EVALUATION OF ANAEROBIC CO-DIGESTION OF PARA-GRASS AND PIG MANURE ON METHANE PRODUCTION EFFICIENCY AND MICROBIAL COMMUNITY

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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สุเมธ เดชรักษา: การประเมินประสิทธิภาพการผลิตมีเทนและกลุ่มประชากรจุลินทรีย์ จากระบบหมักร่วมของหญ้าขนกับมูลสุกร. (EVALUATION OF ANAEROBIC CO-DIGESTION OF PARA-GRASS AND PIG MANURE ON METHANE PRODUCTION EFFICIENCY AND MICROBIAL COMMUNITY อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. สุเมธ ไชยประพัทธ์, 174 หน้า.

การศึกษาศักยภาพการผลิตมีเทนของการหมักร่วมระหว่างหญ้าขนกับมูลสุกร ที่อุณหภูมิ 35 องศาเซลเซียส พบว่า เชื้อตั้งต้นจากระบบบำบัดไร้อากาศของฟาร์มสุกร สามารถผลิตก๊าซชีวภาพจากหญ้าขนได้เท่ากับ 332.4, 475.0, 519.5 และ 521.9 มิลลิลิตรมีเทนต่อกรัมของแข็งทั้งหมดที่เติม ที่สัดส่วนของเชื้อตั้งต้นต่อวัสดุอินทรีย์ (ISR) เท่ากับ 1, 2, 3 และ 4 ตามลำดับ ดังนั้น ค่า ISR ที่เหมาะสมสำหรับการศึกษาศักยภาพการผลิตมีเทนของการหมักร่วมควรมากกว่า 3 ขึ้นไป การศึกษาระบบการหมักแบบไร้อากาศขั้นตอนเดียวที่อุณหภูมิ 35 องศาเซลเซียส กับระบบการหมักแบบไร้อากาศสองขั้นตอนแบบต่างอุณหภูมิ (Temperature-phased anaerobic digestion system, TPAD) ที่อุณหภูมิ 55 กับ 35 องศาเซลเซียส โดยศึกษาที่อัตราภาระบรรทุกสารอินทรีย์อยู่ในช่วง 0.10-3.76 กรัมของแข็งระเหยง่ายต่อลิตรต่อวัน (เท่ากับสัดส่วนผสมของหญ้าขนร้อยละ 0-8 โดยน้ำหนักแห้ง) พบว่า ระบบ TPAD ที่ป้อนหญ้าขนร้อยละ 4 โดยน้ำหนักแห้ง สามารถ ผลิตมีเทนได้สูงสุด เท่ากับ 158.6 มิลลิลิตรมีเทนต่อกรัมของแข็งระเหยง่ายที่เติม มีองค์ประกอบของมีเทนร้อยละ 55 คิดเป็นผลผลิตก๊าซชีวภาพสูงสุด เท่ากับ 66.3 ลูกบาศก์เมตรก๊าซชีวภาพต่อตันน้ำหนักหญ้าสด การวิเคราะห์กลุ่มประชากรจุลินทรีย์โดยวิธีดีจีจีอี (DGGE) พบว่าการเพิ่มขึ้นของหญ้าขนส่งผลต่อการเปลี่ยนชนิดของแบคทีเรีย แต่ไม่มีผลต่อการเปลี่ยนแปลงของกลุ่มอาร์เคีย สำหรับผลการศึกษารูปแบบของการป้อนวัสดุอินทรีย์ ภายใต้อัตราภาระบรรทุกสารอินทรีย์ที่ต่างกัน เข้าระบบเอเอสบีอาร์ที่อุณหภูมิ 35 องศาเซลเซียส พบว่าการเติมหญ้าขนร้อยละ 2 โดยน้ำหนักแห้ง แบบช่วงเวลา ให้ผลผลิตมีเทนสูงสุดเท่ากับ 169.8 มิลลิลิตรมีเทนต่อกรัมของแข็งระเหยง่ายที่เติม มีองค์ประกอบมีเทนเร้อยละ 53.3 การป้อนแบบช่วงเวลาให้ก๊าซชีวภาพสูงกว่าแบบการเติมปกติและมีข้อดีที่สามารถประหยัดเวลาและต้นทุนการจัดการระบบ

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SUMETH DECHRUGSA: EVALUATION OF ANAEROBIC CO-DIGESTION OF PARA-GRASS AND PIG MANURE ON METHANE PRODUCTION EFFICIENCY AND MICROBIAL COMMUNITY. ADVISOR: ASSOC. PROF. SUMATE CHAIPRAPAT, 174 pp.

The study was focused on the co-digestion of para-grass and pig manure, which was divided into 3 parts. The first part was to evaluate the biochemical methane potential of para-grass conducted at 35^{o}C at various inoculum to substrate ratio (ISR). The maximum methane yields derived were 332.4, 475.0, 519.5 and 521.9 mL/gTS_{added} from pig farm digester inoculum at ISR 1, 2, 3, and 4, respectively. It suggested the use of ISR higher than 3 for BMP assay of this substrate. In the second part, anaerobic single-stage mesophilic 35°C, and temperature-phased anaerobic digestion (TPAD) system (thermophilic 55^{o}C – mesophilic 35°C) were compared under different solid loadings from 0.10-3.76 gVS/L/d (0-8% dry para-grass mixing ratios, PG). Results showed the highest methane yield of 158.6 $mLCH_4/gVS_{added}$ was obtained from TPAD at 4 %PG with a methane content of around 55%. The highest biogas yield in terms of grass addition was 66.3 m^3_{biogas}/ton_{fresh} at 4 %PG. Analysis of microbial communities by DGGE indicated that an addition of para-grass had shifted the domination of bacteria while achaea were rather stable. Third part was the investigation of effects of digester feeding scheme. Anaerobic sequencing batch reactor (ASBR) was operated at 35°C under increasing solid loadings in a regular ASBR feeding (RF) and a periodic feeding (PF) patterns. Results showed the highest methane yield of 169.8 $mLCH_4/gVS_{added}$ was obtained from periodic feeding at 2 %PG with a methane content of 53.3%. PF pattern could be advantageous since it could save time and labor to operate.

Field of Study: Environmental Management Student's Signature .....

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LIST OF ABBREVIATIONS

BMP	Biochemical methane potential
SMA	Specific methanogenic activity
ISR	Inoculum to substrate ratio
RLD	Rubber latex digester
PFD	Pig farm digester
PM	pig manure
PG	para-grass
TS	total solid
VS	volatile solid
VFA	volatile fatty acid
HPr	propionic acid
HAc	acetic acid
HBu	butyric acid
i-HBu	iso-butyric acid
HVa	valeric acid
i-HVa	iso- valeric acid
AD	anaerobic digestion
ASBR	anaerobic sequencing batch reactor
TPAD	temperature-phased anaerobic digester
HRT	Hydraulic retention time
OLR	organic loading rate
Meso-Single	single stage of mesophilic
RF	regular feeding
PF	periodic feeding
Thermo-1st	first stage thermophilic of TPAD
Meso-2nd	second stage mesophilic of TPAD
DGGE	denaturing gradient gel electrophoresis
TCD	thermal conductivity detector
wt	weight

CHAPTER I

INTRODUCTION

1.1 Backgrounds

The environmental consciousness has become an important policy in all countries around the world. Thai communities have increasingly given an importance on environmental issues among the first priority in local development. The energy and environmental crisis has evolved in recent years because we consume great amount of non-renewable resources with the lack of good and long term management planning. There is an ever growing amount of organic waste and wastewater produced annually**.** This has alarmed all parties to concern about the use of renewable resources. At the present time many studies showed that the anaerobic process is a good option to treat various kinds of waste from industrial wastewaters and solid organic wastes including livestock manure and any degradable wastes (Appels *et al*., 2008; Dugba and Zhang, 1999; Lansing *et al*., 2010; Xie *et al*., 2011) since environmental issue and renewable energy can be matched.

Agricultural residues from both the agricultural and agro-industry are usually used as feed materials in the anaerobic digestion systems in Thailand. This can be used as the raw materials for biogas production as environmentally friendly renewable energy. The amount of agricultural residues is about 61 million tons a year. The most residues are rice husk, oil palm residue and rubber wood residue. Biogas resources from industrial wastewater and livestock manure have a potential of 7,800 and 13,000 TJ/year (Terajoule $=10^{12}$ Joules), respectively (EforE, 2011).

