yu *et al.*, 2006; Klocke *et al.*, 2008; Lee *et al.*, 2009; O'Reilly *et al.*, 2009; Kim *et al.*, 2010; Bialek *et al.*, 2011). More recently, Kim *et al.* (2011) developed new real-time PCR primer/probe sets for detection of common key acidogens (i.e., *Aeromonas* and *Clostridium sticklandii*) in three anaerobic reactors treating different wastewaters. However, comparative microbial studies remain scarce (Klocke *et al.*, 2008; O'Reilly *et al.*, 2009; Bialek *et al.*, 2011), especially for studies on microorganisms in acidogenic phase of two-phase anaerobic process.

Many studies had focused on either acidogens in the acidogenic phase/period (Shin *et al.*, 2010a; Shin *et al.*, 2010b; Kim *et al.*, 2011) or methanogens in methanogenic phase/single-phase process (Klocke *et al.*, 2008; Lee *et al.*, 2009; Kim *et al.*, 2010; Shin *et al.*, 2010a). However, as indicated in previous sections, methanogens, especially hydrogenotrophic methanogens, can also play an important role in the acidogenic phase. Some researchers have found high content of methane in biogas of acidogenic reactors at pH as low as 5.5. Wu *et al.* found that at pH 5.6, the methane content could reach 13% in acidogenic reactor (Wu *et al.*, 2010). Studies of Yu and Fang found even higher content of methane in an acidogenic reactor (Yu and Fang, 2002, 2003). Almost 30% of methane was observed in an acidogenic reactor treating gelatin-rich wastewater at pH 5.5 (Yu and Fang, 2003). All these researches indicated the existence of methanogens in acidogenic reactor.

Wu et al. observed a substantial decrease in soluble microbial products (including VFAs and ethanol) with increase of methane content in the biogas. The authors attributed this phenomenon to the consumption of extra carbon to produce methane. However, such decrease in VFAs and ethanol with increasing production of methane was not observed in studies reported by Yu and Fang (Yu and Fang, 2002, 2003). On the contrary, VFA production improved with higher methane content in the biogas of the acidogenic reactor. Results of Shimada et al. (2011) also showed that the methane generation did not necessarily result in lower production of HAc. The authors found that hydrogenotrophic methanogens constitute the major archaeal microbes in the acidogenic reactors of two-phase anaerobic digesters (Shimada *et al.*, 2011). These studies suggested the existence of methanogens in acidogenic reactor might well have a positive impact on acidogenesis.

2.8 Knowledge gap and concerns about methanogens in acidogenic reactors

Hydrogen and pH has been acknowledged to be affect the acidogenesis processes in acidogenic reactors, but a few concerns associated with the effects of these two factors remain.

Firstly, there were contradictory results on the effects of hydrogen on fermentation of organic matters in acidogenic reactors. Wu *et al.* (2010), found in an acid-phase anSBR treating liquid swine manure with glucose supplement the optimal HAc/HBu ratio was associated with the highest hydrogen content and production. However, with a different seed sludge treating liquid swine manure with supplement using a similar anSBR, the same research group derived completely different conclusion (Li *et al.*, 2010). It was found that at the optimal pH when hydrogen production was highest, HAc/HBu ratio was lowest.

Secondly, for effects of pH on anaerobic fermentation in acidogenic reactor, similar confliction in research of this filed was found. Yu and Fang (2002, 2003) found that increasing pH improved acidogenesis by the fraction of HAc in effluent organic products. Results from other reports, however, suggested otherwise (Li *et al.*, 2010; Wu *et al.*, 2010). Li *et al.* (2010) found no specific relationship between VFA production and pH from pH 4.7 to 5.9 in an acidogenic reactor. And Wu *et al.* (2010) found that for pH of 4.4 to 5.0 VFA concentration did not change much but decreased substantially when pH further increased to 5.6.

Thirdly, a few studies had confirmed the existence of methanogens in acidogenic reactor and their existence might have a positive impact on acidogenesis. Observable amount of methane (13%-30%) had been found in Wu *et al.* and Yu and Fang's studies on acidogenic reactors. Shimada *et al.* (2011) even showed that the methane generation did not necessarily result in lower but higher production of HAc.

Methanogens not only keep a proper hydrogen partial pressure in the acidogenic phase, but also serve as syntrophic partners for acetogens and acetate-oxidizing bacteria in various biological reactions. Moreover, as mentioned previously, while acidogens, due to their heterogeneous nature, are more robust and adaptable compared to methanogens; methanogens, especially in an acidogenic phase where conditions favor the acidogens, are likely to be more vulnerable and thus more sensitive to process fluctuations. Any changes, like loading fluctuations or changes in operating parameters (e.g., pH), may have a stronger adverse impact on methanogens than acidogens in the acidogenic reactor, thus changing the microbial structure of methanogenic community and eventually affecting performance of the reactor.

Therefore, this project, instead of conducting more investigation on performance in response to pH change, tended to explore the performance of an acidogenic

reactor in response to methanogen population changes. This project would provide a better understanding on the essential role of methanogens in the acidogenic phase.

CHAPTER 3 TWO-PHASE ANAEROBIC DEGRADATION OF HIGH-STRENGTH ORGANIC WASTEWATER

3.1 Introduction

Since first proposed by Pohland and Ghosh in 1971 (Pohland and Ghosh, 1971), two-phase anaerobic treatment has aroused extensive researches and application of such technology. The continuous stirred tank reactor (CSTR) is the most widely used type of two-phase anaerobic reactor in researches due to its relatively simple configuration and ease of control (Cooney *et al.*, 2007; Shin *et al.*, 2010a). But with development of automation technology another type of reactor had become preferred.

In 1995, Sung and Dague developed an anaerobic sequencing batch reactor (anSBR) that worked by consecutive cycles of operations which includes: feeding, mixing, settling and decanting processes (Sung and Dague, 1995). One of the most important advantages of anSBR is the presence of a concentration gradient of organic pollutant in the reactor. Such concentration gradient of organic pollutant guarantees a higher substrate level at early stage of the process resulting in a greater growth rate of microbes inside the reactors and hence a higher degradation ability. Other advantages of anSBR over CSTR includes: 1) no primary or secondary settles is required; 2) absence of liquid or solids recycling equipment; 3) high organic matter removal efficiency; 4) high microbial activities (Zaiat *et al.*, 2001).

The degradation of organic pollutant and the formation of methane are achieved by the resident of microbes in anaerobic reactors. Knowledge of microbial communities and their development in the reactor is hence essential for further optimization of two-phase anSBR. Quantitative analysis of microbial communities had been applied to determine the abundance of certain microbes in anaerobic digesters and proved a precise tool for understanding the

relationship between the resident microbes and performance of reactors. (Yu *et al.*, 2005; Yu *et al.*, 2006).

The objective of this chapter was to implement a laboratory-scale two-phase anSBR system treating a synthetic high-strength organic wastewater and evaluate the performance of such system. A group-specific real-time PCR approach was also applied to quantify the methanogens in the acidogenic and methanogenic reactors and to understand the relationship between the performance of the two-phase anSBR and the microbial community's structural dynamics.

3.2 Material and methods

3.2.1 AnSBR setup and substrate

A laboratory-scale two-phase anSBR system comprising an acidogenic reactor (R_A) and a methanogenic reactor (R_M) was operated using a programmable logic controller (PLC) system. The working volumes of the reactors were 5 L for R_A and 10 L for R_M , respectively. Both reactors were jacketed by heating tapes and the temperature were controlled at 40 ± 1 °C. The pH control, feeding (10 min), mixing (for 2 cycles/d 668 min; for 3 cycles/d 428 min), desludging (2 min), settling (30 min) and decanting (10 min) processes were implemented by peristaltic pumps and controlled by the PLC. 2. 2. Mixing was conducted by steel mixing blades fixed on the central mixing arm extending from the cover of each reactors. The schematic diagram of the anSBR is shown in Figure 3.1.

The seed sludge for R_A and R_M was obtained from an anaerobic digester at the Ulu Pandan water reclamation plant, Singapore. After collection, the seed sludge was filtered through a 600 μ m sieve and stored at 4 °C before seeding into the reactors. Both reactors were fed with synthetic feed simulating high strength organic wastewater. The composition of the synthetic feed is as shown in Table 3.1. Detailed operating parameters are shown in Table 3.2.

Figure 3.1 Schematic diagram of the laboratory-scale two-phase anSBR.

3.2.2 Start-up procedures

R_A was seeded with the filtered sludge while purging continuously with N_2 gas over 10 minutes. The initial pH of the reactor was approximately 7.20 – 7.45. Then the pH was adjusted to 5.5 in order to establish the acidogenic condition and the final pH was between 5.30 and 5.50. The pH was controlled by the PLC system with 1 mol/L sodium hydroxide solution and 1 mol/L hydrochloric acid solution. Initial MLVSS of R_A was around 14 g/L with a MLVSS/MLSS ratio of 0.80. Diluted synthetic feed (with COD of 12.5 g/L) was fed into R_A for acclimation of the acidogenic sludge. Since feed for R_M was effluent from R_A , to generate enough feed for R_M , R_M was started 7 d after the start of R_A . The filtered sludge was used to seed R_M which was also purged with N_2 gas over 10 minutes. The pH was controlled at between 7.40 and 7.80 to establish the methanogenic condition. Initial MLVSS in R_M was around 15 g/L with a MLVSS/MLSS ratio of 0.80. Effluent collected from R_A was fed to R_M for sludge. The day when R_M was started was defined as the first day of operation, i.e., Day 1.

Table 3.1 Synthetic feed composition

Component	Concentration, mg/L
Organics:	
Sucrose	20000
Ethanol	1200
Sodium Acetate	1100
Propionic Acid	540
Butyric Acid	260
Starch	1100
Cellulose	1100
Macronutrients (inorganics):	
NH_4HCO_3	1885
NH_4Cl	1276
K_2HPO_4	250
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	125
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	180
CaCl_2	100
Micronutrients (inorganics):	
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	2.5
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	2.5
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.5
H_3BO_4	0.5
Others (inorganics):	
Na_2SO_4	800
ZnCl_2	1
$\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$	2
CdNO_3	0.3
PbCl_2	1.4
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	1.8

Table 3.2 Two-phase anSBR operating parameters

Parameters	R _A	R _M
Working volume (L)	5	10
Total volume (L)	6	12
Feed	Synthetic feed	Effluent from R _A
Feed SCOD (g/L)	25.3±1.9 ^a	NA ^b
Feed VFA (g/L)	1.4±0.1	NA
Feed HAc (g/L)	0.9±0.1	NA
pH	5.4±0.1	7.6±0.2
T (°C)	40±1	40±1
ORP (mV)	-400±50	-500±50
SRT(d)	25	50

a During startup (Period I) the feed for R_A is diluted synthetic feed with COD of around 12.5 g/L.

b Not available (The value equals to the COD of R_A effluent and changes with performance of R_A).

3.2.3 Operation of the two-phase anSBR system

From Day 1 to Day 15 (Period I), R_A feed was diluted synthetic feed with COD of about 12.5 g/L for acclimation and HRT_{RA} at startup of R_A was 5 d. As performance of R_A was relatively stable, from Day 16 on, R_A feed was changed to COD of 25 g/L. From Day 16 to 53 (Period II), HRT_{RA} was still 5 d; from Day 54 to 80 (Period III), HRT_{RA} was 2.5 d; and from Day 81 to 114 (Period IV), HRT_{RA} was 1.7 d. Operational details are listed in Table 3.3. (Since working volume of R_M is two times that of R_A and all effluent from R_A goes to R_M, HRT_{RM} equals to 2HRT_{RA})

Table 3.3 Operation schedule of R_A^a

Period	Day	Feed COD, g/L	Vol/Cycle, L	Cycle	HRT _{RA} , d	OLR g/L/d ^b
I	1-15	12.5	0.5	2	5	0.8
II	16-53	25	0.5	2	5	1.7
III	54-80	25	1	2	2.5	3.3
IV	81-114	25	1	3	1.7	5

a R_M feed was effluent from R_A, and HRT_{RM} was two times of HRT_{RA}.

b Calculated based on working volume of (R_A + R_M). $OLR = \text{Feed COD} \times (\text{Vol/Cycle}) \times \text{Cycle} / \text{Vol}(R_A + R_M)$

3.2.4 Analytical methods

3.2.4.1 Composition of VFAs

During the operation of the anSBR system, influent and effluent samples from the system were collected from feed tanks and reactors routinely for chemical analysis. VFAs (i.e., HAc, HPr, Iso-HBu, HBu, Iso-HVa, HVa, Iso-HCa, HCa and HHe) were measured using gas chromatography (Agilent Technologies 7890A GC system, US) with Zebron ZB-FFAP 30 m × 320 μm × 0.5 μm column and a flame ionization detector (FID). The injector and detector temperatures were set at 260 °C and 250 °C respectively. The oven temperature was initially 60 °C, ramped up to 120 °C at a rate of 20°C/min and held for 1 min. Finally, the temperature reached 240 °C at 20 °C/min and held for 2 min. Helium was the carrier gas with a constant pressure of 80.96 kPa. The injector was in a split mode with split ratio of 20:1. Prior to analysis, 0.1 mL of 10% formic acid was added to each 0.9 mL of samples and standards to acidify the samples. MLSS/MLVSS inside the reactors and in the discharged solution was tracked in order to monitor the SRT. COD, MLSS and MLVSS were determined in accordance with Standard Methods (APHA, 1998).

