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CHANGES IN MICROBIAL COMMUNITY ASSOCIATED WITH THE DETERIORATION OF METHANE FERMENTATION BY INCREASING ORGANIC LOADING RATE

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Changes in microbial community associated with the deterioration of methane fermentation by increasing organic loading rate

A dissertation
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Chapter 1

Introduction

1.1. BACKGROUND

Recently, the energy crisis and global warming are considered as two severe problems all over the world (Chang et al., 2003). Since the dawn of the industrial revolution, and the fossil fuels have been the driving force behind the industrialized world and its economic growth (Höök and Tang, 2013). At present, about 80% of all primary energy in the world is derived from fossil fuels with oil accounting for 32.8%, coal for 27.2%, and natural gas for 20.9% (IEA, 2011). On the contrary, combustible biomass and waste (10.2%), nuclear power (5.8%), hydroelectric dams (2.3%), and other alternative energy sources such as geothermal, wind, solar and other sources (0.8%) are the largest contributors to the global energy system after fossil energy, but they account for only a minor share of the global primary energy supply (IEA, 2011). Likewise, the energy is mostly dependent on fossil fuels in the present. However, when the fossil fuels are consistently used like nowadays, it can lead to the depletion of fossil fuels and the environmental crisis. Climate change such as global warming is one of the most serious environmental problems by the usage of fossil fuels. Especially, the concentration of carbon dioxide in the atmosphere will continue rising unless major changes are made in the way, the fossil fuels are used to provide energy (Hoffert et al., 1998). Global warming is a problem in which the combustion of coal, oil and other fossil fuels cause the increase in the atmospheric concentrations of greenhouse gases (GHGs) such as carbon dioxide and methane. These results mounting global air temperatures that lead to climate changes and especially, the global warming will cause a rise in sea levels, the changes in the rainfall patterns, and the climate change (Vijaya VenkataRaman et al., 2012). Both carbon dioxide and methane are significant greenhouse gases and their presence in the upper atmosphere decrease irradiative heat losses from the earth's surface, effectively trapping heat, which may result in a warming of the plant and cause the climate changes. Therefore, it is absolutely necessary to reduce the use of fossil fuels and find the resources that can be replaced to the fossil fuels. It is necessary to apply biomass in the production of alternative energy to solve the energy and environmental crisis. Because, biomass resources

include various natural and waste-derived materials mainly categorized as agricultural residues, wood and wood wastes, animal dung, sludge, municipal solid waste. In other words, demand-supply of biomass resources is not limitative like fossil fuel materials. Besides, biomass energy can play an important role in reducing greenhouse gas emissions, since when produced and utilized in a sustainable way, the use of biomass for energy offsets fossil fuel greenhouse gas emissions (Hoogwijk et al., 2003). Hall et al. (1997) reported that biomass has the potential to become one of the major global energy sources and modernized bioenergy systems are suggested to be important contributors to future sustainable energy systems and sustainable energy systems. The contribution of biomass to global energy will fluctuate from 100 to 400 EJ $year^{-1}$ which is about 15% of the global primary energy supply (Berndes et al., 2003) and Hoogwijk et al. (2003) reported that biomass energy remains the primary source of energy for more than half the world's population, and accounts for 14% of the total energy consumption in the world.

Biomass energy technologies can produce such as ethanol, methanol, and methane. Among them, methane is easy to produce and emits few atmospheric pollutants, while other fuels such as ethanol and methanol are not well developed commercially for production and are technically more difficult to be produced from biomass (Chynoweth et al., 2001). Anaerobic treatment technology for producing methane is ideal treatment for biomass among diverse technologies. Sekiguchi et al. (2001) reported that methane fermentation does not need aeration and produces methane, hence it is a cost-effective energy-yielding process. Moreover, methanogenic reactions in methane fermentation process produce less excess sludge than aerobic treatment system and the energy stored within organic matters can be recovered as biogas. Likewise, methane fermentation technology has a lot of advantages and is an attractive method; therefore, this technology should be widely accepted in the worldwide. Table 1.1 represents the advantages and disadvantages of methane fermentation. The application of methane fermentation has the environmental benefit of reducing the potential for global warming. The carbon dioxide emitted from burning the biogas comes from the carbon in the organic wastes, which ultimately came from the atmosphere, and is part of a closed carbon cycle; therefore, it does not contribute to increasing atmospheric carbon dioxide levels. Moreover, methane fermentation can reduce potential global warming by reducing methane emissions because, by applying methane fermentation to biomass and capturing and utilize the biogas, emission of methane to the atmosphere and the greenhouse

effects of methane are avoided (Wilkie, 2005). While methane fermentation has some disadvantages such as installation of high cost, professional skill for operation.

Table 1. 1. Advantages and disadvantages of methane fermentation (Kangle et al., 2012)

Advantage	**Disadvantage**
Generation of biogas using diverse biomass	Requirement of initial installation cost
Reduction of greenhouse gas emissions	Control of sensitive microorganisms
Combined treatment of diverse organic wastes and wastewaters	Experts are required for the design, construction, operation, and maintenance
Reduction of pathogen	
Reduction of sludge generation	

So far, extensive researches have been carried out to investigate the operational conditions of methane fermentation with the consideration of diverse factors. Those researches investigated about substrate concentration (Sánchez et al., 2001), hydraulic retention time (Sasaki et al., 2006), organic loading rate (Patel and Madamwar, 2002), pH (Zhang et al., 2005), volatile fatty acid (Wang et al., 2009) and trace metals (Qiang et al., 2013). Especially, the increase of organic loading rate can induce the large amount of methane production; therefore the high organic loading rate is attractive and important for methane production. However, sometimes methane production suddenly diminishes with increasing organic loading rate. Previous studies reported that high organic loading rate caused the deterioration of methane fermentation (Patel and Madamwar, 2002; Teerachark and Rachdawong, 2012) but they did not clarify the effect of organic loading rate on the deterioration of methane fermentation by considering the deterioration mechanism. Moreover, so far it has not been studied what happens in methane fermentation by the increase of organic loading rate. Therefore, it is urgently required to study about the relationship between the performance of the methane fermentation and organic loading rate for successful methane fermentation. Besides, it is important to clarify the mechanism of methane fermentation by changes of microbial community because methane fermentation is a complex microbial system. Previous studies which addressed the microbial community structures in methane-

producing reactors fed with substrates such as acetate (Shigematsu et al., 2003), propionate (Shigematsu et al., 2006), butyrate (Tang et al., 2007), and bovine serum albumin (Tang et al., 2005). However, the knowledge about the changes of microbial community with deterioration of methane fermentation still rather limited even though it is necessary to understand the deterioration mechanism for successful methane fermentation.

In the present study, an investigation of the deterioration mechanism of methane fermentation with increasing organic loading rate was carried out during the pilot-scale and the laboratory-scale methane fermentation processes. Moreover, changes in microbial community associated with the deterioration of methane fermentation were also elucidated.

1.2. GOAL AND OBJECTIVES OF DISSERTATION

The goal of this study is to elucidate the deterioration mechanism of methane fermentation by physico-chemical analyses and biological analyses during methane fermentation.

To achieve the overall objective, several specific objectives are taken into account in this study as follows:

1. To investigate the deterioration mechanism of methane fermentation treating syrup wastewater with increasing organic loading rate.

2. To determine the effects of hydraulic retention time and substrate concentration on the deterioration of methane fermentation when organic loading rate of the syrup wastewater into the reactor increased with the progress of fermentation.

3. To ascertain the deterioration mechanism of methane fermentation using peptone as a substrate instead of syrup wastewater for generalization of the deterioration mechanism of methane fermentation.

The detailed discussion of each specific objective is consecutively presented in the following chapters 3, 4, and 5.

1.3. THE OUTLINE OF DISSERTATION

CHAPTER 1: INTRODUCTION

This chapter represents a statement of global concern on the energy consumption and environmental crisis with their solutions. Besides, an alternative source as biomass and sustainable energy system as methane fermentation process were briefly introduced with the necessities of the study. Furthermore, the significant goal and the specific objective of the dissertation as the investigation of the deterioration mechanism of methane fermentation stated.