Animal manures have been used as a source of excellent material for anaerobic digestion with clear environmental benefit. Especially for pig manure, Thailand raises an average of 9 million pigs a year, manure generating approximately 5 billion kilograms annually (DLD, 2012). Natural degradation of pig manure in open lagoons is leading to emissions of methane gas during storage to the atmosphere. This

contributes to global warming resulted from the release of greenhouse gases (Moller *et al*., 2004; Pattanapongchai and Limmeechokchai, 2011). With a more strict regulation on pig farms, treatment of wastewater from pig production operations is nowadays of very important to farm's survivability. Anaerobic digester has been widely accepted in pig business as a way to treat their waste and produces biogas as a by-product. This by-product is generally converted to electricity and heat for use mainly in cooling and heating systems in the farm. These pig farms become energy intensive with modern animal growing technology.

Bioresource within the farm boundary is of interest since bringing in the off-farm feedstock is against a farm disease control protocol. In-farm feedstock should be used as co-substrate to produce energy with pig manure. Co-digesting para-grass with pig manure in the farm's existing digester becomes a valid approach to enhance biogas production. Para-grass (*Branchiria mutica*) is the tropical weed that pervasive around the farm. It needs to be cut down and removed frequently for fire hazard, and disease and vector controls. Addition of grass can help raise C:N of the feedstock to be suitable for metabolic activities in anaerobic digestion system (Xie *et al*., 2011). However, due to the different characteristics of both substrates (pig manure and para-grass), the effect of this para-grass mixing with the farm wastewater needs to be known.

Four steps in anaerobic digestion that take place in an oxygen free environment are hydrolysis, acidogenesis, acetogenesis and methanogenesis. First step of hydrolysis, the complex molecules of carbohydrates, fats and proteins were broken into soluble organics by the enzymatic action of hydrolytic fermentative bacteria. Second step, the soluble organics were converted by acidogenic bacteria to organic acids or volatile fatty acids (VFAs), alcohols, H_2 and CO_2. The VFAs and alcohols were converted to acetic acid by the H_2-producing acetogenic bacteria which are subsequently transformed into CH_4 by methanogens (Angelidaki *et al*., 2009; Lozano *et al*., 2009). The effectiveness of this digestion process is mostly based on the volume of methane produced that can be expressed per dry mass of the substrate

(added or destroyed) for solid digestion, or per mass of substrate COD (added or destroyed) for liquid digestion.

Anaerobic sequencing batch reactor (ASBR) is one of promising high-rate anaerobic process and has been used for treating organic wastewaters, activated sludge, swine manure, leachate and dairy. ASBR operation consists of four steps; fill, react, settle, and decant (Sarti *et al*., 2007). It is able to attain a high solid retention because of its settling phase. Therefore, it can maintain high concentration of slow-growing anaerobic bacteria in the reactor (Dugba and Zhang, 1999).

Conventionally, the acid-forming and methane forming microorganisms are kept together in a single reactor. The two groups of organisms need a delicate balance because of the difference in terms of physiology, nutritional, growth kinetic and environmental condition. Therefore, the two stage reactor is one that separation of acid-formers and methane-formers to the optimum environmental conditions for each group of microorganisms. These could provide the optimum condition to enhance the overall process stability (Nasr *et al*., 2012). Temperature-phased anaerobic digestion (TPAD) system is a two-stage anaerobic digestion. It combines thermophilic (55 ^{o}C) and mesophilic(35 ^{o}C) process within a system. The front reactor is thermophilic reactor. It has high digestion rate and pathogen destruction. Second stage is mesophilic reactor that requires low energy and gives high quality of effluent (Dugba and Zhang, 1999, Song *et al*., 2004). The advantages of this system are enhancing the efficiency in the effluent quality, good performance of the organic matter removal, high digestion rate, methane yield, volatile solid reduction, process stability, and pathogen control (Riau *et al*., 2010, De La Rubia *et al.,* 2009). The TPAD process could be operated at higher loading rates than single-stage processes. The feedstock is fed into thermophilic digester and then the digestate is transferred into mesophilic digester. Therefore, the four steps of biomethanation process can be divided into hydrolysis and acidogenesis hypothetically occurring mainly in the thermophilic digester, while acetogenesis and methanogenesis occurring in the mesophilic digester (Riau *et al*., 2010).

This study is emphasizing on the utilization of para-grass grown on farm in co-digesting with pig manure, the unlimited resource on farm. Because of a high buffering capacity and richness in nutrients of pig manure, the addition of an extra carbon source to it was justified in enhancing the biogas production from the existing mono digestion of manure alone. Responses of the digester systems in different configuration and operation regime were undertaken in this research program. Moreover, the attempts to analyze the microbial community were performed in order to collect the necessary information to understand the behaviors of the systems at microbial scale.

1.2 Objectives

The objectives of this study are:

1.2.1 To evaluate the potential of anaerobic digestion of pig manure with para-grass

1.2.2 To evaluate the benefit of a two-stage thermophilic-mesophilic system versus single stage mesophilic system in co-digesting grass and pig manure.

1.2.3 To assess the applicability of periodic feeding scheme in the anaerobic sequencing batch reactor at various loading conditions.

1.2.4 To characterize microbial communities in anaerobic digesters operated in different modes of co-digestion.

1.3 Scope of the study

1.3.1 Biogas production potential of different substrate mixture ratios and inoculum to substrate ratios (ISR) were performed using biochemical methane production potential assay (BMP assay).

1.3.2 Experiments to monitor the performance of anaerobic reactor in single stage of mesophilic versus the temperature-phased anaerobic digestion (TPAD) system, and feeding patterns were conducted in lab-scale systems.

1.3.3 Sludges from the systems operated at different conditions were collected and analyzed for dominant species in the microbial community in the reactors using DGGE technique.

1.4 Expected Outcomes

1.4.1 The potential of anaerobic digestion of pig manure with para-grass as co-substrate is revealed.

1.4.2 The performance of co-digestion in anaerobic digestion of single stage versus two-stage thermophilic-mesophilic (TPAD) systems and feeding pattern for pig manure and grass co-digestion are known.

1.4.3 The microbial communities in an anaerobic digester are verified using DGGE.

1.5 Structure of the Dissertation

This dissertation consists of the following chapters.

Chapter 1	An introduction of this research
Chapter 2	Literature review of the fundamental and basic knowledge
Chapter 3	Materials and the experimental set-up that were used in this research.
Chapter 4	Research report on the effects of inoculum, substrate ratio and inoculum source on biochemical methane potential (BMP) assay
Chapter 5	Research report on the performance of single stage mesophilic and TPAD system
Chapter 6	Research report on the performance of single stage of mesophilic with difference feeding patterns
Chapter 7	Conclusion of this research program

CHAPTER II

BACKGROUND AND LITERATURE REVIEW

2.1 Quantity of livestock waste

Normally, manure includes excreted material from the animal (feces and urine), used bedding, wasted feed, water (drinking and washing), and hair. Which the quantity of manure produced depends on several factors including: animal type (ruminant or non-ruminant), diet (forage-based or grain-based or formulate feed), animal age (which can influence the amount of feed consumed) and animal environment

Animal waste from farms and livestock that include of pig manure, beef cattle, dairy cattle, and chicken manure can negative effects on the environment if doesn't have a good managed. All waste or manure impact on: water pollution by nitrates and eutrophication, air pollution, especially ammonia and greenhouse gases emissions, and soil pollution because of nutrient accumulation (Martinez *et al*., 2009). The quantity of manure can estimate in the Table 2.1 as shown below:

2.2 Quality and characteristic of manure

The most important parameters for characterizing of slurries are total solids content (TS) and volatile solids content (VS). VS content is an indicator of potential methane production. While chemical oxygen demand (COD) could also give a measure of the organic of slurries and showed the efficiency of anaerobic bacteria to convert substrate into biogas (Florida, 2011). Wastes from livestock farms have high organic matter content. Generally, manure can classified as liquid, slurry, semi-solid, or solid depending on the total solids content of the manure. Liquid manure contains up to 4% solids content, slurry manure contains 4% to 10% solids, semi-solid manure contains between 10 and 20% solids, and solid manure contains 20% solids content or

more (Organization, 2011). For most manure and sludge this occurs at TS of 10-15%. Waste with a higher TS content may need the dilution if it is to be treated as slurry.