3.2.4.2 Composition of biogas

The biogas was collected using a 3 L or 10 L gas bag (TEDLAR, US), and the volume of biogas generated each day was estimated from the gas bag. The methane, carbon dioxide and hydrogen in the biogas was analyzed using gas chromatography (Agilent Technologies 7890A GC system, US) with (1) an Agilent HayeSep R 0.9 m × 1/8" × 2.0 mm packed column, (2) an Agilent HayeSep C 3.0 m × 1/8" × 2.0 mm packed column, (3) an Agilent MolSieve 5A 3.0 m × 1/8" × 2.0 mm packed column, (4) an Agilent HayeSep Q 0.9 m × 1/8" × 2.0 mm packed column, (5) an Agilent MolSieve 13 × 3.0 m × 1/8" × 2.0 mm packed column and two thermal conductivity detectors (TCD, one front detector for measuring methane and carbon dioxide and one back detector for measuring hydrogen). The injector and detector temperatures were set as 120 °C and 150 °C respectively. The oven temperature was 115 °C and held for 12.5 min. Helium was the reference gas for Column 1-3 for detection of methane and carbon dioxide; argon was the reference gas for Column 4-5 for detection of hydrogen. Helium cannot be used for detection of hydrogen, because the thermal conductivities of helium and hydrogen are too similar to each other. Both flow speeds was controlled at 45mL/min. Three valves were installed to control the flow of gases in the columns.

3.2.4.3 DNA extraction

0.5 mL sludge samples were collected in 2 mL plastic tubes, centrifuged at 10000 rpm for 60 sec, followed by decantation of the supernatant. The sludge was then washed with 1 mL phosphate buffer solution (PBS 1×). This process was repeated again and the tubes were stored at 4 °C in preparation for DNA extraction. Before extraction, the sludge samples were diluted 5 times. Total DNA was then extracted from the samples using an automated nucleic acid extractor (MagNA Pure Compact, Roche, Germany). The purified DNA was then stored at -20 °C.

3.2.4.4 16S ribosomal RNA gene real-time quantitative PCR (qPCR)

16S rRNA gene quantifications of the DNA samples were performed on LightCycler 480 II (Roche, Germany). The primer and probe sets specific for two domain: Bacteria (BAC) and Archaea (ARC); two order-level Archaea: Methanomicrobiales (MMB) and Methanobacteriales (MBT); and two family-level Archaea: *Methanosarcinaceae* (MSC) and *Methanosaetaceae* (MST) were used (Table 3.4). Most bacteria and methanogens in anaerobic reactors would be covered by these primer and probe sets as described in (Yu *et al.*, 2005; Bialek *et al.*, 2011). MMB and MBT are hydrogenotrophic methanogens, which utilize only H₂ and CO₂ or formate to produce methane; MST only utilize acetate, and MSC utilize acetate as well as various other methyl compounds and hydrogen (Madigan and Brock, 2009). The reaction was performed with a total volume of 20 µL mixture: 10 µL of 2× LightCycler 480 Probes Master, 4 µL of PCR-grade water, 2 µL of TaqMan probe (final concentration 200 nM), 1 µL of each forward and reverse primer (final concentration 500 nM) and 2 µL of template DNA. The operation processes consisted of a predenaturation step of 10 min at 95 °C, amplification of 55 cycles (10 s) at 95 °C and 30 s at 60 °C, and cooling of 10 s at 40 °C. Standard curves were constructed using those strains corresponding to primer and probe sets used in this experiment (Table 3.4). A 10-fold dilution series from 10¹-10¹⁰ copies/µL of standard solution was established and analyzed by qPCR in duplicate to construct the standard curve for the corresponding primer and probe set.

Table 3.4 Primer and probe sets for qPCR (Yu *et al.*, 2005; Bialek *et al.*, 2011)

Name	Function	Target group	Sequence (5'-->3')	Representative strains
ARC787F	F primer	Archaea	ATTAG ATACC CSBGT AGTCC	MMB+MBT+MSC+MST
ARC915F	TaqMan		AGGAA TTGGC GGGGG AGCAC	
ARC1059R	R primer		GCCAT GCACC WCCTC T	
BAC338F	F primer	Bacteria	ACTCC TACGG GAGGC AG	Escherichia coli K12
BAC516F	TaqMan		TGCCA GCAGC CGCGG TAATA C	
BAC805R	R primer		GACTA CCAGG GTATC TAATC C	
MMB282F	F primer	Methanomicrobiales	ATCGR TACGG GTTGT GGG	Methanospirillum hungatei JF1 (DSM 864)
MMB749F	TaqMan		TYCGA CAGTG AGGRA CGAAA GCTG	Methanomicrobium mobile BP (DSM 1539)
MMB832R	R primer		CACCT AACGC RCATH GTTTA C	
MBT857F	F primer	Methanobacteriales	CGWAG GGAAG CTGTT AAGT	Methanobacterium formicicum M.o.H. (DSM 863)
MBT929F	TaqMan		AGCAC CACAA CGCGT GGA	Methanobrevibacter arboriphilicus DH1 (DSM 1536)
MBT1196R	R primer		TACCG TCGTC CACTC CTT	
Mst702F	F primer	Methanosaetaceae	TAATC CTYGA RGGAC CACCA	Methanosaeta concilli GP6 (DSM 3671)
Mst753F	TaqMan		ACGGC AAGGG ACGAA AGCTA GG	
Mst862R	R primer		CCTAC GGCAC CRACM AC	
Msc380F	F primer	Methanosarcinaceae	GAAAC CGYGA TAAGG GGA	Methanosarcina acetivorans C2A (DSM 2834)
Msc492F	TaqMan		TTAGC AAGGG CCGGG CAA	Methanosarcina barkeri MS (DSM 800)
Msc828R	R primer		TAGCG ARCAT CGTTT ACG	Methanosarcina mazei Go1 (DSM 3647)

3.3 Results and discussion

3.3.1 Performance of two-phase anSBR

For better comparison of COD data and VFA data, the mass of VFA was converted to equivalent COD of VFA. The COD of each VFA was calculated based on the theoretical chemical oxygen demand of the VFA as is shown in Table 3.5. To evaluate performance of the respective reactors of the two-phase anSBR system, several performance parameters were defined and calculated. The acidified organic fraction (AOF) is determined by the ratio of VFAs produced in R_A effluent and the COD of the influent fed into R_A. The value of AOF is:

$$AOF = \frac{COD_{VFA\ Effl\ A} - COD_{VFA\ Infl\ A}}{COD_{Infl\ A}} \quad (8)$$

where VFA Effl A is the VFA in effluent from R_A; VFA Infl A is the VFA in the influent into R_A; and Infl A is the influent into R_A.

Table 3.5 Theoretical equivalent COD of respective VFA.

Unit	HAc	HPr	HBu	HVa	HCa	HHe
mol/L	1	1	1	1	1	1
g/L	60	74	88	102	116	130
g/L COD	64	112	160	208	256	304

Figure 3.2 to 3.6 shows the COD, VFA and biogas data for Period I-IV. In Period I, influent into R_A was diluted synthetic feed with COD of around 12.5 g/L; effluent COD from R_A was 11.01 ± 0.48 g/L and effluent COD from R_M was 4.8-5.0 g/L. COD removal rate of R_A and R_M was 11.9 ± 3.8% and 53.7 ± 1.7%, respectively. The low COD removal rate of R_A was expected because in the acidogenic phase the main process was conversion of organic compounds into fatty acids (Lema *et al.*, 1988). Correspondingly, a sharp increase of TVFAs was observed in effluent from R_A with a high value of AOF (61.2 ± 1.6%). The COD of TVFAs

increased from 1.25 g/L to 10.15 ± 0.20 g/L which was almost equal to the effluent COD from R_A . For R_M , the low COD removal rate was probably because the methanogens therein were still acclimating and the additional COD might then have been from cell lysis in the seed sludge. Only a very limited amount of biogas was collected from R_M in this period.

In Period II and III, when feed COD concentration was doubled and HRT was further shortened, COD removal rate for R_A was $31.2 \pm 4.0\%$ and AOF was $53.5 \pm 7.1\%$ with an AOF_{HAC} of $15.2 \pm 4.2\%$. For HPr, the concentration declined from 3.1 ± 0.9 gCOD/L to 1.4 ± 0.5 gCOD/L; while for HBU the concentration declined from 3.4 ± 1.0 gCOD/L to 2.6 ± 0.8 gCOD/L from Period II to Period III, respectively. It was then noted that a considerable amount of methane was produced in R_A , at 30-45% of the total volume of biogas (about 1 L/d). This indicated the presence of methanogens in R_A even when the pH was controlled at 5.5. Similar phenomenon was reported in the study of Yu and Fang where almost 30% of methane was observed in an acidogenic reactor treating gelatin-rich wastewater at pH 5.5 (Yu and Fang, 2003). In R_M , COD removal rate increased to as high as $97.7 \pm 0.9\%$. Around 8-10 L of biogas with 70% of methane could be collected daily by the end of this period. This indicated successful phase separation in the respective reactors in the anSBR system.

As OLR was further increased to 5.0 g/L/d in Period IV, satisfactory performance was achieved for both R_A and R_M with high VFA conversion in the acidogenic phase (AOF was $55.1 \pm 3.9\%$ with an AOF_{HAC} of $15.5 \pm 2.2\%$), high COD removal (98%-99%) and high biogas production in the methanogenic phase. Though loading was increased in R_A , concentration of HPr further declined to 0.49 ± 0.17 gCOD/L. Such performance indicated a favorable VFA composition for feed into R_M (Klocke *et al.*, 2008). In R_M the biogas composition remained stable. Since the OLR was higher, daily production of biogas increased to around 15 L with a methane content at 70%. After 114 d of operation, stable and successful phase

separation (i.e., acidogenesis and methanogenesis phase) was achieved in R_A and R_M , respectively.

Figure 3.2 COD and VFA of R_A during Period I-IV. (a): COD of influent into R_A (Infl A), effluent from R_A (Effl A), theoretical COD of VFA in influent into R_A (VFA Infl A), effluent from R_A (VFA Effl A) and HAc in effluent from R_A (HAc Effl A); (b): Theoretical COD of effluent HPr (HPr Effl A) and HBu (HBu Effl A) from R_A .

Figure 3.3 Biogas of R_A during Period I-IV: CH₄ and CO₂ content of biogas from R_A.

Figure 3.4 Removal rate and AOF in R_A.

Figure 3.5 COD and VFA of R_M during Period I-IV: COD of influent into R_M (Infl M), effluent from R_M (Effl M), theoretical COD of VFA in influent into R_M (VFA Infl M), effluent from R_M (VFA Effl M) and HAc in effluent from R_M (HAc Effl M).

Figure 3.6 Biogas of R_M during Period I-IV: CH₄ and CO₂ content of biogas from R_M .

3.3.2 Microbial community dynamics in two-phase anSBR – based on qPCR results

To evaluate the relative dominance of respective methanogens in the archaea domain, relative dominance of methanogens (RDM) is introduced and determined by the ratio between logarithm forms of gene concentration of methanogens to that of archaea:

$$\text{RDM} = \frac{\log c(\text{Methanogen})}{\log c(\text{Archaea})} \quad (9)$$

where if $c(\text{Methanogen})$ equals to zero, $\text{RDM} = 0$.