CHAPTER 2: LITERATURE REVIEW

This chapter reviews the literature on the mechanisms of methane fermentation processes, the role of microbial activity during methane fermentation, the adaptable types of biomass and various types of methane fermentation reactors were discussed. Furthermore, important parameters and inhibiting factors in the operation of methane fermentation were also reviewed.

CHAPTER 3: IDENTIFICATION OF MICROORGANISMS IN THE GRANULES GENERATED DURING METHANE FERMENTATION OF THE SYRUP WASTEWATER

In this chapter, the deterioration mechanism of methane fermentation was elucidated when the organic loading rate of the syrup wastewater into the reactor increased with the progress of fermentation. A commercial-scale reactor was operated during methane fermentation and the microbial community was detected by the PCR-DGGE and real-time PCR methods. The characteristic microorganisms were identified through a comparison between the performances and the microbial community.

CHAPTER 4: EFFECTS OF OPERATION CONDITIONS ON THE PERFORMANCE OF THE ASBR METHANE FERMENTATION OF SYRUP WASTEWATER

This chapter aimed at clarifying the effects of hydraulic retention time and substrate concentration on the performance of the methane fermentation using the syrup wastewater from fruit canning process. The effects of hydraulic retention time and substrate concentration simultaneously were evaluated in the deterioration of methane fermentation.

CHAPTER 5: RELATIONSHIP BETWEEN THE CHANGES IN MICROBIAL COMMUNITY AND THE DETERIORATION OF METHANE FERMENTATION WHICH TREATS SYNTHETIC PEPTONE WASTEWATER

In this chapter, ascertainment the deterioration mechanism of methane fermentation with peptone as a substrate for the generalization of the deterioration mechanism in methane fermentation.

CHAPTER 6: GENERAL CONCLUSIONS

With the results obtained in chapters 3, 4, and 5, the general conclusions associated with the clarification of the deterioration mechanism of methane fermentation with the changes of microbial community during methane fermentation process were presented together with the recommendation for further studies in this chapter.

Chapter 2

Literature review

This chapter represents the comprehensive review on the present strong demand for alternative energy and clean energy produced in the methane fermentation. The mechanisms of overall methane fermentation processes were reviewed. Moreover, the adaptable types of wastes in methane fermentation and various types of methane fermentation reactors were discussed. Lastly, overviews on the important parameters and inhibiting factors in methane fermentation were described in this chapter.

2.1. METHANE FERMENTATION

Methane fermentation is the degradation of biodegradable organic materials by microorganisms without oxygen. It is multi-step biological processes where organic carbon is mainly converted to the methane and carbon dioxide. The processes can be divided to four steps; hydrolysis, acidogenesis, acetogenesis and methanogenesis. Figure 2.1 shows the process flow of the degradation of organic material through methane fermentation and table 2.1 represents the types of microorganisms involved in each step of methane fermentation.

2.1.1. Hydrolysis

Complex polymers cannot be directly utilized by fermentative microorganisms (Kangle et al., 2012). During the hydrolysis step, complex polymers such as carbohydrates, proteins and lipids are hydrolyzed by extra-cellular enzymes into products such as sugars, amino acids and fatty acids (Parawira et al., 2005). Proteases, secreted by proteolytic microbes, convert proteins into amino acids, hydrolyze cellulose and xylose (both complex carbohydrates) into glucose (sugar); lipases, created by lipolytic microbes, convert lipids (fats) into long-chain fatty acids and glycerol (Salminen and Rintala, 2002). Hydrolysis is a rather slow and energy-consuming process and is considered as the overall rate-limiting step for the methane fermentation of complex polymers (McCarty and Mosey, 1991; Gallert and Winter, 1999). When the substrate as complex polymer is readily degradable, the rate-limiting step will be acetogenesis and methanogenesis (Björnsson et al., 2000).

2.1.2. Acidogenesis

In the acidogenesis step, the soluble organic molecules produced by the hydrolysis are utilized by fermentative bacteria or anaerobic oxidizer (Garcia-Heras, 2003). Acidogenesis involves the conversion of the sugars, amino acids and fatty acids to hydrogen, acetate, carbon dioxide, volatile fatty acids and lactic acid by anaerobic bacteria. This degradation pathway gives energy for the microorganisms and the products can be utilized by methanogenic microorganisms (Schink, 1997).

2.1.3. Acetogenesis

Acetogenesis is the conversion of products such as volatile fatty acids with more than two carbon atoms, alcohols and aromatic fatty acids into acetate and hydrogen by hydrogen-producing acetogenic bacteria (Boe, 2006). In acetogenesis, the acetate is mainly created.

2.1.4. Methanogenesis

In methanogenesis step, finally, acetate, carbon dioxide and hydrogen are converted to methane and carbon dioxide by archaea. Archaea are able to grow on acetate, carbon dioxide, hydrogen and one-carbon compound (Schink, 1997). Acetate is converted into methane and carbon dioxide in acetoclastic methanogenesis, and hydrogen and carbon dioxide are converted into methane. In this step, approximately 70% of methane is produced from acetate while the remaining 30% is produced from carbon dioxide and hydrogen (Klass, 1984).

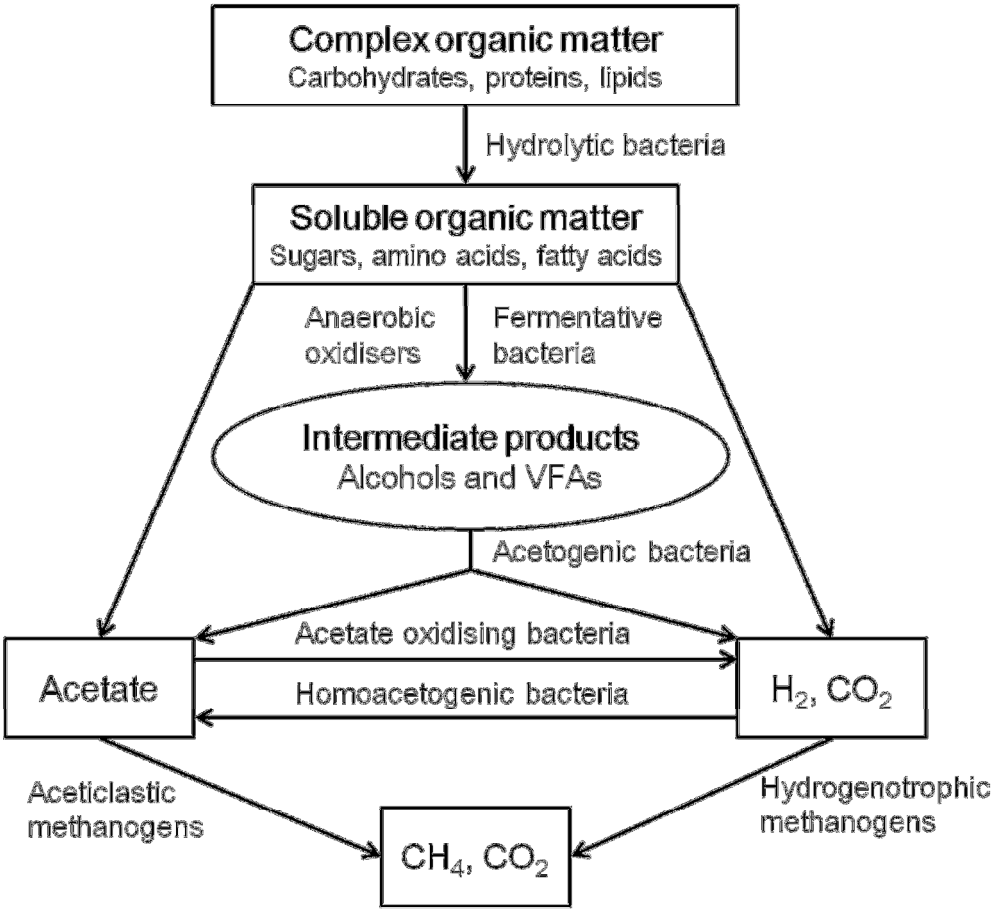

Figure 2. 1. The process flow of the degradation of organic material through methane fermentation.