Table 2.1 Production of fresh manure, total solids, and volatile solids for typical swine per day

Farm type	Average weight (kg/PU)	Manure production (kg/AU/day)	Total solids (kg/AU/day)	Volatile solids (kg/AU/day)
Farrow-to-Wean	196.40/sow	27.22	2.68	2.04
Nursery	13.61 /pig	38.10	4.99	3.86
Farrow-to-Feeder	236.77/sow	29.23	3.04	2.31
Feeder -to- Finish	61.23/hog	38.10	4.99	3.86
Farrow-to-Finish	64.74/sow	34.93	4.22	3.27

AU is Animal Unit which equal 453.59 kg of live weight

PU is Production Unit. The production unit is a sow, pig, or hog as shown

Source: (modified from John, 2003)

Table 2.2 Characteristic of livestock manure

Characteristics/ substrates	Pig manure		Cattle manure		Dairy manure	
pH	7.12		7.4		7.0	
TS	0.459	%	41	g /l	39,854	mg/l
VS	0.248	%	22	g / l	28,372	mg/l
TKN	918	mg/l	2,200	mg/l	2,198	mg/l
NH4+-N	709.1	mg/l	1,100	mg/l	716	mg/l
BOD	3,780	mg/l	-	mg/l	-	mg/l
COD	7,040	mg/l	4,200.1	mg /l	48,026	mg/l
Reference	(Lv *et al*., 2010)		(Maranon *et al*., 2008)		(Rico *et al*., 2007)	

2.3 Waste management

From the past, there are many ways to manage the manure which is a valuable material. Manure can be used as a source of organic matter and fertilizer for crop or plant. Small amount of it can handle by collected, stored, and spread on the land field. For the slurry manure used a deep-pit storage system. Methods of treating livestock wastewater involve lagoon retention and subsequent spreading on fields (Harrington and McInnes, 2009). Whether, it can also be used as source of energy on the farm through anaerobic digestion to produce biomethane or thermochemical to produce heat or electricity as the renewable energy for power generating unit, burner, boiler, and lamps. Some of the by-products (sludge) can be use as organic fertilizer for plant. Normally is treated by using anaerobic digestion (Organization, 2011).

Table 2.3 Inventory of livestock materials and biogas energy potential

Manure	TS (%)	VS (%) of TS	Biogas yield (m^3/tonnes)	Energy potential (PJ/y)	Methane content (%)
Beef cattle	8-12	80-85	19-46	20-48	53
Hog (grower-finisher)	9-11	80-85	28-46	1.4-2.3	58
Dairy	12	80-85	25-32	2.0-2.6	54
Poultry	25-27	70-80	69-96	3.3	60

PJ = Petajoule = 10^{15} joules

Source: (Sarmah, 2009)

2.4 Anaerobic digestion (AD)

2.4.1 The principle and definition of anaerobic digestion (Biogas)

Anaerobic digestion technology is an ideal biological treatment for the removal of organic pollutants in waste and wastewater. This treatment is without input of oxygen. The most organic compounds can be transformed in the smaller

reactor volume and produce the methane which is a potential energy source (Tchobanoglous *et al.*, 2004). Therefore, it has been widely applied to treat the waste from agricultural and industrial operations (Chen *et al*., 2008) as shown in Table 2.4.

Table 2.4 Examples of type wastewater treated by anaerobic process

Alcohol distillation	Landfill leachate
Breweries	Pharmaceuticals
Chemical manufacturing	Pulp and paper
Dairy and cheese processing	Slaughterhouse and meatpacking
Domestic wastewater	Soft drink beverages
Fish and sea food processing	Sugar processing

Source: (Tchobanoglous *et al.,* 2004)

Table 2.5 General operating and loading conditions for anaerobic digestion

Temperature	Optimum	36.7 ^{o}C
	General operating range	29-37 ^{o}C
pH	Optimum	7.0 to 7.1
	General limits	6.7 to 7.4
Gas production	Per pound of VS added	230-340 liters
	Per pound of VS destroyed	450-510 liters
Gas composition	Methane	65-69 %
	Carbon dioxide	31-35 %
	Hydrogen sulfide	Trace to 80 mg/l
Volatile acids concentration	General range	200-800 mg/l
Alkalinity concentration	Normal operation	2,000-3,500 mg/l
Volatile solid reduction	Conventional single stage	50-70 %
	First stage high rate	50 %
Solid retention time	Conventional single stage	30-90 days
	First stage high rate	15-20 days

Source: Adapted from (Hammer, 2004)

The efficiency of AD depends on many factors. Therefore, the general operating and the conditions for treating wastewater showed in Table 2.5. This process require more further treatment with an aerobic process to meet discharge requirements and need more longer start-up time to develop necessary biomass inventory, and the need alkalinity addition (Tchobanoglous *et al.*, 2004).

2.4.2 Anaerobic digestion process

Anaerobic digestion is operated to degrade organic waste components (Rico *et al*., 2007) or to biologically degrade a portion of the volatile solids in sludge (Gerardi, 2003) and produce biogas that contain of carbon dioxide, methane, and water etc. The reactions of this process require the cooperative action of several organisms. It occurs in each stage as the result of the activity of a variety of microorganisms. In the first stage, a variety of primary producers (acidogens) break down the raw wastes into simpler fatty acids. In the second stage, a different group of organisms (methanogens) consumes the organic acids produced by the acidogens, generating biogas as a metabolic by product. On average, acidogens grow much more quickly than methanogens. Finally, the organic acids are converted to biogas. Biogas consists of methane 55 – 70 %, carbon dioxide 30 – 45 %, with the balance being made up of nitrogen, hydrogen and hydrogen sulfide (Deublein and Steinhauser, 2008). But this treatment type has long retention time, slow start-up (granulating reactors), and large area required for conventional digesters (Poh and Chong, 2009).

The anaerobic digestion of organic material basically follows; hydrolysis, acidogenesis, acetogenesis and methanogenesis as shown in Figure 2.1.

The hydrolysis stage: Hydrolytic fermentative bacteria degrade organic compounds and high molecular weight compounds such as fats, polysaccharides, and proteins, into soluble organic substances (e.g. monosaccharides, amino acids and fatty acids). Hydrolysis of the complex molecules is catalyzed by extracellular enzymes such as cellulases, proteases, and lipases (De La Rubia *et al.*, 2009; Themelis, 2002).

However, the hydrolytic phase is relatively slow and can be limiting in anaerobic digestion of wastes such as raw cellulolytic wastes that contain lignin.

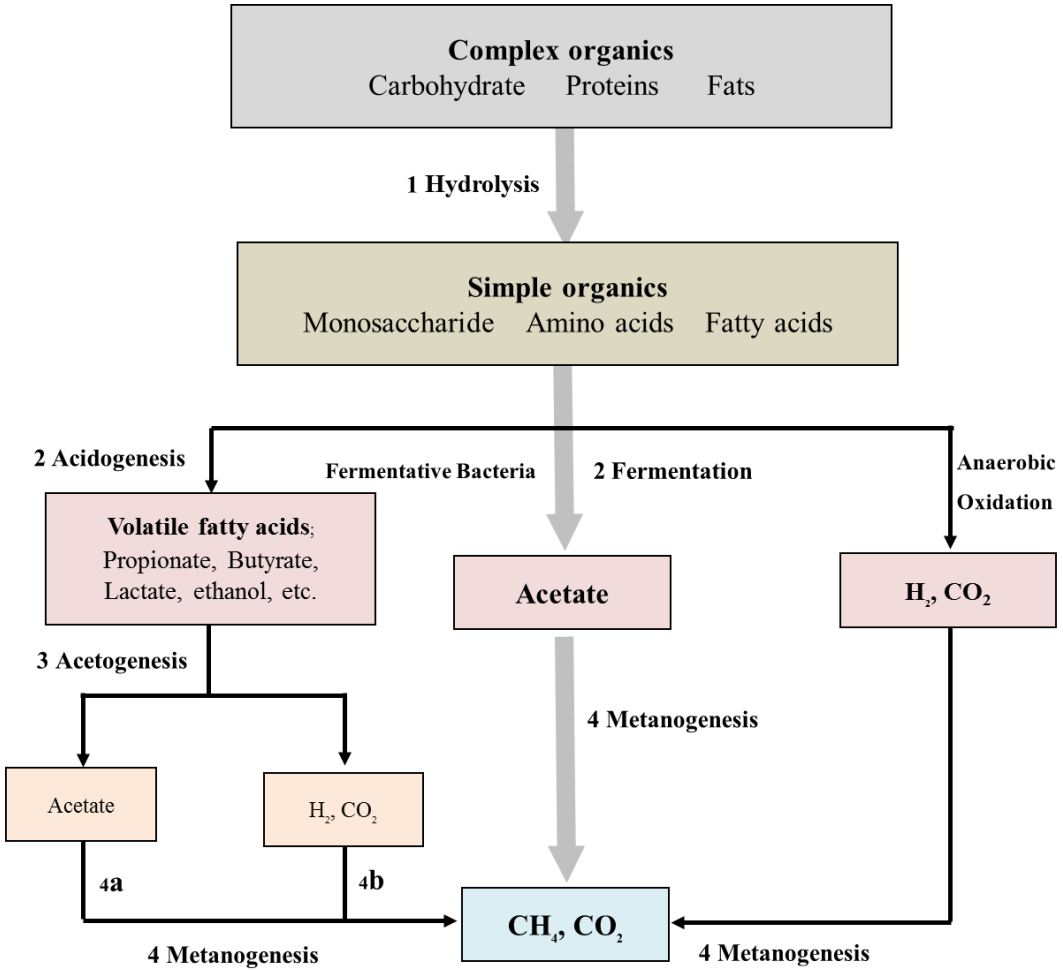

Figure 2.1 Show the conversion steps in anaerobic digestion: 1, hydrolytic fermentative bacteria; 2, acidogenic bacteria; 3, acetogenic bacteria; 4a, acetoclastic methanogens; 4b, hydrogenotrophic methanogens. (Modified from (Thamsiriroj and Murphy, 2011; Speece, 1996)