The qPCR assays revealed 16S rRNA gene concentration of the domain Bacteria (BAC) and Archaea (ARC) in the reactors during Periods I-IV (Figure 4.4-4.6) Bacteria was the predominant domain in both reactors. However, while $c(\text{BAC})/c(\text{ARC})$ (the ratio of BAC population to ARC population) in R_A could reach as high as 3200, that value in R_M was between 11 and 40 throughout the experiment. In Period I, although distinct differences in microbial community structures were not evident between R_A and R_M [for both reactors, $c(\text{BAC}) > c(\text{MMB}) > c(\text{MST}) > c(\text{MBT}) > c(\text{MSC})$], the low pH already had an impact on the 16S rRNA gene concentration of ARC in R_A . At the startup of the anSBR, gene concentrations of BAC in both reactors were almost the same, but concentration of ARC was two orders of magnitude less in R_A than in R_M . The ratio of $c(\text{BAC})/c(\text{ARC})$ in R_A was 543, while the value in R_M was only 40.

As OLR further increased, microbial community structures in R_A and R_M started to show distinctly different profiles. In Period II-IV, as is shown in Figure 4.4, the bacteria population was still the most abundant in R_A with a 16S rRNA gene concentration of $9.84 \pm 4.22 \times 10^9$ copies/mL. Although pH was kept low at 5.5, the gene concentration of ARC did not decline as expected but increased more than one order of magnitude. This could explain why methane content in biogas

from R_A was more than 30%. Meanwhile the hydrogenotrophic order Methanobacteriales (MBT) started to predominate in the archaea domain ($RDM_{MBT} = 100.2 \pm 0.5\%$), while the order Methanomicrobiales (MMB, $RDM_{MMB} = 74.7 \pm 8.8\%$) and acetotrophic families i.e., *Methanosaetaceae* (MST, $RDM_{MST} = 76.5 \pm 6.1\%$) and *Methanosarcinaceae* (MSC, $RDM_{MSC} = 69.7 \pm 9.2\%$) seemed less preferred in R_A . MSC was not even detectable on Day 108 in Period IV.

In R_M , 16S rRNA gene concentration of BAC and ARC remained relatively stable in Period II. Within the Archaea domain, however, acetotrophic methanogens outcompeted hydrogenotrophic methanogens with MST becoming the dominant archaea ($RDM_{MST} = 94.7 \pm 3.4\%$) in Period II-III. The MST mainly used acetate as substrate and would predominate in sludge of anaerobic reactors with good performance (Bialek *et al.*, 2011). With OLR increasing in Period III, population of both BAC and ARC was further increased from $5.10 \pm 2.50 \times 10^9$ to $2.83 \pm 0.15 \times 10^{10}$ copies/mL, and $2.14 \pm 0.47 \times 10^8$ to $1.88 \pm 0.21 \times 10^9$ copies/mL, respectively. RDM of respective methanogens remained relatively stable during Period IV. Microbial profile of the sludge samples coupled with the process performance of R_A and R_M from the reactors indicated successful phase separation in R_A and R_M .

Figure 3.7 Quantification of 16S rRNA gene concentration of bacteria and methanogenic communities in: (a) R_A and (b) R_M of Period I-IV

Figure 3.8 RDM of: (a) R_A and (b) R_M of Period I-IV.

3.4 Conclusions

Bacteria was dominant in both acidogenic and methanogenic reactors during phase separation. Methanogens could exist and produce methane in the in the acidogenic reactor of the two-phase anSBR at pH 5.5. The dominance of hydrogenotrophic methanogen MBT in methanogenic communities in R_A indicated that methane produced in R_A could be produced from hydrogen utilization by hydrogenotrophic methanogens in acidogenic reactor, but such assumption need further investigation on the role of methanogens in acidogenic reactor.

CHAPTER 4 ROLE OF HYDROGENOTROPHIC METHANOGEN IN ACIDOGENIC REACTOR

4.1 Introduction

As the lead phase of two-phase anaerobic degradation, various microorganisms and complex biological conversions are involved (Mosey, 1983). Acidogens, as one of the key microbial groups in the acidogenic phase, have been extensively investigated. Intuitively it would seem reasonable that only limited studies showed interests in methanogens in the acidogenic phase (Mizuno *et al.*, 1998; Shimada *et al.*, 2011). However, methanogens, such as hydrogenotrophic methanogens, do exist and may also play an important role in the acidogenic phase. Results of Shimada (2011) showed that hydrogenotrophic methanogens constitute the major archaeal microbes in the acidogenic reactors of two-phase anaerobic digesters, and the methane generation did not necessarily result in lower production of acetic acids.

Hydrogen, which is an inevitable by-product during the acidogenic process, can affect composition of the acid products generated from fermentation of organic matters (Mosey, 1983; Madigan and Brock, 2009). Mosey (1983) suggested increased concentration of hydrogen slows down glycolysis but speeds up conversion from pyruvate to butyrate and propionate in acidogenesis reactions by lowering the ratio of $[NAD^+]/[NADH]$. It may also inhibit acetogenesis of butyrate and propionate to acetate by acetogenic bacteria due to thermodynamic reasons. So for a well-performing acidogenic reactor, hydrogen concentration in the gaseous phase need to be kept as low as possible.

Hydrogenotrophic methanogen can use hydrogen and carbon dioxide as substrate to produce methane. Its role in single-phase anaerobic degradation has never been underestimated and has been suggested to contribute to 28-34% of methane production in single-phase anaerobic degradation (Conrad, 1999). However, its role in the acidogenic phase, where pH is usually less than 6.0, is

yet to be studied. In the acidogenic phase where conditions favor the acidogens, environmental condition changes, e.g. pH, may have a larger impact on methanogens than the resident acidogens. With microbial changes in methanogenic community, the performance of acidogenic process may also be affected consequently. Therefore, research on methanogens therein is essential for a better understanding of acidogenic reactor performance.

In this chapter, a laboratory-scale acidogenic reactor was studied to investigate the effects of sharp pH changes on the performance of the acidogenic reactor. Microbial community structure was analyzed by quantitative real-time PCR during these periods of stress to understand the role of methanogens in the acidogenic phase.

4.2 Material and methods

4.2.1 AnSBR setup and operation

The laboratory-scale acidogenic reactor (R_A) of the two-phase anSBR system as described in Chapter 3 (Figure 3.1) was operated with a PLC system. The reactor was fed with synthetic feed simulating high-strength organic wastewater and operated under pH 5.4 ± 0.1 for 114 d as described in Chapter 3. The operating parameters were the same as those implemented in Period IV and were kept from Day 115 till Day 300 (Table 4.1).

Table 4.1 Operating parameters of R_A

Parameters	R _A
Working volume (L)	5
Total volume (L)	6
Feed	Synthetic feed
Feed SCOD (g/L)	25.3±1.9
Feed VFA (g/L)	1.4±0.1
Feed HAc (g/L)	0.9±0.1
Feeding Volume (L/d)	3
Feeding Times (/d)	3
pH	5.4±0.1
T (°C)	40±1
ORP (mV)	-400±50
SRT (d)	25
HRT (d)	1.7
OLR g/L/d	5

4.2.2 Batch test

On Day 136, 100 mL sludge aliquots were collected from R_A and seeded into 3 120 mL serum bottles (labeled as I, II and III) leaving 20 mL of headspace. The sludge in the serum bottles was first purged with N₂ and then with biogas collected from R_A to simulate the condition in R_A. The feed was still the synthetic feed as described in Table 3.1 with COD of 25 g/L. The serum bottles were then placed in an incubator with shaking speed of 120 rpm at 35 °C. Each serum bottle was discharged and fed 2 times per day on a 12 h basis (with HRT of 2.5 d). Prior to discharge the sludge was settled for 20 min. Then a total volume of 20 mL of the supernatant was extracted using a 60 mL syringe. After that 20 mL of

the synthetic feed was fed into the serum bottles. The extracted effluent samples and biogas were collected from each serum bottle twice daily for chemical analysis (pH, COD, VFAs and biogas content). A sludge sample was collected at the beginning and the end of the batch test for microbial analysis (DNA extraction and qPCR analysis). The whole batch test lasted for 15 d. From Day 1 to Day 10, pH was not controlled in the serum bottles; from Day 11 to Day 15, five drops of 1 mol/L sodium hydroxide was added to increase the pH in serum bottles.

4.2.3 pH change test on laboratory-scale reactor R_A

In order to suppress the methanogenic population and investigate performance change of R_A, on Day 180, the pH was decreased from 5.5 ± 0.2 to 4.5 ± 0.2 and the culture was then incubated at pH of 4.5 ± 0.2 for 10 d. Then the pH was changed back to 5.5 ± 0.2 to allow R_A to recover. Other operation details were the same as normal during the pH change test.

4.2.4 Analytical methods

4.2.4.1 Chemical analysis

Influent and effluent samples were collected from feed tanks and reactors routinely for chemical analysis. Volatile fatty acids (i.e., HAc, HPr, Iso-HBu, HBu, Iso-HVa, HVa, Iso-HCa, HCa and HHe) were measured using gas chromatography (Agilent Technologies 7890A GC system, US) with Zebron ZB-FFAP 30 m $\times$ 320 μ m $\times$ 0.5 μ m column and a flame ionization detector (FID). Prior to analysis, 0.1 mL of 10% formic acid was added to each 0.9 mL of samples and standards to acidify the samples. COD, MLSS, and MLVSS were determined in accordance with Standard Methods (APHA, 1998). MLSS and MLVSS inside the reactors and in the discharge were tracked in order to monitor the SRT.

Biogas was collected using a 3 L or 10 L gasbag (TEDLAR, US), and the volume of biogas generated each day was estimated from the gas bag. Methane, carbon dioxide and hydrogen in the biogas were analyzed using gas chromatography (Agilent Technologies 7890A GC system, US) with (1) an Agilent HayeSep R 0.9 m × 1/8" × 2.0 mm packed column, (2) an Agilent HayeSep C 3.0 m × 1/8" × 2.0 mm packed column, (3) an Agilent MolSieve 5A 3.0 m × 1/8" × 2.0 mm packed column, (4) an Agilent HayeSep Q 0.9 m × 1/8" × 2.0 mm packed column, and (5) an Agilent MolSieve 13 × 3.0 m × 1/8" × 2.0 mm packed column with two thermal conductivity detectors (TCD, a front detector for measuring methane and carbon dioxide, and a back detector for measuring hydrogen). Helium was the reference gas for Column 1-3 for detection of methane and carbon dioxide and argon was the reference gas for Column 4-5 for detection of hydrogen.

4.2.4.2 Biological analysis

0.5 mL sludge samples were collected in 2 mL plastic tubes, centrifuged at 10000 rpm for 30 sec, followed by decantation of the supernatant. The sludge was then washed twice with 1 mL phosphate buffer solution (PBS 1×). The pellets were stored at 4 °C before DNA extraction. Before extraction, the sludge samples were diluted 5 times to reach cell concentration of around 10^{10} /mL. Total DNA was then extracted from samples using an automated nucleic acid extractor (Magna Pure Compact, Roche, Germany). The purified DNA was then stored at -20 °C before analysis.

16S rRNA gene quantifications of the DNA samples were performed on LightCycler 480 II (Roche, Germany). The primer and probe sets specific for two domains: Bacteria (BAC) and Archaea (ARC); two order-level Archaea: Methanomicrobiales (MMB) and Methanobacteriales (MBT); and two family-level Archaea: *Methanosarcinaceae* (MSC) and *Methanosaetaceae* (MST) were used (Yu *et al.*, 2005; Bialek *et al.*, 2011). The reaction was performed with a total volume of 20 µL mixture: 10 µL of 2 × LightCycler 480 Probes Master, 4 µL of

PCR-grade water, 2 μL of TaqMan probe (final concentration 200 nM), 1 μL of each forward and reverse primer (final concentration 500 nM), and 2 μL of template DNA. The operation processes consisted of a predenaturation step of 10 min at 95 °C, amplification of 55 cycles (10 s) at 95 °C and 30 s at 60 °C, and cooling for 10 s at 40 °C. Standard curves were constructed using those strains corresponding to primer and probe sets used in this experiment (Table 3.4). A 10-fold dilution series from 10^1 - 10^{10} copies/ μL of standard solution was established and analyzed by qPCR in duplicate to construct the standard curve for the corresponding primer and probe set.