Table 2. 1. Types of microorganisms involved in each step of methane fermentation (Nayono, 2010).

Process	Group	Type of conversion	Microorganisms
Hydrolysis	Hydrolytic bacteria	Proteins to soluble peptides and amino acids	*Clostridium, Proteus vulgaris, Peptococcus, Bacteriodes, Bacillus, Vibrio*
		Carbohydrates to soluble sugars	*Clostridium, AcetovibrioCelluliticus, Staphylococcus, Bacteriodes*
		Lipids to higher fatty acids or alcohols and glycerol	*Clostridium, Micrococcus, Staphylococus*
Acidogenesis	Acidogenic bacteria	Amino acids to fatty acids, acetate and NH_3	*Lactobacillus, Escherichia, Staphylococcus, Bacillus, Pseudomonas, Sarcina, Veillonella, Streptococcus, Desulfobacter, Desulforomonas*
		Sugars to intermediary fermentation products	*Clostridium, Eubacteriumlimosum, Streptococus*
Acetogenesis	Acetogenic bacteria	Higher fatty acids or alcohols to hydrogen and acetate	*Clostridium, Syntrophomonaswolfeii*
		Volatile fatty acids and alcohols to acetate or hydrogen	*Sytrophomonaswolfei, Syntrophomonaswolinii*
Methanogenesis	Carbon dioxide-reducing methanogens	Hydrogen and carbon dioxide to methane	*Methanobacterium, Methanobrevibacterium, Methanoplanus, Methanospirillum*
	Aceticlastic methanogens	Acetate to methane and carbon dioxide	*Methanosaeta, Methanosarcina*

2.2. MICROBIAL COMMUNITY COMPOSITION IN METHANE FERMENTATION PROCESS

To achieve an understanding of methane fermentation, knowledge of microbial community in methane fermentation is fundamental because methane fermentation is the consequence of a series of metabolic interactions among various groups of microorganisms. Important microorganisms in methane fermentation are shown below.

2.2.1. Bacteria

Diverse microbial constituents belonging to various bacterial phyla have been detected in anaerobic sludge used to treat various wastewaters under different conditions. Typical 16S rRNA genes frequently detected in these anaerobic sludges are those of the bacterial phyla Proteobacteria, Firmicutes, and Chloroflexi as shown in table 2.2 (Sekiguchi and Kamagata, 2004).

2.2.1.1. Proteobacteria

Important gram-negative sulfate-reducers and syntrophic bacteria belong to the delta-subphylum of Proteobacteria (Garrity, 2001). Among them, species of the genus *Syntrophobactor* are particularly well recognized as syntrophic bacteria capable of degrading propionate in association with hydrogenotrophs (Harmsen et al., 1998), because, propionate is one of the most important intermediates in the anaerobic degradation of organic compounds (Sekiguchi and Kamagata, 2004).

16S rRNA gene sequences affiliated with genera *Desulfovibrio*, *Desulfobulbus*, *Syntrophobactor*, *Syntrophus*, and *Smithella* have been frequently retrieved from methanogenic wastewater sludge. Godon et al. (1997) reported the microbial community structure of methanogenic sludge in a fluidized bed reactor treating vinasses, in which a number of clones closely related with species of the genera *Desulfovibrio*, *Syntrophus* and *Smithella* were detected. Clones related with these microorganisms have also been detected at high frequencies in methanogenic sludge used in the treatment of actual and artificial wastewater (Sekiguchi et al., 1998; Liu et al., 2002).

2.2.1.2. Firmicutes

The majority of well-characterized gram-positive heterotrophs, acetogens, and sulfate-reducing bacteria are known to be part of the bacterial phylum Firmicutes which

capable of degrading butyrate and its analogues in syntrophic association with hydrogenotrophic methanogens (Garrity, 2001). A number of diverse 16S rRNA gene clones within phylum Firmicutes have been found in methane fermentation and clones that are relatively close to cultured species such as those of the genera *Clostridium*, *Eubacterium*, *Streptococcus*, and member of the family Syntrophomonadaceae were frequently found in anaerobic sludge (Sekiguchi and Kamagata, 2004).

One of the important features of phylum Firmicutes is that a relatively high number of 16S rRNA gene clones can be retrieved from thermophilic UASB sludge. When methane fermentation was operated by mesophilic and thermophilic conditions, respectively,16S rRNA gene clones affiliated with Deltaproteobacteria, which are the most frequently retrieved clones among all bacterial phyla from the mesophilic anaerobic sludge were not found from the thermophilic sludge while, clones belonging to Firmicutes are more abundant in the thermophilic sludge (Sekiguchi et al., 1998).

2.2.1.3. Chloroflexi

The bacterial phylum Chloroflexi has been recognized as a typical bacterial phylum represented by a number of diverse environmental 16S rRNA gene clones with only a few cultured representatives (Hugenholtz et al., 1998). The phylum contains cultured microbes belonging to genera *Chloroflexus*, *Oscillochloris*, *Herpetosiphon*, *Sphaerobacter*, and *Roseiflexus*. The phylum Chloroflexi clones were found from hot spring environments, sediments, subsurface environments, aerobic and anaerobic wastewater treatment sludge, and contaminated aquifers (Sekiguchi et al., 1998; Juretschko et al., 2002) and are numerically abundant in methane fermentation and that they are important constituents in sludge granules, in that they maintain the granule structure (Sekiguchi et al., 1999).

2.2.1.4. Other bacterial phyla

A variety of 16S rRNA genes belonging to other bacterial phyla have been found to be present in anaerobic processes, suggesting that anaerobic sludge harbors greater microbial diversity than we ever imagined (Table 2.2). Typical examples of these clones in recognized bacterial phyla are those of the phyla Synergistes, Chlorobi, and Nitrospira.

2.2.2. Archaea

Table 2.3 represents the typical 16S rRNA genes frequently detected archaeal population in methane fermentation. Methanogens compared to other heterotrophic bacteria are relatively well-understood microbes in methane fermentation (Garcia et al., 2000). All of the methanogens recognized so far belong to the phylum Euryarchaeota of the domain archaea. In general, almost all of the archaeal 16S rRNA genes retrieved from anaerobic sludge were found to be those of Euryarchaeota. Most of the studies based on cloning analyses of methanogens suggest that the number of genera of predominant methanogens in methane fermentation process is limited (Hiraishi et al., 1995; Plumb et al., 2001) (Table 2.3). The most frequently retrieved archaeal clones are closely related with members of the genera *Methanosaeta* (Kamagata and Mikami, 1991; Kamagata et al., 1992), *Methanosarcina*, *Methanospirillum*, *Methanobacterium*, and *Methanothermobacter* (Wasserfallen et al., 2000). Among 16S rRNA gene clones closely related with *Methanosaeta* spp. are the clones most frequently retrieved from methanogenic processes.

Table 2. 2. Community analyses of bacterial population in methane fermentation by retrieval of 16S rRNA genes (Sekiguchi and Kamagata, 2004).

		No. A	No. B	No. C	No. D	No. E	No. F
	Temperature (ºC)	35	28	37	55	35	35
	Reactor type	AnFB	AnFB	UASB	UASB	UASB	UASB
	Wastewater type	Wine distillation wastewater	Synthetic wastewater containing acetate, methanol, and trichlorobenzene	Synthetic organic wastewater composed of sucrose, propionate, acetate, and yeast extract	Synthetic organic wastewater composed of sucrose, propionate, acetate, and peptone	Synthetic terephthalate wastewater	Synthetic 4-methylbenzoate wastewater
	Reference	Godon et al., 1997	Von Wintzingerode et al., 1999	Sekiguchi et al., 1998	Sekiguchi et al., 1998	Wu et al., 2001	Wu et al., 2001
Phylum Proteo-bacteria	Class Alphaproteobacteria	●	●	○	○	○	○
	Class Betaproteobacteria	●	●	○	○	○	○
	Class Gammaproteobacteria	●	●	○	○	○	●
	Class Deltaproteobacteria	●●	●	●●	○	●●●	●●●

a. AnFB, anaerobic fluidized bed reactor; UASB, upflow anaerobic blanket reactor.

b. Frequency of sequences assigned with a phylogenetic group in percentage of the total number of sequences analyzed: ○, 0%; ○○, 0.1-0.9%; ●, 1-9%; ●●,10-29%; ●●●,>29%.