Acidogenesis stage: Acidogenic (or fermentative), (acid-forming) bacteria (e.g., Clostridium) convert sugars, amino acids, and fatty acids to organic acids (e.g., acetic, propionic, formic, lactic, butyric, or succinic acids), alcohols and ketones (e.g.,

ethanol, methanol, glycerol, acetone), acetate, CO_2, and H_2. Acetate is the main product of carbohydrate fermentation. The products formed vary with the bacterial type as well as with culture conditions (temperature, pH). Chemical reaction take place in the digester is as follow in Equations. 2.1 -2.6.

Acetogenesis stage: where the higher organic acids and alcohols produced by acidogenesis are further digested by acetogens bacteria (acetate and H_2-producing bacteria) such as *Syntrobacter wolinii* and *Syntrophomonas wolfeito* produce mainly acetic acid as well as propionic acid, butyric acid, CO_2 and H_2. Chemical reactions take place as follow in Equations. 2.7 - 2.10 (Themelis, 2002).

Methanogenesis stage: Methanogenic bacteria can be subdivided into two groups: first group is hydrogenophilic or hydrogenotrophic methanogens, which form methane by the reduction of CO_2 using H_2 as electron donor as Equation 2.11. Most of the methanococcales and methanobacteriales use H_2 and CO_2 (Bitton, 2005). The second group is acetoclastic or acetotrophic methanogens that convert acetate into methane and carbon dioxide. Methanogens can also utilize other substrates to produce methane, such as methanol, methylamines, and formate as Equation 2.14 and 2.15. Figure 2.2 showed the carbon and hydrogen flow in AD that about 72 percent of methane produced in anaerobic reactor is derived from acetate, whereas the remainder is derived from H_2 and CO_2. This group comprises two main genera which have only two acetoclastic genera, *Methanosarcina* and *Methanosaet.* (Appels *et al*., 2008; Speece, 1996; Khanal, 2008; Bitton, 2005). *Methanosarcina* was the dominant acetoclastic methanogen in the bioreactor during thermophilic (58^0C) digestion of lignocellulosic waste (Bitton, 2005).

The biochemical pathways are very important for the formation of methane. The substrates that are used in methanogens are CO_2, H_2, formate, acetate, methanol, methylamines, and carbon monoxide as follow (Tchobanoglous *et al.*, 1993; Hammer, 2004).

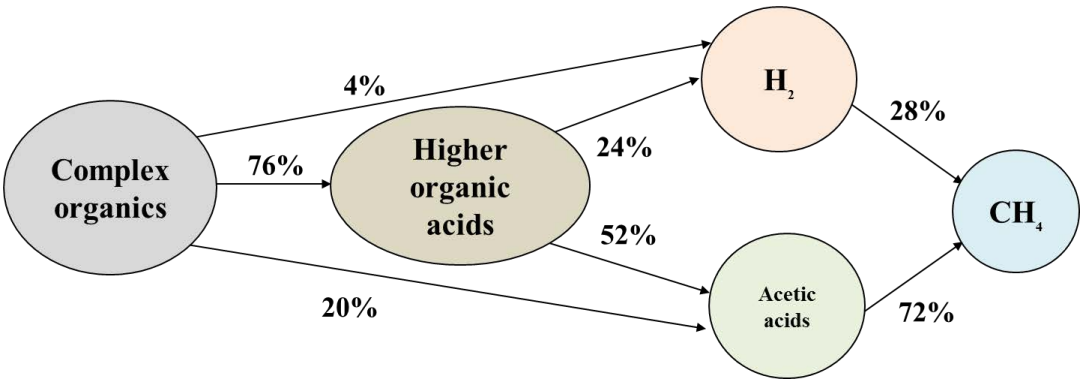

Figure 2.2 Show the carbon and hydrogen flow in anaerobic digestion process (Tchobanoglous *et al*., 2004)

Fermentative reaction:

$C_6H_{12}O_6 + 3H_2O \rightarrow 3CH_4 + 3HCO^-_3 + 3H^+$ (2.1)

$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3CH_2OH + 2HCO^- + 3H^+$ (2.2)

$C_6H_{12}O_6 + 3H_2O \rightarrow CH_3CH_2CH_2COOH + 2H_2 + 2HCO^-_3 + 2H^+$ (2.3)

$C_6H_{12}O_6 \rightarrow 2\text{Lactate} + 2H^+$ (2.4)

$C_6H_{12}O_6 \rightarrow 3\text{Acetate} + 3H^+$ (2.5)

$3\text{Lactate} \rightarrow 2\text{Propionate} + \text{Acetate} + HCO^-_3 + 3H^+$ (2.6)

Acetogenic reaction:

Propionate: $CH_3CH_2COOH + 2H_2O \rightarrow CH_3COOH + CO_2 + 3H_2$ (2.7)

Butyrate: $CH_3CH_2CH_2COOH + 2H_2O \rightarrow 2CH_3COOH + 2H_2$ (2.8)

Ethanol: $CH_3CH_2OH + H_2O \rightarrow CH_3COOH + 2H_2$ (2.9)

Lactate + $2H_2O \rightarrow CH_3COOH + HCO^-_3 + 2H_2$ (2.10)

Methanogenic reaction:

$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$ (2.11)

Formate: $4HCOOH \rightarrow CH_4 + 3CO_2 + 2H_2O$ (2.12)

Acetate: $CH_3COOH \rightarrow CH_4 + CO_2$ (2.13)

Methanol: $4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$ (2.14)

Methylamines $4(CH_3)_{3N} + 6H_2O \rightarrow 9CH_4 + 3CO_2 + 4NH_3$ (2.15)

2.4.3 Microbial community in anaerobic digestion

More recently, the microbial ecology of anaerobic reactor systems has also been investigated in detail. It is obvious that the performance of an anaerobic reactor is primarily determined by the amount of active microorganisms retained within the system. Besides, changes in operational and environmental conditions of the anaerobic reactor and within the microbial populations present in the reactor definitely affect each other mutually (Demirel and Yenigun, 2006). Molecular analysis of microbial cells and communities can, therefore, furnish useful information about structure (who they are), function (what they do), and dynamics (how they change through space and time). The microbial community structure obtained from various molecular techniques (Khanal, 2008). The dominated microorganism during hydrogen production from POME using ASBR at high temperature is *Thermoanaerobactarium* spp. (Demirel and Yenigun, 2006). Bacterial composition in the reactor gradually changed as the HRT decreased. Low HRT and OLR triggered a transition in the bacterial community structure (Prasertsan *et al*., 2009). Demirel and Yenigun, (2006) found that Medium rods and Methanococcus-like species were observed to be the dominant methanogens in the seed sludge prior to inoculation. And Methanococcus-like species were still the most dominant methanogens, with an increase in number, at the end of start-up. While medium rods constituted another dominant methanogen in sludge that seemed to decrease in number.