4.3 Results

4.3.1 Batch tests with biomass from the R_A

4.3.1.1 Performance of batch test

As is shown in Figure 4.1, performance of the biomass in the three serum bottles showed good consistency. The change in the TVFAs showed some fluctuation during the batch test. TVFA concentration decreased in the first 5 d, but built up on Day 6 and then decreased until pH was manually increased on Day 11. The HBu concentration trend was very similar to that of TVFA. However, HAc concentration had decreased from the Day 1 till Day 8 and then remained low till the end of the batch test even after adjustment of pH.

Methane content of the biogas remained above 30% and hydrogen was almost 0% in all serum bottles in the first 4 d of the batch test. But as pH further decreased to around 4.5 (since pH was not controlled in all serum bottles), methane content decreased to 20% on Day 5 and to only a few percent on Day 6 while hydrogen content increased to 20% on Day 5 and built up to 35-55% in the serum bottles (I, II and III) on Day 6. After that, the hydrogen content remained high with methane content remaining at 0%. The increase of pH caused a substantial decrease in hydrogen content in III (57.3% to 28.9%), but

Figure 4.1 COD, VFA, biogas and pH information during batch test: (a) COD of TVFAs in effluent (TVFA Effl), COD of effluent (COD Effl), COD of HBU and HAc in effluent (HBU Effl, HAc Effl); (b) CH₄ and H₂ content of biogas from serum bottles and pH of respective effluent. CO₂ data are not shown, since the content of CO₂ = (100% - [CH₄] - [H₂]). The dash line marked the day when NaOH was added to increase the pH in serum bottles (Day 11).

did not cause significant change in I and II. However, methane content had remained unchanged at 0%.

4.3.1.2 Real-time qPCR analysis of microbial community

Figure 4.2 shows the microbial structure of the biomass before and after the batch test. After 15 d of the batch test, the microbial community in the serum bottles had changed substantially. At the start of the batch test, 16S rRNA gene concentration of BAC and ARC was 1.22×10^{10} and 1.22×10^8 copies/mL, respectively. At the end of the batch test gene concentration of BAC was $1.50 \pm 0.42 \times 10^{10}$ copies/mL and this had remained relatively unchanged during the batch test, but ARC had declined substantially to $1.07 \pm 0.06 \times 10^5$ copies/mL. Within the archaea domain, all methanogen populations reduced substantially and especially so for the hydrogenotrophic MMB and MBT. Gene concentration

Figure 4.2 Quantification of 16S rRNA gene concentration of bacteria and methanogenic communities in serum bottle I-III at the start of the batch test (No. 0) and at the end of the batch test (No. I-III).

of MBT declined from 1.26×10^8 to $1.55 \pm 0.82 \times 10^5$ copies/mL while MMB had become undetectable (less than 9×10^4 copies/mL) after 15 d of operation. The gene concentration of MST also had a one magnitude of order decrease in gene concentration.

4.3.2 Effect of pH change on R_A

4.3.2.1 Performance of R_A before pH change

After 114 d of operation, stable acidogenesis performance was achieved in R_A . From Day 115 to Day 179, TVFA concentration in the effluent was 16.2 ± 1.4 gCOD/L, with 4.7 ± 0.6 gCOD/L of HAc. Soluble COD in the effluent was 17.1 ± 1.4 gCOD/L (Fig. 4.3 a). This indicated a healthy acidogenesis performance in R_A . Production rate of biogas was around 5 L/d with $39 \pm 4\%$ of methane and $61 \pm 4\%$ of carbon dioxide (Fig. 4.3 b). Such performance was consistent with the performance of R_A before Day 114 (Chapter 3).

4.3.2.2 Performance of R_A after pH change

After pH was manually adjusted to 4.5, there was no significant change in the reactor's performance during the first 10 d (Fig. 4.3 a and b) (Day 180 – Day 190). However, from Day 202, hydrogen content in the biogas increased sharply from 0 to 22%. Meanwhile, COD in the effluent increased from 17.1 ± 1.0 to 21.5 ± 1.8 g/L with substantial reduction in TVFA (15.1 ± 2.5 to 4.0 gCOD/L) and HAc (6.1 ± 1.0 to 1.6 gCOD/L) concentrations (Fig. 4.1 a) which indicated that acidogenesis was inhibited in R_A . Biogas production increased to around 11 L/d but with hydrogen content of 25% to 38% till Day 281. On Day 281, hydrogen content decreased to 0% and methane content increased to 17%. Meanwhile, TVFA and HAc concentration in the effluent also increased. TVFA concentration increased from 8.90 ± 2.15 gCOD/L to 21.1 gCOD/L (Day 294) and HAc concentration increased from 2.14 ± 0.48 gCOD/L to 7.0 gCOD/L respectively. Increased organic acids production indicated acidogenic performance of R_A was recovering from the pH stress.

Figure 4.3 Performance and microbial community structure of RA. (a) COD, VFA variation: COD of influent into RA (Infl A) and effluent from RA (Effl A); COD of TVFA in influent into RA (VFA Infl A) and effluent from RA (VFA Effl A); COD of HAc in effluent (HAc Effl A); (b) Biogas: CH₄, CO₂ and H₂ content of biogas from RA; (c) Quantification of 16S rRNA gene concentration of methanogenic communities and bacteria in the RA.

4.3.2.3 Microbial community dynamics in R_A

The qPCR assays revealed 16S rRNA gene concentration of the domain Bacteria (BAC) and Archaea (ARC) in the acidogenic reactor (Fig. 4.1 c). BAC was predominant and remained relatively stable with an average gene concentration of $3.9 \pm 1.9 \times 10^{10}$ copies/mL during the whole process. For the domain of Archaea, hydrogenotrophic MBT was dominant while MMB and MST constituted very small portion of the Archaea population. From Day 135 to Day 179, no substantial change in the Archaea domain was observed. However, after 10 d of pH change, a major change in community structure took place (Fig. 4.1 c). While the BAC population remained relatively stable, ARC population decreased significantly. 16S rRNA gene concentration of ARC decreased from 7.53×10^7 to 1.83×10^7 copies/mL and kept decreasing to 9.43×10^5 copies/mL till Day 230. Within the domain of Archaea, population of the two hydrogenotrophic methanogens declined greatly. Gene concentration of MBT decreased from 8.06×10^7 to 5.64×10^5 copies/mL on Day 230, and MMB became undetectable (less than 9×10^4 copies/mL). Population of MST was less affected by the pH change, but also became undetectable (less than 1.7×10^4 copies/mL) after Day 230. After Day 230, ARC population started to recover and kept increasing till the end of the operation. However, only MBT was detectable in the domain of Archaea since then.

4.4 Discussions

4.4.1 Effects of pH on performance and microbes in serum bottle batch study

With decreasing pH, TVFA concentration decreased for 5 d but increased on Day 6, while HAc concentration kept decreasing. It was also noted that the decrease in TVFA came mostly from the decrease in HAc instead of the other VFAs before Day 6. After Day 6, with presence of hydrogen in the serum bottles, increased concentration of TVFA was not accompanied by increased concentration of HAc but by the increase in H₂. This indicated that a shift in the

fermentative type from HAc-type to HBU-type fermentation occurred during the decrease of pH and increase of hydrogen content in biogas. Such phenomenon was in accordance with previous studies in which it was found that the presence of hydrogen in acidogenic reactor encouraged HBU production rather than HAc production (Mosey, 1983; Mu *et al.*, 2006).

With pH adjusted upwards in the serum bottles after Day 11, TVFA and HBU concentration increased but HAc concentration remained low. This could probably be due to high hydrogen content still present in the biogas of the serum bottles. HAc production could not then be recovered. Therefore both pH and the presence of hydrogen could have affect the acidogenic process. While the pH could be adjusted to an optimal value, hydrogen in the headspace could not be as readily resolved.

The drastic change in the microbial profile after 15 d of experiment may explain the existence of hydrogen in the serum bottle. Total ARC concentration decreased substantially by 3 orders of magnitude during the experiment. And within the archaea domain, the gene concentration of hydrogenotrophic methanogens i.e., MMB and MBT, decreased most significantly. Meanwhile, bacteria population remained almost unchanged during the experiment. This indicated that the low pH could have substantially brought down the population of hydrogenotrophic methanogens. And the existence of high content of hydrogen in the biogas could be caused by the lack of hydrogen consumers (i.e., MMB and MBT). The manually increased pH could help alleviate the stress brought by low pH, but could not bring up the gene concentration of the hydrogenotrophic methanogens immediately. This could explain why the hydrogen content in biogas remained high. And such high content of hydrogen then consequently inhibited HAc production even when pH was recovered to a relatively optimal level. To verify such assumption further test were done to the laboratory-scale acidogenic reactor.

4.4.2 Effects of pH on performance and microbes in R_A

The results suggested that the pH stress could have caused the following performance changes to the acidogenic reactor: (1) decrease in methane content in biogas; (2) increase in hydrogen content in biogas; (3) decrease in TVFA production; and (4) decrease in HAc production. The performance change (1) was observed during the pH change test. This was because methanogens were sensitive to pH change, especially under acidic environment. However, performance changes (2)-(4) was observed not during the pH change test but 12 d after that. Similarly, the performance of R_A changed on Day 280 when pH was stabilized at 5.5 ± 0.2 . This suggested that pH was not the direct cause of performance change (2)-(4) in present study.

pH was reported to be closely related to substrate degradation and organic acid products distribution (Ren *et al.*, 1997; Yu and Fang, 2002, 2003). Yu *et al.* (2003) showed that overall acidogenesis performance improved with increasing pH in the pH range of 4.0 - 6.0. As shown in Table 4.2, at pH 4.5, concentrations of HAc and TVFA were 47% and 26% lower as compared to those at pH 5.5. However, data from other reports suggested otherwise (Li *et al.*, 2010; Wu *et al.*, 2010). Results of Wu *et al.* (2010) showed concentration of TVFA was 28% higher at pH 4.5 than pH 5.5, while that of HAc was 3% lower (Table 4.2). For the present study, as shown in Table 4.2 and Figure 4.3, in the same pH range (5.5), performance of R_A was drastically different before and after the pH change whereby the system was in steady-state in both cases. These results indicated that other than pH, there may still be other factors influencing the acidogenesis process, even when the pH was the unique operating variable.

Table 4.2 Effects of pH on acidogenesis

Reference	VFA	Conc. at pH 5.5 mg/L	Increment →	Conc. at pH 4.5 mg/L	Increment →	Conc. at pH 5.5 mg/L
Yu et al, 2003	HAc	373	-47%	196	N.A. ^b	N.A.
	TVFA	1404	-26%	1034	N.A.	N.A.
Wu et al, 2010	HAc	594 (pH 5.6)	-3%	578 (pH 4.4)	N.A.	N.A.
	TVFA	1083 (pH 5.6)	28%	1388 (pH 4.4)	N.A.	N.A.
Present study	HAc	5889 ^a	1%	5951	-68%	2007
	TVFA	10110	0%	10098	-46%	5480

a Averaged by data from Day 170 to Day 180.

b Not available.

From Fig. 4.3 c, after the pH change, a substantial reduction in the hydrogenotrophic methanogen population was observed in R_A. This observation was in accordance with the performance of the acidogenic reactor when methane content decreased to 0% and hydrogen content built up to 20-30%. As the hydrogenotrophic methanogen population declined, consumption of hydrogen was also substantially reduced. As the hydrogenotrophic methanogen population gradually recovered (between 10⁷ to 10⁸ copies/mL in present study), consumption of hydrogen was regained, leading to decrease in the hydrogen content and increase in methane content from Day 281. This indicated that the pH change (5.5 to 4.5) had a profound impact on the hydrogenotrophic methanogens which then led to the performance change of R_A.

4.4.3 Effect of hydrogen on acidogenesis

It was observed during operation of R_A under normal conditions, more than 60% COD was converted to VFA COD, indicating healthy acidogenesis in R_A. However, production of TVFA and HAc in R_A declined after the pH of the system was changed.

This may be attributed to two reasons: low pH and/or high hydrogen content in biogas. Low pH may affect fermentation by the acidogens; hydrogen content can

affect the redox balance within the acidogenic phase. However, as is shown in Figure 4.3 a and b, pH had no immediate impact on production of TVFA and HAc. Furthermore, substantial reduction in TVFA and HAc was observed once hydrogen content started to build in the system on Day 202; similarly, as hydrogen decreased on Day 280, TVFA and HAc started to increase in the system when pH remained unchanged. This indicated that the reduction of TVFA and HAc was more likely to be caused by the accumulated hydrogen in R_A . Hydrogen was reported to have impact on acidogenic and acetogenic bacteria which are two important bacteria groups involved in the production of HAc (Mosey, 1983). This phenomenon was also in consistency with the results of batch study as stated in section 4.4.1.