Table 2. 2. Community analyses of bacterial population in methane fermentation by retrieval of 16S rRNA genes (Sekiguchi and Kamagata, 2004) (continued).

	No. A	No. B	No. C	No. D	No. E	No. F
Phylum Chloroflexi	●	●●	●●	●●	●	●
Phylum Firmicutes	●●●	●●	●	●	○	●
Phylum Spirochaetes	●	●●	●	○	○	○
Phylum Bacteroides	●●	○	●	○	○	●
Phylum Actinobacteria	●	○○	●	○	○	○
Phylum Synergistes	●	○	○○	●	○○	○
Phylum Planctomycetes	●	○	○○	●	○	●
Phylum Chlorobi	○	○○	●	●	○	○
Phylum Nitrospira	○	○	○○	●●	○	○
Phylum Acidobacteria	○	●●	○	○	○	○
Phylum Verrucomicrobia	●	○	○	○	○	○

Table 2. 3. Community analyses of archaeal population in methane fermentation by retrieval of 16S rRNA genes (Sekiguchi and Kamagata, 2004).

	No. 1	**No. 2**	**No. 3**	**No. 4**	**No. 5**	**No. 6**	**No. 7**	**No. 8**	**No. 9**
Temperature (ºC)	35	35	55	37	30	37	37	55	10-14
Reactor type	FB	UASB	ABR	FB	DF	IC	HR	HR	HR
Wastewater type	Wine distillation wastewater	Synthetic terephthalate wastewater	Industrial dye wastewater	Citric acid production wastewater	Milk processing wastewater	Potato processing wastewater	Synthetic wastewater containing VFAs	Molasses wastewater	Synthetic wastewater composed of glucose and peptone
Reference	Godon et al., 1997	Wu et al., 2001	Plumb et al., 2001	McHugh et al., 2003	McHugh et al., 2003	McHugh et al., 2003	McHugh et al., 2003	McHugh et al., 2003	McHugh et al., 2003
Order Methanosarcinales	●●●	●●●	●●●	●●●	●●●	●●●	●●●	●●●	●●●
Order Methanomicrobiales	○	●●	●	○	○	○	○	○	●
Class Methanobacteria	●●●	●●	●	●●●	●●	●●	○	●●●	●●

a. FB, fluidized bed reactor; UASB, upflow anaerobic blanket reactor; ABR, anaerobic buffled reactor; DF, downflow filter reactor; IC, internal circulation reactor primarily based on UASB-type system; HR, hybrid reactor primarily based on UASB-type system.

b. Frequency of sequences assigned with a phylogenetic group in percentage of the total number of sequences analyzed: ○, 0%; ○○, 0.1-0.9%; ●, 1-9%; ●●, 10-29%; ●●●,>29%.

2.3. TYPES OF WASTES IN METHANE FERMENTATION

A waste as a substrate is a carbon source in the biochemical reactions that take place in methane fermentation. When there is not sufficient substrate for microbial growth and maintenance, process performance will become impaired. Likewise, the substrate is one of important factors in methane fermentation. Commonly used wastes as substrates in methane fermentation are shown below.

2.3.1. Municipal wastes

Municipal wastes produced in households and infrastructure facilities, such as those related to trade, services, education, market and municipal wastes are divided into three groups; biodegradable wastes (kitchen refuse, paper, green wastes); combustible wastes (plastics, packages, textile wastes); inert wastes (glass, mineral wastes, metals) (Frac and Ziemiński, 2012). The value of municipal wastes as a potential renewable energy source and biodegradability are high; therefore, there is increasing interests in the methane fermentation of the municipal wastes (Chen et al., 2008). Likewise, municipal wastes are appropriate for methane fermentation but the biodegradable wastes have to be separated before supplying to reactors. Because, municipal wastes contain diverse combustible and inert wastes thus, if methane fermentation operates with non-biodegradable waste, efficiency of operation will be decreased and finally will be deteriorated. For these reasons, it is necessary to operate the separation processing when methane fermentation operates with municipal wastes.

2.3.2. Commercial organic wastes

Commercial organic wastes consist of food waste from restaurants, catering facilities, schools, hospitals, and universities. Commercial catering waste represents a large, easily targetable amount of food waste, which should be seen as a resource. Resource separation and collection from large institutions such as these would be an easier and cheaper way to divert organics from landfill, than source separated collection from households. These commercial organic wastes can be applied effectively in methane fermentation (Monson et al., 2007).

2.3.3. Agricultural wastes

Most of agricultural wastes contain all components necessary for the development of microorganisms, such as carbohydrates, proteins, fats, microelements and vitamins (Frac and

Ziemiński, 2012). Methane fermentation of agricultural wastes has advantages such as elimination of agriculturally hazardous compounds as organic acids from fertilizers, and an increase of fertilizer value owing to the improved digestibility of the compounds and the prevention of contamination thus it is useful materials to produce methane by methane fermentation (Frac and Ziemiński, 2012).

2.3.4. Slaughterhouse wastes

Slaughterhouse wastes are the energy-rich wastes stream of meat industry. Waste from a slaughterhouse arises from different steps of the slaughtering process such as washing of animal, bleeding out, skinning, cleaning of animal bodies, cleaning of rooms, etc. The waste contains blood, particles of skin, meat, excrements and other pollutants. As such, it is attractive material to treat through methane fermentation for the production of methane while operation of methane fermentation is difficult by high production of ammonia and volatile fatty acids (Ek et al., 2011).

2.4. TYPES OF METHANE FERMENTATION REACTORS

Conventional methane fermentation reactors are in batch, semi-continuous or continuous operations. Semi-continuous or continuous operations are preferable as maximum growth rate can be achieved constantly at steady-state by controlling the feed rate. In batch reactor, the steady-state cannot be achieved as the concentrations of components in the reactor are changing with time (Klass, 1984). Choice of reactor type in the continuous operation is determined according to the substrate characteristics, especially particulate solid contents. Solids and slurry wastes are treated in continuous flow stirred tank reactor (CSTR), while soluble organic wastes are treated using upflow anaerobic sludge blanket (UASB), expanded granular sludge bed (EGSB) and anaerobic sequencing batch reactor (ASBR) (Angelidaki et al., 2002). Commonly operated reactors in methane fermentation are shown below.

2.4.1. Batch reactor

The batch reactor is operated by filling the reactor with granule, letting the reactions that take place in the reactor proceed to completion, and then removing some or all of the contents of the reactor. Stirring may or may not be part of the operation of a batch reactor.

Advantages of a batch rector include: ease of operation, absence of mechanical mixing, and high removal efficiency of an individual contaminant.

2.4.2. Continuous flow stirred tank reactor

The CSTR is the most basic methane fermentation reactor. In CSTR, the substrate is fed continuously to a reactor and immediately mixed with granular sludge and substrate. Therefore, the concentrations of effluent and inside reactor are the same. The advantage of CSTR is high mixing ability; thus, the contents of biomass inside the reactor have relatively uniform properties. In CSTR, the biomass is suspended in the liquid and is removed together with the effluent; therefore, it is necessary to operate the CSTR with longer HRTs compared to other type of reactors. Boe (2006) reported that CSTR operated with the HRT in the range of 10 to 20 days could avoid washing out the granule and could keep the methanogenic microorganisms (Boe, 2006).