Table 2.6 Applications of molecular techniques for microbial community analysis in bio- energy production processes

Type of bio-energy	Molecular techniques utilized	Major findings
Methane	PCR-DGGE and FISH	Microbial community change with operational time
Methane	SSCP, qPCR, and FISH	Significant effect of volatile fatty acids concentrations on methanogenic community
Hydrogen	PCR-DGGE, PCR-RISA, cloning, and T-RFLP	Identify dominant species and contaminant

Table 2.7 Microbial findings of methane and hydrogen producers using molecular techniques

Substrate/Product	**Swine manure /methane**	**Starch in wastewater /hydrogen**	**Palm oil mill effluent/hydrogen**
Reactor type	Anaerobic Membrane Reactor (AnMBR)	Batch	Anaerobic Sequencing Batch Reactor
Dominated Microorganisms	*Methanosarcinaceae* sp. *Methanosaetaceae* sp. *Methanomicrobiales* sp	*Thermoanaerobacteriaceae* *Saccharococcus* sp.	*Thermoanaerobacterium* *Thermosaccharolyticumnos*
Molecular techniques	T-RELP	Clone library FISH	PCR-DGGE
References	(Padmasiri *et al.*, 2007)	(Sarmah, 2009)	(Prasertsan *et al.*, 2009)

2.4.4 Advantages and drawback of various AD

Anaerobic reactors can be classified as low rate or high rate (Table 2.8). Low rate anaerobic reactors are unmixed. Temperature, SRT, and other environmental conditions are not regulated. The organic loading rate is low in the range of 1-2 kg COD/m^3/day. High rate anaerobic systems maintain a very high biomass level in the bioreactor. The organic loading rates vary from 5-30 kg COD/m^3/day or higher.

The principle types of reactors used for the treatment of wastewaters are (Tchobanoglous *et al*., 2004).

2.4.4.1 Anaerobic Filters

Anaerobic filters are the anaerobic trickling filters. As wastewater flows through the filter, and the particles are trapped by filter material. The filter material commonly used rock, gravel, or plastic pieces with the space of approximately 50 percent or more. The bulk of anaerobic microorganisms grow attached to the filter medium. The up-flow of wastewater through the reactor helps retain suspended solids in the column. This process is particularly efficient for wastewaters rich in carbohydrates. The loading rate varies with the type of waste and with the type of support medium (Bitton, 2005).

2.4.4.2 Fluidized bed reactor

The reacting stream is expanded by the upward movement of fluid (air or water) through the bed. The expanded porosity can be varied by controlling the flow rate of the fluid. This type can apply for fluidized-bed reactors for anaerobic biological treatment and up-flow sludge blanket reactor (Reynolds and Richards, 1996).

Table 2.8 Classification of anaerobic reactors

Low rate anaerobic reactors	High rate anaerobic reactors
• Anaerobic pond	• Suspended growth
• Septic tank	- High rate anaerobic digester
• Imhoff tank	- Anaerobic contact process
• Standard rate anaerobic digester	- Upflow anaerobic sludge blanket (UASB)
	- Anaerobic sequencing batch reactor (ASBR)
	• Attached growth
	- Anaerobic filter (AF)
	- Fluidized/expanded bed reactor
	• Other
	- Static granular bed reactor (SGBR)
	- Anaerobic membrane reactor (AnMBR)
	- Hybrid reactor

Source (Khanal, 2008)

2.4.4.3 Up-flow Sludge Blanket (UASB)

The UASB-type digester consists of a bottom layer of packed sludge, a sludge blanket and an upper liquid layer. Wastewater flows upward through a sludge bed, which is covered with a floating blanket. Settler screens separate the sludge flocs from the treated water and gas is collected at the top of the reactor (Bitton, 2005).

2.4.4.4 Plug flow reactor

Plug flow has the geometric shape of a long tube or tank. The reactants enter at the upstream end of reactor and the products leave at the downstream end. The particles remain in the reactor for a time equal to the theoretical detention time. This type of flow is approximated in long open tanks with a high length to width ratio. There is no induced mixing between elements of fluid along the direction of flow (Reynolds and Richards, 1996).

2.4.4.5 Completely Stirred Tank Reactor (CSTR)

This reactor consists of stirred tank that has feed stream of the reactants and discharge stream. Mixing is employed to improve their performance. Therefore, this can apply for aerated lagoons (Reynolds and Richards, 1996).

2.4.4.6 Anaerobic Sequencing Batch Reactor (ASBR)

Anaerobic sequencing batch reactors are currently used for the treatment of wastewaters with amounts of particulate organic matter such as swine manure, leachate and dairy. The process of ASBR consists of five step of fill, react, settle, draw and idle. This system, anaerobic step is adapted in total process (Sarti *et al*., 2007).

Zupancic *et al*.(2007) reported that ASBR experiments were conducted for the treatment of brewery slurry under different organic loading rates (OLR) from 3.23 to 8.57 kgCOD/m^3/day of reactor and control was conducted with OLR of 3.0 kgCOD/m^3/day. The ASBR COD removal efficiency was from 79.6% to 88.9%, control experiment efficiency was 65%. ASBR VSS removal efficiency was from 78.5% to 90.5%, control experiment efficiency was 54%. The ASBR methane production yield was from 371 to 418 L_{CH4}/kg/COD_{added}, control experiment methane yield was 248 L_{CH4}/kg COD_{added}.

Table 2.9 Advantages and disadvantages of various anaerobic treatment methods

Type	Advantages	Drawbacks
Anaerobic filtration (Poh and Chong, 2009)	- Small reactor volume - Producing high quality effluent - Short hydraulic retention times - Able to tolerate shock loadings - Retains high biomass concentration in the packing	- Clogging at high OLRs - High media and support cost - Unsuitable for high suspended solid wastewater
Fluidized bed (Poh and Chong, 2009)	- Most compact of all high-rate processes - Very well mixed conditions in the reactor - Large surface area for biomass attachment - No channeling. It make plugging for gas hold-up - Faster start-up	- High power requirements for bed fluidization - High cost of carrier media - Not suitable for high suspended solid wastewaters - Normally does not capture generated biogas
UASB (Demirel *et al*., 2005)	- Suitable for treating wastewaters - Can treat large volume of wastewater - Producing high quality effluent - Low HRT, small reactor - High loading rate - High methane production	- Performance dependant on sludge settle ability - High level of operating skill required - Costly

Table 2.9 Advantages and disadvantages of various anaerobic treatment methods (continued)

Type	Advantages	Drawbacks
Plug Flow (Nijaguna, 2002)	- High solid loading - High gas yield	- Low gas delivery pressure - High land requirement
CSTR (Poh and Chong, 2009)	- Provides more contact of wastewater with biomass through mixing - Increased gas production compared to conventional method	- Less efficient gas production at high treatment volume - Less biomass retention
ASBR (Khanal, 2008), (Luo *et al*., 2009)	- Suitable to treat high and medium solids content (TS 1-4%) - Suitable for bioenergy production from animal manure and biowastes - High efficiency for both COD removal and gas production	- Low performance efficiency if overloaded - Gas storage require

2.5 Influence of factors on performance of anaerobic digestion

Many factors are very important on the performance of anaerobic digestion system. Manure quality, temperature, and storage time in swine houses can significantly affect the solid-liquid separation efficiency due to changes in manure composition caused by biological activity during manure storage (Zhu, 2000). Microorganism growing rate is also important in the AD process. The operating parameters of the digester must be controlled to enhance the microbial activity and increase the anaerobic degradation efficiency of the system. Some of these parameters are discussed in the following.

2.5.1 Temperature

In an anaerobic system, there are three optimal temperature ranges for methanogenesis: psychrophilic, mesophilic, and thermophilic. Anaerobic conversion has its highest efficiency at 5-15 ^{o}C for psycrophiles, 35-40 ^{o}C for mesophiles, and 55 ^{o}C for thermophiles (Khanal, 2008; Speece, 1996) and the retention time are over 100, over 20, and over 8 days, respectively. Effect of temperature on the performance of anaerobic digestion was investigated. Yu *et al*.(2002) found that substrate degradation rate and biogas production rate at 55 ^{o}C was higher than operation at 37 ^{o}C. Studies have reported that thermophilic digesters are able to tolerate higher OLRs and operate at shorter HRT while producing more biogas. Because of the temperature has an important effect and also influences the growth rate and metabolism of micro-organisms and the population dynamics in the anaerobic reactor (Appels *et al*., 2008).