Accumulation of HAc in the acidogenic reactor of a two-phase system, which would be the carbon source for the following methane generation process, is a key indicator of a robust acidogenic reactor. Therefore in order to increase the HAc production, it is crucial to create an environment with low hydrogen presence and so guarantee an efficient performance of acidogenic reactor.

4.4.4 Role of methanogens in acidogenic reactor

As shown in Fig. 4.3 c, hydrogenotrophic methanogen MBT comprised majority of methanogen population in R_A , while acetotrophic methanogens (MST and MSC) comprised only minor population of methanogen. This is because short HRT, low pH and high HAc concentration conditions are more inhibitory to acetotrophic methanogens. All acetotrophic methanogens belong to the order Methanosarcinales comprised of the two families, *Methanosaetaceae* and *Methanosarcinaceae*. Their growth rates are rather low with minimum doubling time of 2-3 d, and they are very sensitive to pH change. *Methanosaetaceae* is adapted for utilizing low concentrations of acetate (threshold < 600 µg/L), while *Methanosarcinaceae* is only able to convert acetate at higher concentrations (threshold of 12-72 mg/L) (Fey and Conrad, 2000; Klocke *et al.*, 2008; Shimada

et al., 2011). In the present study, however, pH was lower than 5.5 and HAc concentration in acidogenic reactor was always above 1 g/L, which is inhibitory to the *Methanosaetaceae* family. On the other hand, unlike acetotrophic methanogens, hydrogenotrophic methanogens' growth rates are relatively faster with minimum doubling times of around 6 hours. Previous studies have also confirmed the existence of hydrogenotrophic methanogen in acidogenic reactors (Raskin *et al.*, 1995; Liu *et al.*, 2002b; Shimada *et al.*, 2011). These suggested that methane production from acetate was not favored in the acidogenic phase, even though this process is considered to be responsible for most methane production (66-72%) in single-phase (combining acidogenic phase and methanogenic phase) anaerobic degradation (Schink, 1997).

In this sense, the role methanogens played in R_A was more likely to convert hydrogen to methane (by hydrogenotrophic methanogen) and was not to use HAc to produce methane (by acetotrophic methanogen). This may also explain why higher population of methanogen did not result in lower HAc production but higher HAc production in acidogenic reactor. It was also noted that acetotrophic methanogens (MST and MSC) both became undetected after Day 230. Therefore, keeping a proper methanogenic activity in acidogenic phase can enhance the acetic acid production by consuming hydrogen without interfering with the acidogenesis process.

4.5 Conclusions

In summary, methanogens, especially hydrogenotrophic methanogens existed and played an important role in the acidogenic reactor. While production of hydrogen weakened acidogenesis, methane production in the acidogenic reactor, which was always ignored, can effectively remove the hydrogen leading to higher organic acid production, HAc production in particular. With more HAc produced in the acidogenic phase, which is the desired substrate for the following

methanogenic phase, the overall stability and efficiency of a two-phase anaerobic process is expected to be effectively improved.

CHAPTER 5 IMPROVED PERFORMANCE OF ACID-REACTOR

5.1 Introduction

Hydrogen is involved in many major processes in acidogenesis of anaerobic degradation, e.g., the formation of pyruvate, production of various organic acids and conversion between these organic acids. As an inevitable byproduct of anaerobic degradation, it can affect the decomposition of VFAs such as propionic acids and butyric acids. For example, propionic acids can only be converted to acetic acids when the hydrogen partial pressure is lower than 10.1 Pa (Fukuzaki *et al.*, 1990). In real life practice, however, to control the hydrogen partial pressure under such low value would not be easy, especially for degradation of a substrate rich in organic components (Fennell *et al.*, 1997).

Gas sparging had been suggested to be a useful method to keep low hydrogen partial pressure in anaerobic digestors (Siriwongrungson *et al.*, 2007). However, such sparging techniques require high purity inert gases (e.g., nitrogen) and constant sparging. For digestors which are designed to produce and utilize biogas, such techniques not only require additional energy input but also dilute the produced biogas. Alternatively, existence of hydrogenotrophic methanogens in the acidogenic reactor can greatly alleviate the problem caused by hydrogen (Huang *et al.*, 2014). However, hydrogenotrophic methanogens could only exist in a pH higher than 5.0, if pH dropped further, low pH could inhibit hydrogenotrophic methanogens in the acidogenic reactor leading to increase in hydrogen content in the biogas and weakened acidogenic performance (Ren *et al.*, 1997; Yu and Fang, 2002, 2003; Huang *et al.*, 2014). Therefore, to implement biological conversion of hydrogen in an acidogenic reactor where the resident hydrogenotrophic methanogens are inhibited, the incorporation of an external source of hydrogenotrophic methanogen may be helpful.

In this chapter, a laboratory-scale reactor designed to enrich hydrogenotrophic methanogens (R_H) was added to the two-phase anSBR (R_A and R_M). The pH of

R_A was maintained at 4.5 to inhibit hydrogenotrophic methanogens and encourage hydrogen production. R_H was studied to check whether external hydrogenotrophic methanogen for R_A could help in alleviating the hydrogen stress on VFA production in R_A .

5.2 Material and methods

5.2.1 AnSBR setup and operation

A laboratory-scale anSBR system comprising an acidogenic reactor (R_A), a methanogenic reactor (R_M) and a hydrogenotrophic methanogenic reactor (R_H) was operated using a programmable logic controller (PLC) system. The working volume of the reactors were 5 L for R_A and R_H ; and 10 L for R_M , respectively. All reactors were jacketed with heating tapes and the temperature was controlled at 40 ± 1 °C. The pH control, feeding, mixing, desludging, settling and decanting processes were implemented with peristaltic pumps and controlled with the PLC. The schematic diagram of the anSBR is shown in Figure 5.1.

5.2.1.1 Operation before connecting R_A and R_H

R_A was operated for 310 d as described in Chapter 3 and Chapter 4. The seed sludge for R_M was obtained from an anaerobic digester at Ulu Pandan wastewater treatment plant, Singapore. After collection, the seed sludge was filtered through a 600 μ m sieve and stored at 4 °C before seeding in the reactors. Seed sludge for R_H was taken from R_A (pH of 5.5) and R_M (pH of 7.5) with v/v ratio of 1:1. R_A was fed with synthetic feed simulating high strength organic wastewater; R_M was fed with effluent from R_A ; and effluent from R_M was fed into R_H as feed solution. The composition of the synthetic feed is as shown in Table 5.1.

Figure 5.1 Schematic diagram of the R_H augmented two-phase anSBR: a) before the Connection; b) after the Connection.

The pH of R_A was adjusted to 4.5 to inhibit hydrogenotrophic methanogen in the reactor. R_M was started as described in section 3.2.2. R_H was seeded with the mixed sludge while purging continuously with N_2 gas for 10 minutes. The initial pH of the reactor was approximately 6.90 – 7.10. The pH was controlled by the PLC system with 1 M sodium hydroxide solution and 1 M hydrochloric acid

solution. Initial MLVSS of R_H was around 5 g/L with a MLVSS/MLSS ratio of 0.66. The day when R_H was started was defined as the first day of operation, i.e., Day 1. Detailed operating parameters are shown in Table 5.2.

Table 5.1 Synthetic feed composition

Component	Concentration, mg/L
Organics:	
Sucrose	20000
Ethanol	1200
Sodium Acetate	1100
Propionic Acid	540
Butyric Acid	260
Macronutrients (inorganics):	
NH_4HCO_3	1885
NH_4Cl	1276
K_2HPO_4	250
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	125
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	180
CaCl_2	100
Na_2SO_4	800
Micronutrients (inorganics):	
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	2.5
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	2.5
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.5
H_3BO_4	0.5

5.2.1.2 Operation after connecting R_A and R_H

From Day 115 on, the headspaces of R_A and R_H were connected as shown in Figure 5.1 b. The mixed liquors of R_A and R_H remained separated. Other operations remained unchanged. The time when R_A and R_H headspaces became connected was termed as “Connection” for short; after Connection the shared gaseous phase of R_A and R_H was termed “R_{AH}” for short.

Table 5.2 Operating parameters of R_H augmented two-phase anSBR

Parameters	R _A	R _M	R _H
Working volume (L)	5	10	5
Total volume (L)	6	12	6
Feed	Synthetic feed	Effluent from R _A	Effluent from R _M
Feed SCOD (g/L)	24.1±2.4	NA	NA
Feed VFA (g/L)	1.4±0.1	NA	NA
Feed HAc (g/L)	0.9±0.1	NA	NA
pH	4.5±0.1	7.6±0.2	7.0±0.2
T (°C)	40±1	40±1	40±1
ORP (mV)	-400±50	-500±50	-500±50
SRT(d)	25	50	50
HRT(d)	1.7	3.4	1.7

5.2.2 Analytical methods

5.2.2.1 Chemical analysis

Influent and effluent samples were collected from feed tanks and reactors routinely for chemical analysis. Volatile fatty acids (i.e., HAc, HPr, Iso-HBu, HBu, Iso-HVa, HVa, Iso-HCa, HCa and HHe) were measured using gas chromatography (Agilent Technologies 7890A GC system, US) with Zebron ZB-FFAP 30 m × 320 µm × 0.5 µm column and a flame ionization detector (FID).

Prior to analysis, 0.1 mL of 10% formic acid was added to each 0.9 mL of samples and standards to acidify the samples. COD, MLSS, and MLVSS were determined in accordance with Standard Methods (APHA, 1998). MLSS and MLVSS inside the reactors and in the discharge were tracked in order to monitor the SRT.

Before Connection, volume of biogas produced by R_A , R_M and R_H was tracked by gas mass flow controllers (0.50 to 50.0 mL/min, Cole-Parmer, USA); after Connection, the produced gas from R_{AH} was collected in gas bag AH (Figure 5.1 b) and the volume was determined by a drum-type wet gas flow meter (Ritter, Germany). The gas volume produced from R_M was tracked by gas mass flow controllers (0.50 to 50.0 mL/min, Cole-Parmer, USA) throughout the whole experiment.

Methane, carbon dioxide and hydrogen of biogas in the gas bag A, gas bag M, gas bag H and gas bag AH were analyzed using gas chromatography (Agilent Technologies 7890A GC system, US) with (1) an Agilent HayeSep R 0.9 m $\times$ 1/8" $\times$ 2.0 mm packed column, (2) an Agilent HayeSep C 3.0 m $\times$ 1/8" $\times$ 2.0 mm packed column, (3) an Agilent MolSieve 5A 3.0 m $\times$ 1/8" $\times$ 2.0 mm packed column, (4) an Agilent HayeSep Q 0.9 m $\times$ 1/8" $\times$ 2.0 mm packed column, and (5) an Agilent MolSieve 13 $\times$ 3.0 m $\times$ 1/8" $\times$ 2.0 mm packed column with two thermal conductivity detectors (TCD, a front detector for measuring methane and carbon dioxide, and a back detector for measuring hydrogen). Helium was the reference gas for Column 1-3 for detection of methane and carbon dioxide and argon was the reference gas for Column 4-5 for detection of hydrogen.

5.2.2.2 Molecular biology analysis

0.5 mL sludge samples were collected in 2 mL plastic tubes, centrifuged at 10000 rpm for 30 sec, followed by decantation of the supernatant. The sludge was then washed twice with 1 mL phosphate buffer solution (PBS 1 $\times$). The pellets were

stored at 4 °C before DNA extraction. Before extraction, the sludge samples were diluted 5 times to reach cell concentration of around 10^{10} /mL. Total DNA was then extracted from samples using an automated nucleic acid extractor (MagNA Pure Compact, Roche, Germany). The purified DNA was then stored at -20 °C before analysis.