2.4.3. Upflow anaerobic sludge blanket

The UASB reactor is widely used for treatment of several types of wastewaters (Shastry et al., 2010). In anaerobic system, the flocculation and settling characteristics are important for effective control of physical and chemical conditions of the production of methane (Bal and Dhagat, 2001). In the advantage of UASB, the active biomass in the form of sludge granules is retained in the reactor by direct settling process for achieving high mean cell residence time (MCRT) thereby achieving high cost-effective designs. Among notable disadvantages, it has a long start-up period along with the requirement for a sufficient amount of granular seed sludge for faster startup, and significant wash-out of sludge during the initial phase of the process is likely and the reactor needs skilled operation (Saleh and Mahmood, 2004). The successful operation of the UASB relies on the establishment of a dense sludge bed at the bottom of the reactor. UASB is basically formed by accumulation of the incoming suspended solids and the active microorganisms (Seghezzo et al., 1998).

2.4.4. Expanded granular sludge bed

The EGSB is a modified form of UASB in which a slightly higher superficial liquid velocity is applied (de Man et al., 1988). Because of the higher upflow velocities, mainly granular sludge will be retained in an EGSB system, whereas a significant part of granular sludge bed will be in an expanded or possibly even in a fluidized state in the higher regions of

the bed (Saleh and Mahmood, 2004). As a result, the contact between the wastewater and sludge is excellent in an EGSB.

2.4.5. Anaerobic sequencing batch reactor

The ASBR containing granular biomass have been widely studied for wastewater treatment due to the advantages over continuous process, including better solids retention, efficient operation control (Rodrigues et al., 2003). The ASBR takes place in a single tank in an operational sequence of filling, reacting, settling, and decanting. Main advantages of ASBR comparing to other reactor systems are, relatively easy operation, system flexibility and usage of the same reactor for both reacting and setting of wastewater (Kayranli and Ugurlu, 2011). Moreover, the most important advantage of operating ASBR for wastewater treatment is the maintenance of a high active biomass concentration which enables application of high organic loading rates for high performance (Zupancic et al., 2007). An efficient contact between the substrate and granular sludge is very important for the improvement of the performance and mixing during the reaction step is very important.

2.5. OPERATING PARAMETERS IN METHANE FERMENTATION

The operating parameters of the methane fermentation must be controlled so as to enhance the microbial activity and thus increase the degradation efficiency of methane fermentation. Some of important parameters are discussed in the following section.

2.5.1. pH

The pH value of the digester is an important indicator of the performance and the efficiency of anaerobic digester. The pH levels are different depend on groups of microorganisms participating in methane fermentation. Acidogenic bacteria can perform their decomposition activity when the pH is above 5, and archaea require pH value from 7.5 to 8.0 for their optimal activity. It has been known that the activity of archaea is sensitive in acid concentration of digester. Generally, the optimum pH values are from 5.5 to 8.5 in methane fermentation and the pH will decrease as organic matters undergo acetognensis, then, methanogens rapidly consume those acids, resulting in the increase in pH and digester performances is stabilized (Kangle, et al. 2012).

2.5.2. Temperature

Methane fermentation can be operated at any temperature from psychrophilic to extreme thermophilic temperature. Mesophilic digestion takes place optimally at around 30 to 39 ºC, or at ambient temperatures between 20 and 45 ºC, and thermophilic digestion takes place optimally at around 49 to 57 ºC, or at temperatures up to 70 ºC (Kangle, et al. 2012).

Higher temperature in anaerobic digester can produce more biogas because when methane fermentation operates with increasing temperature, it can increase solubility of organic compounds; increase chemical and biological reaction rates; improve diffusivity of soluble substrate (Boe, 2006). However, thermophilic process is less attractive from the energy point of view since it requires more energy for heating.

2.5.3. Hydraulic retention time

The hydraulic retention time (HRT) is a measure to describe the average time that a certain substrate resides in a digester. A short HRT produces higher biogas per volume owing to the increase of substrate concentration, but less organic matter will be degraded. Although a short HRT is desired for reducing the digester volume, a balance between HRT and the digester volume must be considered to achieve the desired operational conditions (Kangle et al., 2012).

2.5.4. Organic loading rate

The organic loading rate (OLR) is defined as the amount of organic matter supplied to the reactor in a certain time. OLR is a measure of the biological conversion capacity of methane fermentation system. Feeding the substrate to the reactor above its sustainable OLR results in low biogas yield due to the accumulation of inhibiting substances such as fatty acids in the digester slurry (Kangle et al., 2012). Increasing the organic loading leads to higher biogas production, however, extremely high loading induces the failure of methane fermentation.

2.5.5. Nutrients

The nutrients are necessary and important to methanogenic microbial cell growth. It has been demonstrated that macro-nutrients such as carbon, nitrogen, potassium phosphorus, and sulphur (Kayhanian and Rich, 1995) and micro-nutrients such as nickel, cobalt, molybdenum and iron are necessary for optimal growth and methane production because

trace metals stimulate methanogenic activity (Kangle et al., 2012). Furthermore, an appropriate carbon-to-nitrogen ratio (C/N) is essential for successful methane fermentation. It has been known that optimum range of C/N ratio is between 15 and 30. High C/N ratio cause the acid accumulations which decrease the methanogenesis activity, while when C/N ratio is low, nitrogen is converted to ammonium-N readily, resulting in the ammonia accumulation, which is toxic to methanogenic microorganisms.

2.6. INHIBITING FACTORS IN METHANE FERMENTATION

Literature on methane fermentation shows considerable variation in the deterioration/toxicity levels reported for most substances in this section. The major reason for these variations is the complexity of the methane fermentation process where mechanisms such as antagonism, synergism, acclimation, and complex could significantly affect the deterioration of methane fermentation. Table 2.5 shows a summary of deterioration of methane fermentation.

2.6.1. Volatile fatty acid

Volatile fatty acid (VFA) is the most important intermediates in the methane fermentation process, where they are degraded by proton-reducing acetogens in association with hydrogen consuming methanogenic bacteria (Mechichi and Sayadi, 2005). However, the VFA can be toxin to microorganisms. Accumulation of VFA is induced by the process imbalances which can be caused by variation in temperature, organic overloading, toxic compounds, etc (Mechichi and Sayadi, 2005). In such cases, the methanogens are not able to consume the hydrogen and VFA effectively. As a result, the acids accumulate and the pH decreases to such a low value that the hydrolysis and acetogenesis can be inhibited.

The toxicity is due to the increase in the undissociated form of the VFA (Appels et al., 2008). They can flow freely through the cell membrane where they dissociate and hence cause a pH reduction and a disruption of homoeostasis (Bok, 2006). Siegert and Banks (2005) reported that the presences of VFA beyond 3,000 mg L^{-1} have a different effect on the metabolically distinct phases of hydrolysis, acidogenesis and biogas generation. High VFA concentrations inhibit the degradation of microorganisms and can enhance the inhibitory effect of pH on methane production in anaerobic digesters (Rehm et al., 2000).

2.6.2. Ammonia

Ammonia originates from the influent and from the degradation of protein and other compounds such as urea in methane fermentation. There are two forms of ammonia, namely the ammonium ion and free ammonia form and it is generally accepted that ammonia is responsible for the deterioration of methane fermentation. A wide range of inhibiting ammonia concentrations has been reported in the literature, with the inhibitory ammonia concentration that caused the reduction in methane production ranging beyond 1,500 mg L^{-1} (McCarty, 1964; Angelidaki and Ahring, 1994). It has been indicated that free ammonia is strong toxin due to the fact that it can pass through the cell membrane (Chen et al., 2008; Sung and Liu, 2003). An increased temperature has a positive effect on the microbial growth rate but also results in a higher free ammonia concentration. It is found that high concentration of ammonia was more easily inhibited at thermophilic temperatures than at mesophilic temperatures (Sung and Liu, 2003; Hansen et al., 2003).

An increase in pH would result in a higher toxicity level due to the shift to a higher ratio of free to ammonia. The resulting instability of the process often leads to an increase in the large amount of VFA which leads to a decrease in pH and consequently to a lower free ammonia concentration (Chen et al., 2008). As a result, the methane yield is reduced while free ammonia concentration is decreased.