2.5.2 pH

Khanal (2008) reported anaerobes can be grouped into two separate pH groups: acidogens and methanogens. The optimum is 5.5-6.5 for acidogens and 7.8-8.2 for the methanogens that nearly neutral pH (Speece, 1996). The optimum pH for the combined cultures ranges from 6.8-7.4. The microbial community in anaerobic digesters are sensitive to pH changes and methanogens are affected to a greater extend (Appels *et al*., 2008). The methane bacteria cannot function if the pH is drop below 6.2. If the pH as low as 5, resulted in a sustained rate of methane production was about 25 % of that for control at neutral pH (Speece, 1996; Khanal, 2008). Furthermore the alkalinity should be normal range from 1,000 to 5,000 mg/L and the volatile fatty acid should be less than 250 mg/L (Tchobanoglous *et al*., 1993). As such, methanogenic activity will decrease when pH in the digester deviates from the optimum value. Several cases of reactor failure reported in studies of wastewater treatment are due to accumulation of high volatile fatty acid concentration, causing a drop in pH which inhibited methanogenesis (Parawira *et al.*, 2006). Thus, volatile fatty acid concentration is an important parameter to monitor to guarantee reactor performance.

2.5.3 Volatile fatty acid (VFAs)

The changes in VFA production can also be explained by the type of substrate (Demirel and Yenigun, 2006). VFA accumulation accompany to pH falling. So, this is the main cause of toxicity and reactor failure in the AD process. The toxicity of VFAs is also pH dependent, since only the non-ionized forms are toxic to microorganisms. That mean excessive VFAs accumulation can inhibit methanogenesis. The concentrations of acetic, propionic, and butyric acids are considered to be the best indicators of the metabolic state of the most sensitive microbial groups in the anaerobic system and are important in process monitoring (Ralph and Gu, 2010).

2.5.4 Mixing

Mixing provides good contact between microbes and substrates, increasing the mass transfer, reduce the buildup of intermediates and stabilize environmental conditions. When mixing is inefficient, overall rate of process will be reduced by mass of material at different stages of digestion whereby every stage has a different pH and temperature. Mixing can be accomplished through mechanical mixing, biogas recirculation or through slurry recirculation (Karim *et al*., 2005). It was found that mixing improved the performance of digesters treating waste with higher concentration while slurry recirculation showed better results compared to impeller and biogas recirculation mixing mode. Mixing also improved gas production as compared to unmixed digesters. Rapid mixing is not encouraged as methanogens can be less efficient in this mode of operation. Examples of systems with optimal flow include the continuously stirred tank reactor (CSTR) where incoming material is dispersed evenly throughout the vessel by perfect mixing and the plug flow reactor (PFR) where material moves through the vessel (Ward *et al*., 2008).

2.5.5 Toxicity

Anaerobic microorganisms are inhibited by the substances that include in the waste influence. All kinds of substance are ammonia, heavy metal, halogenated

compounds, and cyanide (Khanal, 2008; Hammer, 2004). Ammonia is concerned for the anaerobic treatment of wastewater. Free ammonia (NH3) will be toxic to methanogenic bacteria if it has toxicity concentrations range of 1,500 to 3,000 mg/L. Because of ammonia is a weak base and dissociates in water to form ammonium (NH^{+}_{4}) and hydroxyl ion (Tchobanoglous *et al*., 2004).

2.5.6 C/N Ratio

Animal manure is used as feedstock for most of the digesters for around the world to produce biogas (methane) for energy. Although it has been recognized that using animal manure alone may not be represent the most efficient way to produce biogas due to its inherent deficiency of carbon. The carbon/nitrogen (C/N) ratio for swine manure is around 6 to 8 which is too low for an anaerobic digestion to function efficiently to utilize the nutrients in swine manure and maximize the methane yields. Optimum C/N ratios in anaerobic digesters are 20-30 (Themelis, 2002). The C/N ratio of 20/1 was found to be the best in terms of biogas productivity (Wu *et al*., 2010). If the C/N ratio is high, there is a risk of nutrient deficiency, and a low buffering capacity will result in a more sensitive process, whereas if the nitrogen content is high, ammonia inhibition problems may arise. It can inhibit the rate of digestion. The digestibility of carbohydrate-rich wastes can be improved by mixing them with those containing high amounts of nitrogen to improve the C/N ratio (Ralph, 2010).

2.5.7 Hydraulic retention time (HRT)

In the anaerobic digester can classify the retention time in two. Firstly, Hydraulic retention time (HRT) is the time that waste (liquid) remains in the reactor (Speece, 1996). Waste that contain the simple compound require low HRT but if contain a complex compound are slowly degradable need longer HRT. HRT equals the volume of the reactor divided by the daily flow (HRT=V/Q). The hydraulic retention time is important since it establishes the quantity of time available for bacterial growth and subsequent conversion of the organic material to gas. Secondly, the solids retention time (SRT) is the time that controls the microbial mass (biomass)

in the reactor. SRT is a measure of biological system's capability to achieve specific effluent standards (Khanal, 2008).

2.5.8 Organic loading rate (OLR)

Organic loading rate (OLR) indicates the amount of wastewater that can be treated per unit of reactor volume (kgCOD/m^3/d) (Speece, 1996). OLR can calculate as equation 2.16. Various studies have proven that higher OLRs will reduce COD removal efficiency in wastewater treatment system (Torkian *et al*., 2003; Patel and Madamwar, 2002). OLR is related to substrate concentration and HRT, thus a good balance between these two parameters has to be obtained for good digester operation. Short HRT will reduce the time of contact between substrate and biomass. OLR is the important factors in designing or sizing an anaerobic bioreactor which is given by the following (Khanal, 2008):

$$OLR = C_iQ / V \qquad (2.16)$$

Where: C_i is influent wastewater biodegradable COD concentration (mg/L)

Q is wastewater flow rate (m^3/day), and

V is anaerobic bioreactor volume (m^3)

2.6 Feedstock

Biogas can be generated from a wide range of feedstock that is suitable for anaerobic digestion. It can be made from most biomass and waste materials and over a large range of moisture contents, with limited feedstock preparation. Therefore, feedstock for biogas production may be solid, slurries, and both concentrated and dilute liquids. But the feedstock needs to be a liquid mixture with suitable moisture content. For example, mesophilic complete mix tank digesters typically operate best with a mixture of 4 to 8% solids in water (Faivor, 2010). The range of potential waste feedstock is much broader including: municipal wastewater, residual sludge, food waste, food processing wastewater, dairy manure, poultry manure, and agriculture

wastewater, seafood processing wastewater, yard wastes, and municipal solid wastes (Florida, 2011; Ralph and Gu, 2010). Beside this, feedstocks are energy crops including: sugarcane, sorghum, napier grass, as well as, woody crops, corn, oilseeds, switch grass. The best crops should have low fertility requirements, and low energy costs for planting and harvesting. Biogas production from different feedstock is difficult as performance data for specific types. It is under a wide variety of experimental conditions (Ward *et al.*, 2008).

Table 2.10 Biogas yield from various types of crop residue

Type	Retention time (day)	Dry matter (%)	Gas yield (L/kg DM)	Gas composition (% v/v)	
				CH_4	CO_2
Rice straw	33	46	5.67	22.8	24.8
Para-grass	36	30	5.05	4.3	23.2
Duck weed	41	22	5.46	11.3	32.2
Corn top	32	19	5.43	7.6	28.0
Water hyacinth	46	12	20.30	8.2	16.6

DM is dry matter

Source: (Nijaguna, 2002)

2.6.1 Para-Grass (PG)

Para grass is a common name of *Brachiaria mutica* which is perennial crop that can grow on wet and flooded soils in the higher rainfall areas. It has stems and stolon which grow up to 5 m long and 1 m height. Leaves and leaf sheaths are generally hairy; leaves are 6-20 cm long and 1-2 cm wide. Dry matter yield of 4-7 t/ha has been achieved in pastures with no N fertilizer, if it is used about 10-15 t/ha/year (Cameron, 2011). But dry matter yield of 20 t/ha/year in north-east of Thailand and no production differences between 45-day and 60-day cutting intervals. Absolutely it is found in space farm area. In the way of disease control system in the pig farm, para-

grass was cut every week to keep it clean and good environmental. So, para-grass is the waste as well as organic waste from farm.

2.7 Co-digestion

Co-digestion is a waste treatment method where different types of wastes are treated together. Wu*, et al*.(2010) indicate that significant increases in volumetric biogas production can be achieved by adding carbon rich agricultural residues to the co-digestion process with swine manure. The main reason for co-digestion of feedstock is the adjustment of the carbon-to-nitrogen (C/N) ratio. Microorganisms generally utilize carbon and nitrogen in the ratio of 25–30:1 (Ward *et al.*, 2008). Lansing *et al*. (2010) proved that co-digesting used cooking grease with swine manure in low-cost digesters is a simple way to double energy production. A small volume of grease (2.5%), which corresponded to a 113% increase in organic matter, increased methane production by 124%.