16S rRNA gene quantifications of the DNA samples were performed on LightCycler 480 II (Roche, Germany). The primer and probe sets specific for two domains: Bacteria (BAC) and Archaea (ARC); two order-level Archaea: Methanomicrobiales (MMB) and Methanobacteriales (MBT); and two family-level Archaea: *Methanosarcinaceae* (MSC) and *Methanosaetaceae* (MST) were used (Yu *et al.*, 2005; Bialek *et al.*, 2011). The reaction was performed with a total volume of 20 μ L mixture: 10 μ L of 2 $\times$ LightCycler 480 Probes Master, 4 μ L of PCR-grade water, 2 μ L of TaqMan probe (final concentration 200 nM), 1 μ L of each forward and reverse primer (final concentration 500 nM), and 2 μ L of template DNA. The operation processes consisted of a predenaturation step of 10 min at 95 °C, amplification of 55 cycles (10 s) at 95 °C and 30 s at 60 °C, and cooling for 10 s at 40 °C. Standard curves were constructed using those strains corresponding to primer and probe sets used in this experiment (Table 3.4). A 10-fold dilution series from 10^1 - 10^{10} copies/ μ L of standard solution was established and analyzed by qPCR in duplicate to construct the standard curve for the corresponding primer and probe set.

5.3 Results

5.3.1 Performance of anSBR before Connection

As shown in Figure 5.2 and 5.3, before Connection, with influent COD of 24 g/L, effluent COD was 21.1 ± 1.4 g/L. The concentration of TVFA in the effluent of R_A was 9.55 ± 0.91 gCOD/L, concentration of HAc and HPr was 1.17 ± 0.27 and 0.40 ± 0.08 gCOD/L, respectively. TVFA contributed only 45.2% of effluent COD while HAc contributed only 5.5% of effluent COD - showing relatively weak

acidogenesis. Such performance was expected because pH was controlled at 4.5 ± 0.2 to inhibit hydrogen consumers in R_A and there indeed was high content of hydrogen in the gas phase of R_A ($29.7 \pm 0.44\%$) (Figure 5.5). As stated in previous Chapter, such high content of hydrogen in biogas would consequently lead to weakened acidogenesis performance. For R_M and R_H , effluent TVFA of R_M was 0.04 ± 0.02 gCOD/L and that of R_H was less than 0.01 gCOD/L showing an expected further removal of VFA in the effluent (Figure 5.3). Figure 5.4 shows the concentration of HPr in effluent from R_A and R_M , the concentration were 0.40 ± 0.08 gCOD/L and 0.05 ± 0.03 gCOD/L, respectively, before Connection.

Figure 5.2 COD and VFA of R_A : COD of influent into R_A (Infl A), effluent from R_A (Effl A), theoretical COD of HAc in influent into R_A (HAc Infl A), effluent from R_A (VFA Effl A) and HAc in effluent from R_A (HAc Effl A).

The biogas as shown in Figure 5.5 and 5.6, had high content of hydrogen in R_A ($29.7 \pm 0.44\%$ with 1.03 ± 0.34 L/d of hydrogen was produced). In R_H $4.5 \pm 1.7\%$ with 0.16 ± 0.07 L/d of methane was produced. Though the overall methane production was low in R_H , the existence of methane in the biogas indicated acetotrophic methanogenic activity was present in R_H . For R_M a relatively stable biogas production was observed, and $73.5 \pm 2.9\%$ with 17.92 ± 0.91 L/d of methane was produced from R_M before Connection (Figure 5.7 and 5.8).

Figure 5.3 COD and VFA of effluent from R_M and R_H : effluent from R_M and R_H (Effl M and H), theoretical COD of effluent VFA from R_M and R_H (VFA Effl M and H) and HAc in effluent from R_M and R_H (HAc Effl M and H).

Figure 5.4 Theoretical COD of effluent HPr from R_A and R_M .

Figure 5.5 Biogas of R_A and R_H: H₂ content in R_A and CH₄ content in R_H.

Figure 5.6 Biogas of R_A and R_H: H₂ production rate in R_A and CH₄ production rate in R_H.

Figure 5.7 Biogas of R_M: CH₄ and CO₂ content in R_M.

Figure 5.8 Biogas of R_M: CH₄ and CO₂ production rate in R_M.

5.3.2 Performance of anSBR after Connection

After Connection, only mixed production of biogas from R_A and R_H (R_{AH}) could be measured. From Day 118 to Day 123, high production of hydrogen with no production of methane was observed in R_{AH} indicating no consumption of hydrogen in the system. Starting from Day 125, however, methane started to

build up in the system and reached $21.3 \pm 1.1\%$ after Day 140. Meanwhile hydrogen content gradually declined to 0 on Day 140. These observation demonstrated that R_H as a provider of external hydrogenotrophic methanogen could work as a hydrogen consumer for R_A .

As hydrogen disappeared in R_{AH} , VFA production also started to recover in R_A (Figure 5.2). After Connection, COD of the effluent from R_A did not show significant variation. COD before Connection was 21.1 ± 1.4 g/L, while COD after Connection was 19.9 ± 1.6 g/L. Concentration of HAc and TVFA in the effluent of R_A , however, increased substantially from 1.34 ± 0.13 gCOD/L to 4.14 ± 0.12 gCOD/L and from 8.96 ± 0.95 gCOD/L to 12.02 ± 0.40 gCOD/L, respectively. Meanwhile, concentration of HPr in R_A showed completely different trend compared with that of HAc, the concentration declined from 0.40 ± 0.08 gCOD/L to 0.08 ± 0.05 gCOD/L. Such changes of effluent from R_A led to cleaner effluent both from R_M and R_H . After Day 140, TVFA in R_M decreased from 0.04 gCOD/L to 0.008 gCOD/L and no HPr was detected since then. TVFA in R_H also became undetected after Day 149.

With better quality of R_A effluent, content of methane in biogas produced from R_M slightly increased from $73.5 \pm 2.9\%$ to $77.2 \pm 1.0\%$ while total methane production rate increased from 17.92 ± 0.91 L/d to 18.91 ± 0.55 L/d. This shows a 5.0% of increase in methane content and a 5.5% of increase in methane production daily.

5.3.3 Microbial community dynamics in the R_A and R_H

Similar with the results in previous chapters, bacteria still was the dominant microbe in R_A and R_H . Before Connection, 16S rRNA gene concentration of BAC in both reactors had been relatively stable. The gene concentration in R_A and R_H were $3.80 \pm 2.72 \times 10^{10}$ and $1.01 \pm 0.36 \times 10^9$ copies/mL, respectively. For the Archaea domain, gene concentration of ARC in R_H was relatively stable ($5.56 \pm$

1.79×10^8 copies/mL). Within the Archaea domain, MMB and MBT were the dominant methanogens ($3.14 \pm 1.84 \times 10^7$ copies/mL and $2.34 \pm 0.96 \times 10^7$ copies/mL, respectively), while there were also substantial population of MST and MSC ($5.02 \pm 1.18 \times 10^6$ copies/mL and $5.82 \pm 0.96 \times 10^6$ copies/mL, respectively). The population of the respective methanogens were also very stable with minor variation during the period before Connection. In R_A , however, gene concentration of ARC decreased from 7.38×10^6 (Day 10) to 6.78×10^4 copies/mL (Day 70), and increased back to 7.46×10^5 copies/mL on Day 100. Since only MBT was detectable before Connection, MBT dominated Archaea domain in R_A and the gene concentration of MBT was also very similar to that of ARC.

Figure 5.9 Quantification of 16S rRNA gene concentration of bacteria and methanogenic communities in R_A .

After Connection, population of BAC in R_A did not encounter substantial changes. Gene concentrations of BAC remained between 3.01×10^9 and 2.36×10^{10} copies/mL, of which the difference was less than one order of magnitude. However, gene concentration of BAC in R_H increased substantially from 6.30×10^8 to 6.18×10^{10} copies/mL. For Archaea domain, population of ARC in both

reactors increased. In R_A , the gene concentration increased from 1.11×10^7 to 2.26×10^9 copies/mL; while in R_H the concentration increased from 6.46×10^8 to 1.25×10^{10} copies/mL. In R_A MBT still dominate the Archaea domain with similar gene concentration of ARC, but other methanogens, such as MST and MMB, also became detectable and gradually increased to an evident level. In R_H population trend of different methanogenic groups varied greatly. MBT started to dominate the Archaea domain with a gene concentration very similar to that of ARC. An increase in population of MST was also observed. Gene concentration of MST increased from 5.61×10^6 to 1.41×10^8 copies/mL. For MMB and MSC, the population decreased substantially before increasing. Gene concentration of MMB decreased to 3.76×10^6 copies/mL (Day 130) and increased to 5.60×10^8 copies/mL, while that of MSC decreased to 1.13×10^5 copies/mL (Day 148) and then increased to 1.62×10^7 copies/mL.

Figure 5.10 Quantification of 16S rRNA gene concentration of bacteria and methanogenic communities in R_H .

5.4 Discussions

5.4.1 Conversion of hydrogen to methane

After Connection, conversion of hydrogen to methane did not occur immediately. High content of hydrogen but no detection of methane was observed in R_{AH} in

the first few days. However, observable conversion of hydrogen to methane started on Day 125. Methane content gradually increased and hydrogen content started to decrease while pH and all other operating parameters remained unchanged in both R_A and R_H .

The biological conversion of hydrogen to methane is described as in eqn. 6, Chapter 2. Stoichiometrically, 4 mol of hydrogen is converted to 1 mol of methane. Thus, the theoretical ratio of consumed hydrogen to produced methane ($\Delta H_2/\Delta CH_4$) should be 4. Under the assumption that all methane was converted from hydrogen produced in R_A , after Day 140 with a methane production rate of 1.19 ± 0.16 L/d, the hydrogen potentially produced from R_A would be 5.76 L/d. However, the highest production rate before Connection was only 2.08 L/d. Even when considering the fact that before Connection 0.16 ± 0.07 L/d of methane was produced from acetotrophic methanogens, there was still 1.03 L/d more of methane produced after Connection, so the hydrogen potentially produced from R_A should be revised to 4.12 L/d. That was still two times the highest production rate of hydrogen before Connection. Such observation indicated that after Connection either there were other sources for methane production in R_{AH} (0.5 L of extra methane) or more potential of hydrogen was produced from R_A (2.04 L of extra hydrogen).

5.4.1.1 Methane production from acetotrophic methanogens in R_A

Unlike the microbial gene concentration results from Chapter 4 in which very few or no acetotrophic methanogens were detected in R_A , in Chapter 5 after Connection gene concentration of the acetotrophic methanogen MST increased substantially to 1.41×10^8 copies/mL. Such observation made the conversion of HAc to methane possible in R_A .

Supposing that the extra 0.5 L/d of methane (i.e., 0.02 mole of methane) was produced by HAc, it would mean that 1.28 gCOD/d of HAc was consumed to produce methane in R_A (eqn. 10).

By this pathway, 0.47 gCOD/L more of HAc was consumed in R_A . This meant that for the produced HAc in effluent from R_A , 10.2% of HAc was consumed before fed into R_M . Such reduction in VFA would cause a lower methane production in R_M but would not affect the overall production of methane from the entire anSBR system.

5.4.1.2 Methane production from hydrogen produced in R_A

On the other hand, more hydrogen produced from R_A was also possible with more HAc produced in R_A . From eqn. 1, 4 and 5 (Chapter 2), stoichiometrically, with 1 more mole of HAc produced by conversion from glucose or other VFAs in R_A , at least 1 mole of hydrogen could be produced spontaneously. Considering that concentration of HAc increased from 1.34 ± 0.13 gCOD/L to 4.14 ± 0.12 gCOD/L and HRT was 1.7 d, 8.4 gCOD more of HAc was produced daily after Connection. Supposing that 1 mole more hydrogen was produced with 1 mole more of HAc produced, 8.4 gCOD more of HAc (i.e., 0.13 mole of HAc) per day corresponded to 2.94 L/d of hydrogen. Such amount of more potential hydrogen produced per day in R_A could well compensate for the methane converted in R_H .

From a biogas point of view, this pathway is more interesting than the pathway described in 5.4.1.1. Such pathway, unlike the previous pathway, was completed without further consumption of carbon source (HAc) and produced hydrogen in the process. Moreover, it utilized hydrogen to convert an inorganic carbon source carbon dioxide to methane. Therefore, this pathway would be more likely to improve the overall methane production of the whole anSBR.

From above analysis, though the extra amount of methane produced in R_{AH} could be explained in both pathways, it was hard to determine which one was the main pathway. However, it could be concluded that by either pathway, the overall methane production would not be compromised. Meanwhile, with more HAc produced in the effluent from R_A after Connection, the overall performance of the anSBR could be enhanced.