2.6.3. Sulfide

Sulfate is used as an electron acceptor and hence reduced to sulfide by sulfate reducing bacteria (SRB). Two groups of SRB are responsible for the reduction, the incomplete and the complete oxidizers. The first group oxidizes compounds like lactate to acetate and carbon dioxide, whereas the second one converts acetate to carbon dioxide and bicarbonate (Chen et al., 2008).

Inhibition occurs at two different levels: the primary inhibition caused by the competition for substrates from SRB, whereas secondary inhibition is due to the toxicity of sulfides for the different groups of microorganisms. The inhibitory sulfide levels reported in the previous study were in the range of from 100 to 800 mg L^{-1} dissolved sulfide or approximately from 50 to 400 mg L^{-1} undissociated hydrogen sulfide (Parkin et al., 1990). SRB compete with the fermentative, acetogenic or methanogenic archaea for hydrogen, acetate, propionate and butyrate in the digester. Generally, the inhibition through competition does not occur in the first stage of digestion since the SRB are not capable of degrading

biopolymers. They depend on the fermentative microorganisms to degrade these organics thus they can metabolize the degradation products (Rehm et al., 2000; Chen et al., 2008). Nevertheless the acetogenic and the methanogenic microorganisms are affected by the presence of SRB since they compete for the same fermentation product (Appels et al., 2008). Methanogenesis and sulfate reduction can happen simultaneously, but the hydrogenotrophic methanogens are easily undermined by the SRB for hydrogen.

2.6.4. Sodium and potassium

Various cationic elements, including sodium, potassium and other, are found in the digester influent, where they can be released due to the degradation of organic material or with compounds added for pH adjustment (Chen et al., 2008). Although they are required for microbial growth, they can be toxin or inhibition to the activity of the microorganisms when present in high concentrations.

The presence of low concentrations of sodium is essential for the methanogenic archaea, presumably because it is important for the formation of ATP or the oxidation of NADH (Appels et al., 2008). High concentrations of sodium, however, inhibit the activity of the microorganisms and interfere with their metabolism (Feijoo et al., 1995; Chen et al., 2008). The optimal growth conditions of hydrogenotrophic methanogens occur at concentrations of 350 mg L^{-1}. Inhibitory effects initiate at concentrations between 3,500 and 5,500 mg L^{-1} causing a rather moderate inhibition, whereas a concentration of 8,000 mg L^{-1} is strongly inhibitory to methanogenic archaea during the mesophilic digestion (Chen et al., 2008). VFA degrading bacteria have a different resistance to sodium toxicity: it caused 50% inhibition of propionic acid, acetic acid and butyric acid utilizing bacteria at concentrations 10,500, 7,000, and 19,000 mg L^{-1}, respectively (Feijoo et al., 1995). This is in agreement with the results of previous study, which explained that acetate-utilizing bacteria are more susceptible to the toxicity of sodium than propionate-utilizing and hydrogen/carbon dioxide-utilizing microorganisms.

The simultaneous addition of calcium and potassium in suitable concentrations was found to be beneficial in improving the efficiency of the anaerobic treatment process by reducing sodium toxicity to methanogens and potassium and magnesium were also found to be very effective in reducing the toxicity of sodium when present in the optimum concentration (Appels et al., 2008).

High concentrations of potassium can lead to the passive influx of potassium ions, thereby neutralizing the membrane potential (Chen et al., 2008). When the concentration of potassium is below 400 mg L^{-1} functioning in both mesophilic and thermophilic temperature ranges are improved, however, higher potassium levels induce an inhibitory effect for the thermophilic organisms (Kugelman and McCarthy, 1964; Chen et al., 2008). The bacteria can exhibit an acclimation effect, which depends on both concentration of potassium and exposure time. If allowed a sufficient time of exposure, the anaerobic bacteria can acclimate to the toxic cation and their activity is not affected significantly, while beyond a certain level of the toxic cation, the bacteria can no longer tolerate (Appels et al., 2008). Table 2.4 shows the information about the effects of other cations.

2.6.5. Heavy metals

Industrial contributions are the primary source of heavy metals in MSW and account for up to 50% of the total metal content in sewage sludge (Appels et al., 2008). Industrial contaminants include zinc, copper, chromium, nickel, cadmium and lead. Domestic sources are mainly associated with leaching from plumbing materials (Cu and Pb), gutters and roofs (Cu and Zn) and galvanized materials, use of detergents and washing powders containing Cd, Cu and Zn, and use of body care products containing Zn (Appels et al., 2008). The presence of heavy metals can often cause difficulties in the nitrification/denitrification step of the wastewater treatment processes due to inhibition (Juliastuti et al., 2003). Some values of inhibitory concentrations of metals are listed in Table 2.4.

Many enzymes and co-enzymes depend on a minimal amount of certain traces of metals for their activation and activity and when present in large amounts, they cause an inhibitory or toxic effect to micro-organisms (Appels et al., 2008). The chemical binding of heavy metals to the enzymes and subsequent disruption of the enzyme structure and function are the main cause of this toxic effect (Li and Fang, 2007).

Table 2. 4. Inhibitory concentrations of various compounds in methane fermentation (Turovskiy and Mathai, 2006).

Substance	Moderately inhibitory concentration (mg L^{-1})	Strongly inhibitory concentration (mg L^{-1})
Na^+	3,500-5,500	8,000
K^+	2,500-4,500	12,000
Ca^{2+}	2,500-4,000	8,000
Mg^{2+}	1,000-1,500	3,000
NH_4^+	1,500-3,500	3,000
S^{2-}	200	200
Cu^{2+}		0.5 (soluble), 50-70 (total)
Cr^{6+}	10	3.0 (soluble), 200-250 (total)
Cr^{3+}		2.0 (soluble), 180-240 (total)
Ni^{2+}		30 (total)
Zn^{2+}		1.0 (soluble)
Arsenate and arsenite	>0.7	
Cyanide	1-2	
Lead-containing compounds	5	
Iron-containing compounds	>35	
Cupper-containing compounds	1	
Potassium chloride	>10,000	
Chloride	6,000	

2.7. CHANGES IN MICROBIAL COMMUNITY ASSOCIATED WITH THE DETERIORATION OF METHANE FERMENTATION

In case of operating methane fermentation process, focus has most been on reactor performance neglecting the community structure, which is also one of the key factors for successful operation of methane fermentation. Therefore, the investigation of the microbial community structure would facilitate the optimization of methane fermentation operation at different conditions. Especially, the investigation of microbial community associated with the deterioration of methane fermentation is urgently required to maintain the stable conditions and to prevent the deterioration of methane fermentation. In this section, the changes in microbial community associated with the deterioration of methane fermentation by diverse factors are introduced.

Tang et al. (2011) investigated microbial community of thermophilic methanogenic reactor with change of feeding from paper-based waste to garbage stillage. After changing the substrate methane fermentation deteriorated by accumulation of VFAs and ammonium ion. They found that accumulation of VFAs as well as ammonium ion led to the deterioration of microbial activity with dominances of protein degrading and fatty acid degrading bacteria. Akarsubasi et al. (2012) reported that effect of wastewater composition on archaeal population. They observed that accumulation of VFAs caused the deterioration of methane fermentation with the decrease of acetoclastic methanogenic activity when substrate changed from a glucose containing wastewater to a pharmaceutical wastewater.

Abbassi-Guendouz et al. (2013) researched that changes in microbial community with high solid contents and observed that methane fermentation deteriorated at high total solids contents beyond 30%. High total solids contents, methane production was affected by the overall mass transfer limitation and leaded to deterioration of methane fermentation, and methanogenesis was inhibited as a consequence of VFAs accumulation and the enrichment in *Clostridium* sp. was associated to low methanogenic activity. A specific bacterial structure was observed with main dominant bacteria related to *Clotridium* sp., known for their ability to grow at low pH. Additionally, archaeal community was gradually impacted by total solid content and archaeal structure was observed with a gradual shift from *Methanobacterium* sp. to *Methanosarcina* sp., according to the total solid content. The predominance of *Methanosarcina* sp. in methane fermentation was probably due to the resistance of methanogen to high acid concentration, since the total amount of VFA accumulated at total solid of 30%.