2.8 Biochemical methane potential (BMP)

The biochemical methane potential test is normally conduct in laboratory setting using replicated serum bottles. It is conducted in the batch sample to monitoring biomass conversion. So, only biogas production and methane are monitoring. The digesters could not be opened to obtained organic matter samples prior to the experimental period (Lansing *et al*., 2010). All cumulative biogas productions are measured using displacement method. The biogas composition was determined by gas chromatography. The modified Gompertz equation (Equation 2.17) is employed to fit the cumulative methane production data (Ho and Sung, 2010):

$$M = P \times \exp\{-\exp[\frac{R_m \times e}{P}](\lambda - t) + 1)\} \quad (2.17)$$

Where; M = Cumulative methane production (ml)

e = exp(1); 2.71828

R_m = Maximum specific methane yield production rate (ml/day)

P = Methane production potential (ml)

λ = Lag phase time (days)

The advantages of BMP are realistically measure anaerobic biodegradability, realistically measure of residual organic pollution and require minimal labor to set up (Speece, 1996).

BMP assays are a method to evaluate the potential to produce biogas. BMPs are a practical, lab-based approach to identifying and evaluating potential feedstock for anaerobic digestion. Potential anaerobic digestion feedstock are commonly evaluated by three criteria (Faivor, 2010).

Feedstock characterization: Both before and after BMP assay, includes pH, chemical oxygen demand (COD), total solids (TS), and volatile solids (VS). Characterization results found prior to the experiment are used to determine the quantity of feedstock needed to maintain the BMP assay for as much as 30 days. Characterization results following the completion of the BMP assay are used to evaluate the anaerobic digestion process in terms of the destruction of the organic material.

Total biogas production: It is measured throughout the BMP either through manual means or continuously by commercial software designed for tracking gas production. Biogas can be scrubbed of the carbon dioxide by running it through a potassium/sodium hydroxide solution to monitor only methane production or can be left it to monitor the total biogas production.

Biogas analysis: Biogas composition can be investigated by means of a gas chromatograph during the BMP assay. Though the capital investment is large, gas chromatographs provide accurate measurements of the constituents of the biogas produced during the BMP. Gas chromatographs can be set up to determine the concentrations of methane, carbon dioxide, nitrogen, and hydrogen sulfide gases.

Table 2.11 Show the importance remarks for the BMP assay

Items	Descriptions
Substrate	- should be characterize TS, VS, COD, N, and P - for the energy crop and agro-waste should characterize lignin, cellulose, and hemicelluloses
Inoculums	- should be fresh from active anaerobic digester - should be homogenous - should be degassed or pre incubation until no significant methane production
Medium	- necessary nutrients/ micronutrient/ vitamins are needed for optimal function of anaerobic microorganisms
Blank and control	- determined in blank assays with medium or water and no substrate - blank should be done in triplicate - determined in control with cellulose standard for crops or agro-waste and with gelatin for meat - control can be one or more vessels
Replicates	- should be at least three for each dilution
Mixing	- prevent the accumulation of substrates and intermediate and make the homogenous condition

Source: (Angelidaki *et al,* 2009)

Raposo *et al.* (2006) studied in difference ratio of I/S of 3, 2, 1.5, and 1 from maize. The reactor was incubated at 35 ^{o}C through the experiment. The result showed that I/S ratio of 1 gave the maximum methane production rate 23 mlCH_4 g/VS/day. This result was the same in batch anaerobic test of *Microcystis* spp. that studied in difference I/S ratio of 2, 1, and 0.5. It was found I/S ratio at 1 gave the maximum yield average 153.66 ±3.31 ml/gVS_{added} (Zeng *et al.*, 2010). According to (Labatut *et al*., 2011) used BMP assay to determine the biomethane potential and biodegradability with mono-and co-digestion samples with dairy manure. Therefore I/S ratio of 1 was used to maximize degradation rates.

Raposo *et al.* (2008) studied to evaluate the BMP of sunflower oil cake (SuOC) at difference of ISRs of 3.0, 2.0, 1.5, 1.0, 0.8, and 0.5. The results showed that the ultimate methane yield decreased from 227 ± 23 to 107 ± 11 ml CH_4/ gVS_{added} when the ISR decreased from 3.0 to 0.5.

2.9 Temperature Phased Anaerobic Digester (TPAD)

TPAD is the one of anaerobic digestion processes that biomethane can hold great potential by enhancing the efficiency in the effluent quality, good performance of the organic matter removal, high digestion rate, methane yield, volatile solid reduction, process stability, and pathogen control (Riau *et al*., 2010; De La Rubia *et al*., 2009). It has two phases of temperature that include of thermophilic (operated at approximately 55 ^{o}C) which this phase require additional energy to heat the digester, the effluent quality of sludge are poor (Song *et al*., 2004). And mesophilic is operated at approximately 35 ^{o}C. The TPAD process could be operated at higher loading rates compared to single-stage processes. Mesophilic digester in a TPAD system has a longer HRT than the thermophilic digester so that sufficient microbial biomass of acetogens and methanogents can be stayed in for long time (Lv *et al.*, 2010). TPAD systems are operated by feeding the feedstock into the first, thermophilic digester and then transferring the digestate into the second, mesophilic digester. So, the four steps of biomethanation process can be separated, with hydrolysis and acidogenesis (or fermentation) primarily occurring in the thermophilic digester, while acetogenesis and methanogenesis takes place mainly in the mesophilic digester (Riau *et al.*, 2010). The digester of the first phase is increased temperature from 35 ^{o}C to 55 ^{o}C. Therefore, Bouskova *et al*.(2005) studied the adaptation of stable mesophilic reactors to thermophilic temperature by applying one-step, or step-wise increase of the temperature. The results showed that one-step is the best in changing from mesophilic to thermophilic operation in anaerobic digestion plants. In addition, the microbes from the thermophilic are transferred to the mesophilic digesters during TPAD operations and many of these microbes can survive and function in the latter digester (Lv *et al*., 2010). The performance of the mesophilic and thermophilic co-phase anaerobic

digestions depend on the sludge exchange rate between the mesophilic and thermophilic digesters (Song *et al.*, 2004).

The optimal thermophilic: mesophilic digester volume ratio may vary for different feedstocks. Lee *et al.*(2009) studied lab-scale TPAD process are operated continuously, and fed with co-substrate composed of dog food and flour. It consisted of two stages, in the first stage, a step feeding reactor was operated at the thermophilic condition, followed by anaerobic sequencing batch reactor (ASBR) operated at the mesophilic condition in the second stage. Each reactor has a working volume of 4.2 L (thermophilic condition) and 11.5 L (mesophilic condition), and a steel stirrer at 200 rpm was used for stirring. Under these conditions, the model predicted reasonably well the dynamic behavior of the TPAD process for verifying the model. De la Rubia *et al*.(2009) demonstrated that TPAD systems with a thermophilic: mesophilic digester volume ratio of 1:5 or smaller is more efficient with respect to VS removal than TPAD systems that has a higher thermophilic: mesophilic digester volume ratio. (Dugba and Zhang, 1999) studied in two-stage anaerobic sequencing batch reactor (ASBR) system for dairy wastewater treatment. The thermophilic: mesophilic digester volume ratio of 1:4 is better than a TPAD system with the volume ratio of 1:2 at the same overall system solid retention time (SRT). Song *et al*.(2004) studied the performance of temperature co-phase anaerobic digestion system consisted of a flow through thermophilic digester (5 L) and mesophilic digester (13.6 L) for sewage sludge is obtained from a municipal wastewater treatment plant in B metro city.

Advantages of TPAD:

- It permits the selection and enrichment of different microorganisms in each digester.
- It increases the stability of the process by controlling the acidification stage in order to prevent over loading.
- The first stage prevents pH shock to the methanogenic population.