5.4.2 Microbial community changes in the reactors

From Figure 5.9 and 5.10, it was observed that the connection of gaseous phase of R_A and R_H did have an impact on the microbial community structure of both reactors.

For R_H , except for MSC, all other tested methanogens together with BAC population increased. The domination of hydrogenotrophic methanogen MBT in Archaea domain and the increase of MMB was due to the feeding of hydrogen from R_A . Increase of MST suggested the possibility of homoacetogenic conversion of hydrogen and carbon dioxide into acetic acid. From Figure 5.10 before Connection, the microbial community structure was quite stable. The feeding of hydrogen after Connection could encourage the growth of hydrogen-utilizers such as MBT and MMB, but not necessarily encourage that of acetic acid-utilizers. The growth of acetotrophic methanogen MST suggested that there might be more acetic acid formed in the reactor. With all operating parameters remaining the same, the extra acetic acid must come from the hydrogen, and the only pathway that can convert hydrogen to acetic acid is performed by homoacetogens (eqn. 7). This may explain why MST population also increased after Connection.

For R_A the most significant changes were the recovery of MST population and the increase of MBT population. Under pH of 4.5, such increase of methanogen

population was unexpected. Considering that none operating parameter changed during the Connection, it would seem that the consumption of hydrogen might be the direct or indirect cause of such significant changes.

For recovery of acetotrophic methanogen MST, the increase of HAc concentration in R_A could be the cause. However, as stated in previous chapter, MST was not favored by low pH or HAc concentration higher than 600 $\mu\text{g/L}$ (Fey and Conrad, 2000; Klocke *et al.*, 2008; Shimada *et al.*, 2011). Meanwhile such recovery of MST was not observed in the experiments conducted in Chapter 4 in which the pH was more favorable to the growth of methanogens (pH of 5.5).

For the increase of MBT population, no previous studies have demonstrated that low hydrogen content could encourage the growth of hydrogenotrophic or acetotrophic methanogen population. There is one study in the literature which suggested high hydrogen partial pressure might suppress the activity of hydrogenotrophic methanogens (Lee *et al.*, 2012). Yet such assumption was derived from the performance and operating settings of an anaerobic reactor for conversion of carbon dioxide and hydrogen, but not from direct evidence of observed changes in microbial community. In the present study, the increase of MBT population and consumption of hydrogen could suggest the possibility of the above assumption. However, the evidences provided in present study are not sufficient to confirm the assumption on the growth of methanogens (neither MBT nor MST) in R_A . Further investigation is needed to confirm the reasons of the unexpected results on microbial changes in R_A .

5.4.3 Improved acidogenesis by external hydrogenotrophic methanogen

Though unexpected microbial community changes occurred in R_A , results of the performance of R_A and R_H still confirmed that the external hydrogenotrophic methanogen could help to alleviate the stress to acidogenesis brought by hydrogen produced in acidogenic reactor. After Connection, observable

reduction of hydrogen content in biogas from R_A was followed by an evident increase of HAc concentration in effluent from R_A . The hydrogen content decreased to 0%, and HAc concentration increased from 1.34 ± 0.13 gCOD/L to 4.14 ± 0.12 gCOD/L. These results together with the results in Chapter 4 confirmed the essential role of hydrogenotrophic methanogen in acidogenic reactor as a hydrogen consumer. Meanwhile, with low population of hydrogenotrophic methanogens in acidogenic reactor, even at a more favorable pH of 5.5, the HAc (2.14 ± 0.48 gCOD/L) and VFA concentration (8.90 ± 2.15 gCOD/L) was not as high as the HAc (4.14 ± 0.12 gCOD/L) and VFA concentration (12.02 ± 0.40 gCOD/L) at pH of 4.5 with external provider of hydrogenotrophic methanogens. These results demonstrated that other than pH, hydrogenotrophic methanogens could also have affected performance of the acidogenic reactor.

Nevertheless, the objective of the present study is not to challenge importance of pH in acidogenic reactor performance. pH still is an important operating parameter in acidogenesis. And it was also noted that though more HAc was produced after Connection, the recovered HAc concentration (4.14 gCOD/L) in Chapter 5 was not as high as that achieved in Chapter 4 (7.0 gCOD/L). Such difference could be caused by the different pH applied to the acidogenic reactor. Low pH could still adversely affect the acidogenesis process (Yu and Fang, 2002, 2003).

5.5 Conclusions

The role of hydrogenotrophic methanogen in the acidogenic reactor is further confirmed by the incorporation of an external provider of hydrogenotrophic methanogen (R_H) to an acidogenic reactor under hydrogen stress (R_A at pH of 4.5). With hydrogen consumed by R_H , HAc and VFA production improved greatly leading to better performance of not only the acidogenic reactor but also the entire anSBR system. The study also provided an alternative method to alleviate

the hydrogen stress to an acidogenic reactor in which hydrogenotrophic methanogen is inhibited inside the reactor.

CHAPTER 6 CONCLUSIONS AND RECOMMENDATIONS

6.1 Major findings

In the present study, it was showed that methanogens could exist and be active in the acidogenic reactor of the two-phase anSBR. However, the microbial profile of the microbes demonstrated that methanogens in the acidogenic reactor was more vulnerable than the bacteria in the acidogenic reactor and the methanogens in the methanogenic reactor.

With a sharp decrease in pH in the acidogenic reactor, methanogens, especially hydrogenotrophic methanogens (Methanobacteriales), underwent significant decrease in population and this caused substantial increase of hydrogen in the biogas. With high content of hydrogen in the gaseous phase, acidogenesis was weakened, as indicated by low acetic acid and VFA production in the acidogenic reactor. It was also shown that the weakened VFA production was mainly caused by the decrease in hydrogenotrophic methanogens which in turn confirmed the important role of hydrogenotrophic methanogens in the acidogenic reactor.

Further, external hydrogenotrophic methanogens were also shown to be capable of alleviating the hydrogen stress to an acidogenic reactor where internal hydrogenotrophic methanogens in the acidogenic reactor had been inhibited.

Figure 6.1 is derived from above findings to represent the possible major pathways in the acidogenic reactor. From the acidogenic reactor, HAc was most desirable product. But since this HAc eventually will be converted to methane in methanogenic reactor, methane could also be desirable in acidogenic reactor, especially when it could help to improve the overall performance of the whole anaerobic system by allowing more HAc production in the acidogenesis phase. Therefore, keeping a proper methanogenic activity internally or externally for the

acidogenic reactor is essential to guarantee an efficient performance of overall two-phase anaerobic system.

Figure 6.1 Major processes occurring in acidogenic reactor.

6.2 Recommendations

In the present study, it was confirmed that hydrogenotrophic methanogens played an essential role in regulating hydrogen and VFA production in the acidogenic reactor. However, as noted from the literature review and in Chapter 5, pH still is a key operating parameter as it not only affected methanogens but also other microbes in the acidogenic reactor, e.g., acidogens, acetogens and homoacetogens. Though these microbes are more robust and more versatile in their roles in the acidogenic reactor, they are the microbes that convert the larger organic matters into smaller VFAs.

For example, for the consumption of hydrogen, it can be achieved by methanogenesis, but can also be achieved by acetogenesis by homoacetogens (eqn. 6 and 7). As is shown in eqn. 6 and 7, methanogenic hydrogen utilization

pathway yields more energy than homoacetogenic hydrogen oxidation pathway at standard conditions. But homoacetogens, due to their metabolic versatility, can have a competitive advantage over hydrogenotrophic methanogens in certain conditions, such as slightly acidic and low temperature (lower than 20 °C) environment (Pohland and Ghosh, 1971). In the present study, process temperature was higher than 30 °C which favored the hydrogenotrophic methanogens. Moreover, a low acetate concentration must be maintained to facilitate the homoacetogenic pathway because at 10 Pa of H₂ and 10 µM acetate, homoacetogenic hydrogen oxidation yields a ΔG of -26 kJ per mol of acetate (Pohland and Ghosh, 1971), which is not the case in the present acidogenic reactor. In this sense, such pathway can be ignored in the present study.

Nonetheless, there are other pathways which cannot be ignored. For example, the oxidization of acetate by acetate-oxidizing bacteria was made possible by the presence of a population of their syntrophic partners – hydrogenotrophic methanogens (eqn. 11). However, although this pathway produce hydrogen from acetate, it is not likely to affect the hydrogen production from the acidogenic reactor because once the hydrogen is produced, it would be consumed instantly by hydrogenotrophic methanogens to produce methane. Therefore, some of the methane produced in the acidogenic reactor might have used this pathway. But further investigation is needed to confirm this assumption.

There is another question which had remained unanswered in the present study. In Chapter 5, unexpected microbial changes in the R_A were observed when the gaseous phase of R_A and R_H were connected. Hydrogenotrophic methanogens

were inhibited at low pH before Connection but increased in population after Connection while pH remained unchanged. Performance-wise, the changes observed in R_A were the disappearance of hydrogen, appearance of methane and increase in VFA production. Thermodynamically, hydrogenotrophic methanogenesis is not encouraged by low hydrogen presence and high methane presence (eqn. 6). However, it was reported low hydrogen presence could promote a syntrophic reaction which involved hydrogenotrophic methanogenesis -- the syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis (SAO-HM) pathway (Hao *et al.*, 2013). This pathway is a combination of eqn. 6 and eqn. 11 (eqn. 12). Though the formation of eqn. 12 is exactly the same as eqn. 10, the microbes involved in these two equations are completely different. Eqn. 10 is performed by acetotrophic methanogens alone but eqn. 12 is conducted by the combination of the acetate oxidation acetogen and hydrogenotrophic methanogen. With lower hydrogen content in the biogas of R_A , hydrogenotrophic methanogenesis is enhanced with promotion of pathway SAO-HM. This pathway might explain the increased population of hydrogenotrophic methanogens. Moreover it might also contribute to the extra methane produced after Connection from R_{AH} as discussed in Section 5.4.1, Chapter 5. However, such assumption also needs further investigation.

REFERENCE

APHA (1998), Standard Methods for the Examination of Water and Wastewater, American Public Health Association, Washington DC.

Bialek, K., Kim, J., Lee, C., Collins, G., Mahony, T., *et al.* (2011), "Quantitative and qualitative analyses of methanogenic community development in high-rate anaerobic bioreactors", Water Research, Vol. 45, No. 3, pp. 1298-1308.

Burgoon, P. S., Kadlec, R. H. and Henderson, M. (1999), "Treatment of potato processing wastewater with engineered natural systems", Water Science and Technology, Vol. 40, No. 3, pp. 211-215.

Chan, Y. J., Chong, M. F., Law, C. L. and Hassell, D. G. (2009), "A review on anaerobic-aerobic treatment of industrial and municipal wastewater", Chemical Engineering Journal, Vol. 155, No. 1-2, pp. 1-18.

Chrobak, R. and Ryder, R. (2005), "Comparison of anaerobic treatment alternatives for brandy distillery process water", Water Science and Technology, Vol. 51, No. 1, pp. 175-181.

Chung, Y. C., Jung, J. Y., Ahn, D. H. and Kim, D. H. (1998), "Development of two phase anaerobic reactor with membrane separation system", Journal of Environmental Science and Health Part a-Toxic/Hazardous Substances & Environmental Engineering, Vol. 33, No. 2, pp. 249-261.

Cohen, A., Breure, A. M., van Andel, J. G. and van Deursen, A. (1980), "Influence of phase separation on the anaerobic digestion of glucose--I maximum COD-turnover rate during continuous operation", Water Research, Vol. 14, No. 10, pp. 1439-1448.

Cohen, A., Breure, A. M., van Andel, J. G. and van Deursen, A. (1982), "Influence of phase separation on the anaerobic digestion of glucose--II: Stability,

and kinetic responses to shock loadings", Water Research, Vol. 16, No. 4, pp. 449-455.

Cohen, A., Zoetemeyer, R. J., Vandeursen, A. and Vanandel, J. G. (1979), "Anaerobic Digestion of Glucose with Separated Acid Production and Methane Formation", Water Research, Vol. 13, No. 7, pp. 571-580.

Conrad, R. (1999), "Contribution of hydrogen to methane production and control of hydrogen concentrations in methanogenic soils and sediments", Fems Microbiology Ecology, Vol. 28, No. 3, pp. 193-202.

Cooney, M., Maynard, N., Cannizzaro, C. and Benemann, J. (2007), "Two-phase anaerobic digestion for production of hydrogen-methane mixtures", Bioresource Technology, Vol. 98, No. 14, pp. 2641-2651.