Previous study (Ahring et al., 2001) reported that the effect of a temperature increase on process performance and microbial population dynamics. After increasing operating temperature from 55 to 65 °C, the level of VFAs increased with the increase of propionate-degrading bacteria while methanogenic activity decreased significantly and finally methane fermentation deteriorated. This previous study was revealed that the increase of temperature deteriorated the growth balance of microorganisms in methane fermentation.

Niu et al. (2013) studied that effect of ammonia in methane fermentation of chicken manure. Accumulation of VFAs and ammonia caused the deterioration of methane fermentation. After methane fermentation deteriorated, evaluation of the microbial diversity and functional bacteria indicated the increase of dominate phylum of Firmicutes, and aceticlastic *Methanosarcina* was not encountered with coincided phenomenal of high VFAs

in the inhibition stage while hydrogenotrophic *Methanothermobacter* was the dominate archaea in the inhibition stage. From these results, aceticlastic methanogens are more sensitive to ammonia inhibition, thus revealing serious VFA accumulation.

Likewise, previous studies investigated the changes in microbial community with the deterioration of methane fermentation by diverse factors but they did not elucidate the changes in microbial community with the deterioration of methane fermentation by increasing OLR. Therefore, this study investigated the deterioration mechanism of methane fermentation by increasing OLR.

Table 2. 5. A summary of selected works focusing on the deterioration of methane fermentation.

No.	Authors	Raw material	Type of reactor	Temp. (°C)	HRT (day)	OLR	pH (-)	VFAs (g L^{-1})	TAN (g L^{-1})	FAN (g L^{-1})	NH_4^+ (g L^{-1})	Results and comments
1	Siegert and Banks (2005)	Cellulose	Batch	35	-	-	7	>2	-	-	-	The VFA caused the inhibition of the cellulotic activity at concentrations ≥2 g L^{-1}.
2	Siegert and Banks (2005)	Glucose	Batch	35	-	-	7	>8	-	-	-	The glucose fermentation was less sensitive to inhibition caused by VFA.
3	Borja et al. (2004)	Protein	FBR	35	2.8	3 g $CODL^{-1}$ d^{-1}		>1.6 as acetic acid	-	-	-	The high OLR was attributed to an inhibition of methanogen which caused an increase in effluent VFAs.
4	González-Fernández and García-Encina (2009)	Swine slurry	Batch	35	-	-	8	>4	0.8-1.4	0.4-0.7	-	No inhibition by FAN reported. Methane fermentation of swine slurry recommended to carried out at the COD:VS ratio of 1, thereby avoiding reactor imbalances due to VFA accumulation.
5	Lin et al. (2011)	Food waste + Fruit vegetable waste	CSTR	35	-	3 kg VS m^{-3} d^{-1}	7	8,800		30	2,300	The methane fermentation was failed to acids accumulation.
6	Salminen et al. (2002)	Slaughterhouse wastes	CSTR	31	13, 25	2.1 kg VS m^{-3} d^{-1}	6.2	>11.3	1.3-2.7			Accumulation in VFA at high OLR and short HRT.
7	Borja et al. (1996)	Cattle manure	UASB	55	15	-	7.9	1.0-3.0	5.0	0.5	-	The ammonia concentrations of above 5 g L^{-1} inhibited the methane fermentation and the methane production was decreased with the concentration of VFA (1 to 3 g L^{-1} as acetic acid).
8	Angelidaki and Ahring (1993)	Cattle manure	Continuously	55	15	-	7.6	7 as acetic acid	>4.0	0.9	-	The specific methanogenic activity of ammonia inhibited reactors (6 g L^{-1}) with acetate or hydrogen as substrate was reduced by 73% and 52%.
9	Koster and Lettinga (1984)	Tomato juice	UASB	14	-	-	7.6-7.95	>2.5	-	-	1.7	Ammonium ion of above 1g L^{-1} and VFA of above 1.4 g L^{-1} inhibited the methanogenic activity.
10	Liu et al. (2012)	Landfill leachate	EGSB	34-36	0.75	44 kg COD m^{-3} d^{-1}	7.4-7.8	-	>1.5	-	-	The TAN at the concentration of above 1.5 g L^{-1} inhibited the biodegradation.
11	van Velsen (1979)	Sludge	Batch	30		-	7.2-7.4	-	-	-	>5.0	The methane forming organisms was inhibited above ammonia of 5 g L^{-1}.
12	van Velsen (1979)	Piggery manure	Batch	30	-	-	7.2-7.4	-	-	-	>3.0	The methane forming organisms was inhibited above ammonia of 3 g L^{-1}.
13	Poggi-Varaldo (1997)	MSW/Sludge	Semi continuous	39	-	-	8	-	2.8	-	-	The critical ammonia concentration was 2.8 g L^{-1} in methane fermentation.
14	Calli et al. (2005)	Synthetic wastewater	UASB	35	4	1.2 kg COD m^{-3} d^{-1}	7.7-8.1	-	6	0.8	-	The propionate degrading acetogenic bacteria were significantly inhibited above 200 mg L^{-1} of FAN.
15	Cuetos et al. (2008)	Slaughterhouse+organic fraction of MSW	CSTR	34	25	3.7 kg VS m^{-3} d^{-1}	7.5	-	4.1	0.33	-	The FAN concentrations were higher by shorter HRT and higher OLR.

HRT: hydraulic retention time, OLR: organic loading rate, VFAs: volatile fatty acids, TAN: total ammonia nitrogen, FAN: free ammonia nitrogen.

Table 2. 5. A summary of selected works focusing on the deterioration of methane fermentation (continued).

No.	Authors	Raw material	Type of reactor	Temp. (°C)	HRT (day)	OLR	pH (-)	VFAs (g L^{-1})	TAN (g L^{-1})	FAN (g L^{-1})	NH_4^+ (g L^{-1})	Results and comments
16	Duan et al. (2012)	Sewage sludge	Semi continuous	35	2.0-12.8	2.0-12.8 kg VS m^{-3} d^{-1}	8	-	3	0.4	-	The FAN concentration higher than 600 mg L^{-1} was the main factor influencing system stability.
17	Angelidaki and Ahring (1994)	Cattle manure	CSTR	45	15	-	7.4-7.9	-	6	0.7	-	The FAN concentration is below a critical level the result, due to the influence of the temperature on growth rates, would be negative.
18	Sung (2003)	Non-fat dry milk	CSTR	55	7	4 g COD L^{-1} d^{-1}	6.5-8.0	2.7	5.8	-	-	The methanogenic activity at lower TAN concentrations (<1.5 g L^{-1}), higher TAN concentrations (>4.0 g L^{-1}) caused an obvious inhibition of methanogenesis.
19	Nakakubo et al. (2008)	Pig manure	CSTR	51	13.3	9.4 g VS L^{-1} d^{-1}	8	>5.7	-	1.4	11	The isobutyric acid, in particular, accumulated initially by ammonia inhibition even before the yield of methane gas was affected.
20	Hansen et al. (1998)	Swine manure	CSTR	37-60	15	-	8	>4 as acetate	-	1.1	-	The methane yield decreased from 188 (at 37 °C) to 22 (at 60 °C) mL CH_4 g^{-1} VS owing to the increase of FAN. The inhibitory methane fermentation was occurred by ammonia of 1.1 gL^{-1}.
21	Nielsen and Angelidaki (2008)	Cattle manure + digested biomass	CSTR	55	15	-	7.6	-	>10	1.2	-	The FAN concentration was 1.2 g L^{-1} when the inhibition was initiated.
22	Aboueleien et al. (2009)	Chiken manure	Batch	37	-	-	7.3-8.8	1.3-9.4	>8	-	-	The total volume of 4.4 L methane kg^{-1} chicken manure, was produced, despite the presence of a high level of ammonia.
23	Rincón et al. (2008)	Olive mill solid residue	Stirred tank	35	15	11 g COD L^{-1} d^{-1}	5.5	6.2	-	-	-	The excessive production of acids resulted in a decrease of pH which leads to a lower efficiency of the methanogenic process.
24	Palatsi et al. (2011)	Fresh pig and cattle slaughterhouse waste	Batch	35	-	15 g COD kg^{-1}	-	3	0.75	-	-	The increase in LCFA through the increase of OLR brought the inhibition of methane fermentation.
25	Lin et al. (2012)	Food waste	CSTR	35	-	3 kg VS m^{-3} d^{-1}	7	8.8	2.3	-	-	The high VFA by increasing OLR caused the deterioration of methane fermentation.
26	Kundu et al. (2013)	Glucose synthetic wastewater	Hybrid reactor	37	5	10 kg m^{-3} d^{-1}	-	>3	-	-	-	The accumulation of VFA caused the inhibition of methane fermentation owing to high OLR.

Table 2. 5. A summary of selected works focusing on the deterioration of methane fermentation (continued).

No.	Authors	Raw material	Type of reactor	Temp. (°C)	HRT (day)	OLR	pH (-)	VFAs (g L^{-1})	TAN (g L^{-1})	FAN (g L^{-1})	NH_4^+ (g L^{-1})	Results and comments
27	Ziganshin et al. (2013)	Cattle manure + Dried distillers grains with solubles	Biogas reactor	54	24	0.5 g VS L^{-1} d^{-1}	7.21	6.5	1.6	-	-	The accumulation of VFA at high OLR caused the deterioration of methane fermentation.
28	Abbassi-Guendouz et al. (2013)	Leachate of MSW	Batch	35	-	-	6	>30	-	-	-	The methane production was inhibited at high total solids contents (>30%).
29	Williams et al. (2013)	Food waste	CSTR	38	60-100	1 kg VS m^{-3} d^{-1}	7.5	2.3	1.6	-	-	The increases of VFAs and ammonium ion concentrations caused the deterioration of methane fermentation.
30	Sawayama et al. (2004)	Acetate and glucose based medium	Batch	35	-	-	-	-	>1.6	-	-	The methane production decreased at ammonium ion concentration of beyond 1,600 mg L^{-1}.

Chapter 3

Identification of microorganisms in the granules generated during methane fermentation of the syrup wastewater

SUMMARY

The wastewater produced in the process of canning fruit contains a syrup that consists mainly of sucrose. This syrup wastewater was treated by methane fermentation in an upflow anaerobic sludge blanket reactor and the deterioration mechanism of methane fermentation with increasing organic loading rate (OLR) was clarified. The changes in the microbial community in the granules were analyzed using PCR-DGGE and real-time PCR during the fermentation process. The OLR of syrup wastewater was increased gradually as fermentation progressed. The high OLR of 30.3 kg COD m^{-3} d^{-1} enhanced the bacterial activity to the increases of volatile fatty acids concentration and dissolved oxygen level which inhibited the archaeal activity, and the iron-reducing bacterium belonging to genus *Geobacter*, which outcompetes methanogens, grew proportionally with the deterioration of methane fermentation.

Chapter 4

Effects of operation conditions on the performance of the ASBR methane fermentation of syrup wastewater

SUMMARY

Methane fermentation of the syrup wastewater was conducted using an anaerobic sequencing batch reactor. An organic loading rate (OLR), which is calculated by dividing the substrate concentration in the wastewater by hydraulic retention time (HRT), was set at the same value in two runs; R1 with long HRT and high substrate concentration, and R2 with short HRT and low substrate concentration. The effects of HRT and substrate concentration on the deterioration of the methane fermentation were tried to be elucidated by comparing two experimental runs. The OLR increased stepwise from 2.6 to 20.4 g COD L^{-1} d^{-1}. No significant difference was observed until the OLR of 10.2 g COD L^{-1} d^{-1}. After the OLR increased to 20.4 g COD L^{-1} d^{-1}, the ORP in R2 with short HRT of 3.1 days increased immediately and the methane production rate drastically decreased. In contrast, the ORP of R1 with long HRT of 6.2 days was in the range from -450 to -350 mV, and the high methane production rate (MPR) was maintained for a while though the OLR in R1 was the same with that in R2. More than 10 days of operation with the OLR of 20.4 g COD L^{-1} d^{-1}, however, the pH in R1 abruptly decreased and the MPR also decreased rapidly. It was revealed that the deterioration of methane fermentation caused by the too short HRT occurred faster than that caused by the too high substrate concentration.

Chapter 5

Relationship between changes in microbial community and the deterioration of methane fermentation which treats synthetic peptone wastewater

SUMMARY

Synthetic peptone wastewater was used as a substrate in a laboratory-scale methane fermentation reactor and the deterioration mechanism of methane fermentation was clarified. Methane production ceased when the hydraulic retention time (HRT) was changed from 4 days to 1 day. A DNA-based method revealed that the amount of acidogenic bacteria increased, while methanogenic archaea decreased under these conditions. The short HRT resulted in a high level of dissolved oxygen in the wastewater, which adversely affected the archaea by stimulating bacterial production of high concentrations of volatile fatty acids and ammonium ion, and growth of the bacterium belonging to *Desulfoglaeba* that competes with methanogens for reducing power.

Chapter 6

General conclusions and recommendation for future studies

6.1. GENERAL CONCLUSIONS

The growth of interest in methane fermentation process is gaining momentum because of the increasing demand for the development of alternative energy and eco-friendly processing technology. Based on the results obtained, the major conclusions associated with the clarification of the deterioration mechanism of methane fermentation from this study are as follows:

1. The short HRT and high substrate concentration for the increase of OLR influenced to the increase of bacterial activity, resulting in the accumulation of VFAs which induced the inhibition of methane fermentation treating syrup wastewater. Moreover, the increase of DO induced the growth of the iron-reducing bacterium, which was competitor of methanogenesis, resulting in the deterioration of methane fermentation.

2. The short HRT induced the increase of DO level in the reactor which strongly stimulated the increase of bacterial activity to produce the large amount of VFAs which indirectly deteriorated methane fermentation and high DO level directly deteriorated methane fermentation. In contrast, high substrate concentration enhanced the bacterial activity to produce the large amount of VFAs which indirectly deteriorated methane fermentation. It was revealed that the short HRT more strongly influenced than the high substrate concentration to the deterioration of methane fermentation at the same OLRs.

3. Operation at short HRT during the methane fermentation which treats peptone wastewater, resulting in the increase of DO which stimulated the bacterial activity to cause the accumulation of NH_4^+ and VFAs that inhibited the archaeal activity. Furthermore, the increase of ORP by the increase of DO caused the growth of

sulfate-reducing bacterium, which intercepts the reducing power that is required by the archaea to produce methane and then finally methane fermentation deteriorated.

4. The increase of OLR by short HRT and high substrate concentration enhanced the bacterial activity to produce the large amount of intermediates such as VFAs and NH_4^+ that inhibited the archaeal activity. Moreover, the increase of DO corresponding to the increase of ORP which directly inhibited the archaeal activity and could not support methanogeneis, and caused the decrease of reducing power with the appearances of the competitor of methanogen and other reducing reaction that caused the deterioration of methane fermentation. It was clarified that the deterioration of methane fermentation was caused by imbalanced activities of bacteria and archaea, and especially low activity of archaea.

6.2. RECOMMENDATION FOR FUTURE STUDIES

The work described in this thesis adds important insights into the changes of microbial community associated with the deterioration of methane fermentation by increasing organic loading rate. For the sake of achieving the stable methane fermentation at high level, when methane fermentation operates with increasing organic loading rate, the highly efficient methane fermentation can be maintained by controlling the organic loading rate on the basis of the concentration of indicator bacterium for the deterioration of methane fermentation. Future work needs to be focused on the changes of microbial community with the deterioration of methane fermentation using a wide variety of raw materials and elucidated fundamental understanding between the physiological performance and the genetic potential of microorganisms with the deterioration of methane fermentation for the maintenance of successful methane fermentation.