Demirel, B. and Scherer, P. (2008), "The roles of acetotrophic and hydrogenotrophic methanogens during anaerobic conversion of biomass to methane: a review", Reviews in Environmental Science and Biotechnology, Vol. 7, No. 2, pp. 173-190.

ElFadel, M., Findikakis, A. N. and Leckie, J. O. (1997), "Environmental impacts of solid waste landfilling", Journal of Environmental Management, Vol. 50, No. 1, pp. 1-25.

EurObserv'er (2010), "Biogas Barometer", Syst Solaires Le Journal des Energies Renouvelables, Vol. No. 200, pp. 104-119.

Fennell, D. E., Gossett, J. M. and Zinder, S. H. (1997), "Comparison of butyric acid, ethanol, lactic acid, and propionic acid as hydrogen donors for the reductive dechlorination of tetrachloroethene", Environmental Science & Technology, Vol. 31, No. 3, pp. 918-926.

Fey, A. and Conrad, R. (2000), "Effect of temperature on carbon and electron flow and on the archaeal community in methanogenic rice field soil", Applied and Environmental Microbiology, Vol. 66, No. 11, pp. 4790-4797.

Fukuzaki, S., Nishio, N., Shobayashi, M. and Nagai, S. (1990), "Inhibition of the Fermentation of Propionate to Methane by Hydrogen, Acetate, and Propionate", Applied and Environmental Microbiology, Vol. 56, No. 3, pp. 719-723.

Guo, X. M., Trably, E., Latrille, E., Carrere, H. and Steyer, J. P. (2010), "Hydrogen production from agricultural waste by dark fermentation: A review", International Journal of Hydrogen Energy, Vol. 35, No. 19, pp. 10660-10673.

Hao, L. P., Lu, F., Shao, L. M. and He, P. J. (2013), "Improving the performance of thermophilic anaerobic digester through recirculation of low hydrogen biogas", Journal of Environmental Science and Health Part a-Toxic/Hazardous Substances & Environmental Engineering, Vol. 48, No. 11, pp. 1431-1436.

Hattori, S. (2008), "Syntrophic acetate-oxidizing microbes in methanogenic environments", Microbes and Environments, Vol. 23, No. 2, pp. 118-127.

Huang, W. H., Wang, Z. Y., Zhou, Y. and Ng, W. J. (2014), "The role of hydrogenotrophic methanogens in an acidogenic reactor", Chemosphere, Vol. 140, No. SI, pp. 40-46.

Ince, O. (1998), "Performance of a two-phase anaerobic digestion system when treating dairy wastewater", Water Research, Vol. 32, No. 9, pp. 2707-2713.

Kim, J., Shin, S. G., Han, G., O'Flaherty, V., Lee, C., *et al.* (2011), "Common key acidogen populations in anaerobic reactors treating different wastewaters: molecular identification and quantitative monitoring", Water Research, Vol. 45, No. 8, pp. 2539-2549.

Kim, W., Lee, S., Shin, S. G., Lee, C., Hwang, K., *et al.* (2010), "Methanogenic community shift in anaerobic batch digesters treating swine wastewater", Water Research, Vol. 44, No. 17, pp. 4900-4907.

Kisaalita, W. S., Pinder, K. L. and Lo, K. V. (1987), "Acidogenic Fermentation of Lactose", Biotechnology and Bioengineering, Vol. 30, No. 1, pp. 88-95.

Klocke, M., Nettmann, E., Bergmann, I., Mundt, K., Soudi, K., *et al.* (2008), "Characterization of the methanogenic Archaea within two-phase biogas reactor systems operated with plant biomass", Systematic and Applied Microbiology, Vol. 31, No. 3, pp. 190-205.

Lee, C., Kim, J., Hwang, K., O'Flaherty, V. and Hwang, S. (2009), "Quantitative analysis of methanogenic community dynamics in three anaerobic batch digesters treating different wastewaters", Water Research, Vol. 43, No. 1, pp. 157-165.

Lee, J. C., Kim, J. H., Chang, W. S. and Pak, D. (2012), "Biological conversion of CO₂ to CH₄ using hydrogenotrophic methanogen in a fixed bed reactor", Journal of Chemical Technology and Biotechnology, Vol. 87, No. 6, pp. 844-847.

Lema, J. M., Mendez, R. and Blazquez, R. (1988), "Characteristics of Landfill Leachates and Alternatives for Their Treatment - a Review", Water Air and Soil Pollution, Vol. 40, No. 3-4, pp. 223-250.

Li, Y. C., Zhu, J., Wu, X. A., Miller, C. and Wang, L. A. (2010), "The Effect of pH on Continuous Biohydrogen Production from Swine Wastewater Supplemented with Glucose", Applied Biochemistry and Biotechnology, Vol. 162, No. 5, pp. 1286-1296.

Liu, W. T., Chan, O. C. and Fang, H. H. P. (2002a), "Characterization of microbial community in granular sludge treating brewery wastewater", Water Research, Vol. 36, No. 7, pp. 1767-1775.

Liu, W. T., Chan, O. C. and Fang, H. H. P. (2002b), "Microbial community dynamics during start-up of acidogenic anaerobic reactors", Water Research, Vol. 36, No. 13, pp. 3203-3210.

Madigan, M. T. and Brock, T. D. (2009), Biology of microorganisms (12th edition), San Francisco, CA : Pearson/Benjamin Cummings,

McCarty, P. L. (2001), "The development of anaerobic treatment and its future", Water Science and Technology, Vol. 44, No. 8, pp. 149-156.

Mizuno, O., Li, Y. Y. and Noike, T. (1998), "The behavior of sulfate-reducing bacteria in acidogenic phase of anaerobic digestion", Water Research, Vol. 32, No. 5, pp. 1626-1634.

Mosey, F. E. (1983), "Mathematical-Modeling of the Anaerobic-Digestion Process - Regulatory Mechanisms for the Formation of Short-Chain Volatile Acids from Glucose", Water Science and Technology, Vol. 15, No. 8-9, pp. 209-232.

Mu, Y., Yu, H. Q. and Wang, Y. (2006), "The role of pH in the fermentative H-2 production from an acidogenic granule-based reactor", Chemosphere, Vol. 64, No. 3, pp. 350-358.

O'Reilly, J., Lee, C., Collins, G., Chinalia, F., Mahony, T., *et al.* (2009), "Quantitative and qualitative analysis of methanogenic communities in mesophilically and psychrophilically cultivated anaerobic granular biofilms", Water Research, Vol. 43, No. 14, pp. 3365-3374.

Pohland, F. G. and Ghosh, S. (1971), "Development in anaerobic stabilization of organic wastes. The two- phase concept", Environmental Letters, Vol. 1, No. 4, pp. 255-266.

Pohland, F. G. and Mancy, K. H. (1969), "Use of Ph and Pe Measurements during Methane Biosynthesis", Biotechnology and Bioengineering, Vol. 11, No. 4, pp. 683-&.

Rajbhandari, B. K. and Annachhatre, A. P. (2004), "Anaerobic ponds treatment of starch wastewater: case study in Thailand", Bioresource Technology, Vol. 95, No. 2, pp. 135-143.

Raskin, L., Stromley, J. M., Rittmann, B. E. and Stahl, D. A. (1994), "Group-Specific 16s Ribosomal-Rna Hybridization Probes to Describe Natural Communities of Methanogens", Applied and Environmental Microbiology, Vol. 60, No. 4, pp. 1232-1240.

Raskin, L., Zheng, D. D., Griffin, M. E., Stroot, P. G. and Misra, P. (1995), "Characterization of microbial communities in anaerobic bioreactors using molecular probes", Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology, Vol. 68, No. 4, pp. 297-308.

Ren, N. Q., Wang, B. Z. and Huang, J. C. (1997), "Ethanol-type fermentation from carbohydrate in high rate acidogenic reactor", Biotechnology and Bioengineering, Vol. 54, No. 5, pp. 428-433.

Schink, B. (1997), "Energetics of syntrophic cooperation in methanogenic degradation", Microbiology and molecular biology reviews : MMBR, Vol. 61, No. 2, pp. 262-280.

Scott, J., Beydoun, D., Amal, R., Low, G. and Cattle, J. (2005), "Landfill management, leachate generation, and leach testing of solid wastes in Australia and overseas", Critical Reviews in Environmental Science and Technology, Vol. 35, No. 3, pp. 239-332.

Sekiguchi, Y., Kamagata, Y. and Harada, H. (2001), "Recent advances in methane fermentation technology", Current Opinion in Biotechnology, Vol. 12, No. 3, pp. 277-282.

Shimada, T., Morgenroth, E., Tandukar, M., Pavlostathis, S. G., Smith, A., *et al.* (2011), "Syntrophic acetate oxidation in two-phase (acid-methane) anaerobic digesters", Water Science and Technology, Vol. 64, No. 9, pp. 1812-1820.

Shin, S. G., Han, G., Lim, J., Lee, C. and Hwang, S. (2010a), "A comprehensive microbial insight into two-stage anaerobic digestion of food waste-recycling wastewater", Water Research, Vol. 44, No. 17, pp. 4838-4849.

Shin, S. G., Lee, S., Lee, C., Hwang, K. and Hwang, S. (2010b), "Qualitative and quantitative assessment of microbial community in batch anaerobic digestion of secondary sludge", Bioresource Technology, Vol. 101, No. 24, pp. 9461-9470.

Siriwongrungson, V., Zeny, R. J. and Angelidaki, I. (2007), "Homoacetogenesis as the alternative pathway for H₂ sink during thermophilic anaerobic degradation of butyrate under suppressed methanogenesis", Water Research, Vol. 41, No. 18, pp. 4204-4210.

Stander, G. J. (1950), "Effluents from Fermentation Industries. Part IV. A New Method for Increasing and Maintaining Efficiency in the Anaerobic Digestion of Fermentation Effluents", Journal The Institute of Sewage Purification, Vol. No. part 4, pp. 447.

Sung, S. W. and Dague, R. R. (1995), "Laboratory Studies on the Anaerobic Sequencing Batch Reactor", Water Environment Research, Vol. 67, No. 3, pp. 294-301.

Tchobanoglous, G., Burton, F. L. and Stensel, H. D. (2003), Wastewater engineering : treatment and reuse / Metcalf & Eddy, Inc, Boston : McGraw-Hill,

c2003. 4th ed. / revised by George Tchobanoglous, Franklin L. Burton, H. David Stensel.,

Won, S. G. and Lau, A. K. (2011), "Effects of key operational parameters on biohydrogen production via anaerobic fermentation in a sequencing batch reactor", Bioresource Technology, Vol. 102, No. 13, pp. 6876-6883.

Wu, X. A., Yao, W. Y. and Zhu, J. (2010), "Effect of pH on continuous biohydrogen production from liquid swine manure with glucose supplement using an anaerobic sequencing batch reactor", International Journal of Hydrogen Energy, Vol. 35, No. 13, pp. 6592-6599.

Yu, H. Q. and Fang, H. H. P. (2002), "Acidogenesis of dairy wastewater at various pH levels", Water Science and Technology, Vol. 45, No. 10, pp. 201-206.

Yu, H. Q. and Fang, H. H. P. (2003), "Acidogenesis of gelatin-rich wastewater in an upflow anaerobic reactor: influence of pH and temperature", Water Research, Vol. 37, No. 1, pp. 55-66.

Yu, Y., Kim, J. and Hwang, S. (2006), "Use of real-time PCR for group-specific quantification of acetoclastic methanogens in anaerobic processes: Population dynamics and community structures", Biotechnology and Bioengineering, Vol. 93, No. 3, pp. 424-433.

Yu, Y., Lee, C., Kim, J. and Hwang, S. (2005), "Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction", Biotechnology and Bioengineering, Vol. 89, No. 6, pp. 670-679.

Zaiat, M., Rodrigues, J. A. D., Ratusznei, S. M., de Camargo, E. F. M. and Borzani, W. (2001), "Anaerobic sequencing batch reactors for wastewater treatment: a developing technology", Applied Microbiology and Biotechnology, Vol. 55, No. 1, pp. 29-35.

Zhang, Q. H.,He, J.,Tian, M.,Mao, Z. G.,Tang, L., *et al.* (2011), "Enhancement of methane production from cassava residues by biological pretreatment using a constructed microbial consortium", Bioresource Technology, Vol. 102, No. 19, pp. 8899-8906.