Disadvantages of TPAD

- The efficient quality and ability to dewater the residual sludge are poor
- Require additional energy to heat the digester

Table 2.12 Operational parameters of TPAD

Substrate	Reactor type	Reactor volume (L)		HRT (d)	OLR (gVS/l/d)	VS remove (%)	Methane yield (m^3 CH_4/Kg VS_{added})	Reference
		1st TP	2nd MP					
Sewage sludge	CSTR	5	13.6	21	-	-	-	Song *et al*., 2004
Raw sludge	CSTR	5	10	20	1.8	78	0.52	Riau *et al.,* 2010
				18		87	0.62	
Dairy wastewater	ASBR	3	12	3	2,3,4,6,	35.06	0.65	Dugba and Zhang,
		5	10	3	8	30.29	0.60	1999
		3	12	6		39.21	0.54	
		5	10	6	2,3,4	12.77	0.45	
Dairy manure	ASBR	3	12	6	1	40.6	0.32	Zhang, 2000
					2	47.5	0.32	
					3	41.7	0.31	
					4	38.9	0.29	
Pig manure	ASBR	3	12	6	1	84.0	0.65	Zhang, 2000
					2	85.9	0.58	
					3	68.7	0.56	
					4	65.5	0.55	

2.10 Denaturing Gradient Gel Electrophoresis (DGGE)

Denaturing gradient gel electrophoresis (DGGE) is a molecular fingerprinting method. It is based on electrophoresis of PCR amplified rRNA gene in polyacrylamide gels. Which used to separates DNA products that generated from polymerase chain reaction (PCR). The polymerase chain reaction of environmental DNA can generate templates of differing DNA sequence that represent many of the dominant microbial organisms. Although, PCR products have the same length (bp), but DGGE can separate PCR products based on sequence differences that results in differential denaturing characteristics of the DNA. PCR products migrate through a polyacrylamide gel and it will begin to denature at which time migration slows dramatically. Differing sequences of DNA (from different bacteria) will denature at different denaturant concentrations resulting in a pattern of bands. By the theoretically, each band represent a different bacterial population that present in the community. The quality of the DGGE is determined by the quality of the PCR products. Therefore this technology has been used widely in environmental microbiology to study diversity and populations.

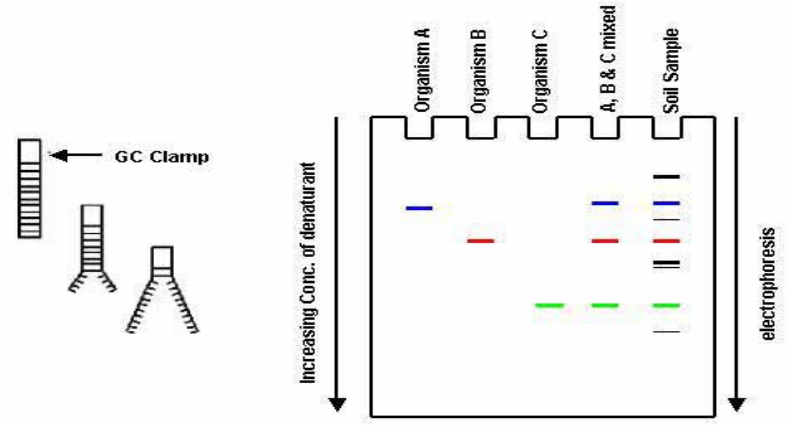

Figure 2.3 Migration of PCR products through a polyacrylamide gel (Ward and Bora 2004)

CHAPTER III

MATERIALS AND METHODS

There are three main experimental parts for co-digestion between pig manure (PM) and para-grass (PG) in this study consisting of biochemical methane potential (BMP assay). Second part is laboratory-scale anaerobic sequencing bath reactor (ASBR) under single stage of mesophilic and temperature-phased anaerobic digestion (TPAD). Last part is comparative of feeding pattern of continuous feeding and periodic feeding under single stage mesophilic reactor. The microbial community in the sludge from each part was tested by DGGE, as shown in Figure 3.1.

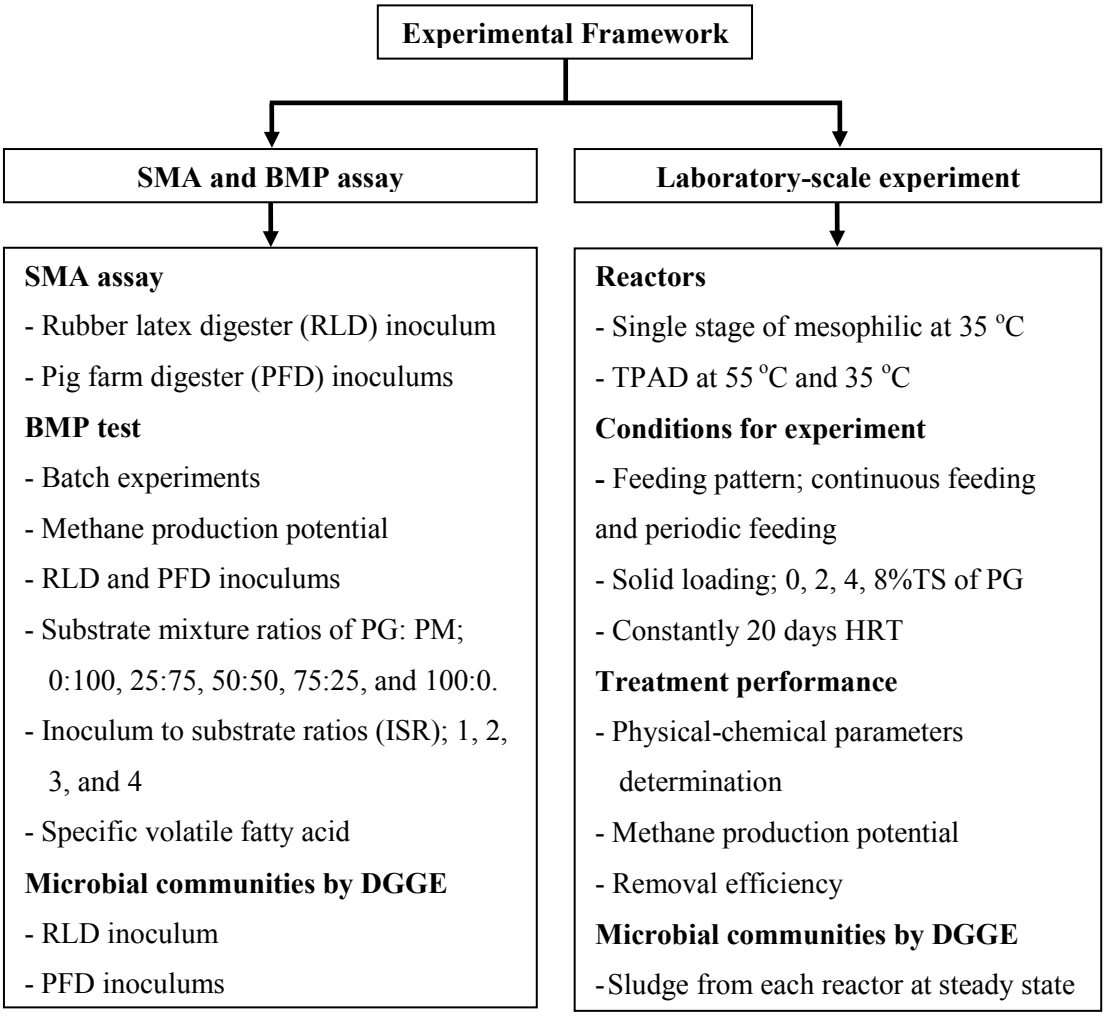

Figure 3.1 Experimental frameworks.

PART I. EFFECTS OF INOCULUM TO SUBSTRATE RATIO, SUBSTRATE MIX RATIO AND INOCULUM SOURCE ON BATCH CO-DIGESTION OF GRASS AND PIG MANURE

3.1 Inocula

Inocula used in this experiment were the anaerobic sludge taken from two different full scale anaerobic digesters; it was conducted from a concentrated rubber latex industry (Figure A.1 in Appendix A) and from a commercial pig farm (Figure A.2 in Appendix A). These anaerobic sludges collected were tested for total solid (TS), volatile solid (VS) concentrations and chemical compositions, and used in the experiments within 24 hours after field collection.

3.2 Substrates

Pig manure (PM) and para-grass (PG) were used as co-substrate in this study. PM was taken from the hog finishing unit. It was dried and pulverized in a mortar. The fresh green para-grass (PG) was harvested from the same commercial pig farm the anaerobic sludge was taken. It was chopped with an agricultural cutting machine, then dried and ground to a small particle. Both substrates were stored at 4 ^{o}C until use in the experiments (Figure A.3 and Figure A.4 in Appendix A).

3.3 Specific methanogenic activity assay (SMA assay) and biochemical methane potential assay (BMP assay)

Inoculum activity test was performed using SMA assay to evaluate the activity of methanogens in the sludge from both sources. Biogas production and its composition were measured every hour for 24 hours with a graduate glass syringe. BMP assay were tested for the methane production potential per dry weight basis. Biogas production was monitored daily during the experiment for 45 days in a similar manner to SMA assay (Figure 3.2).

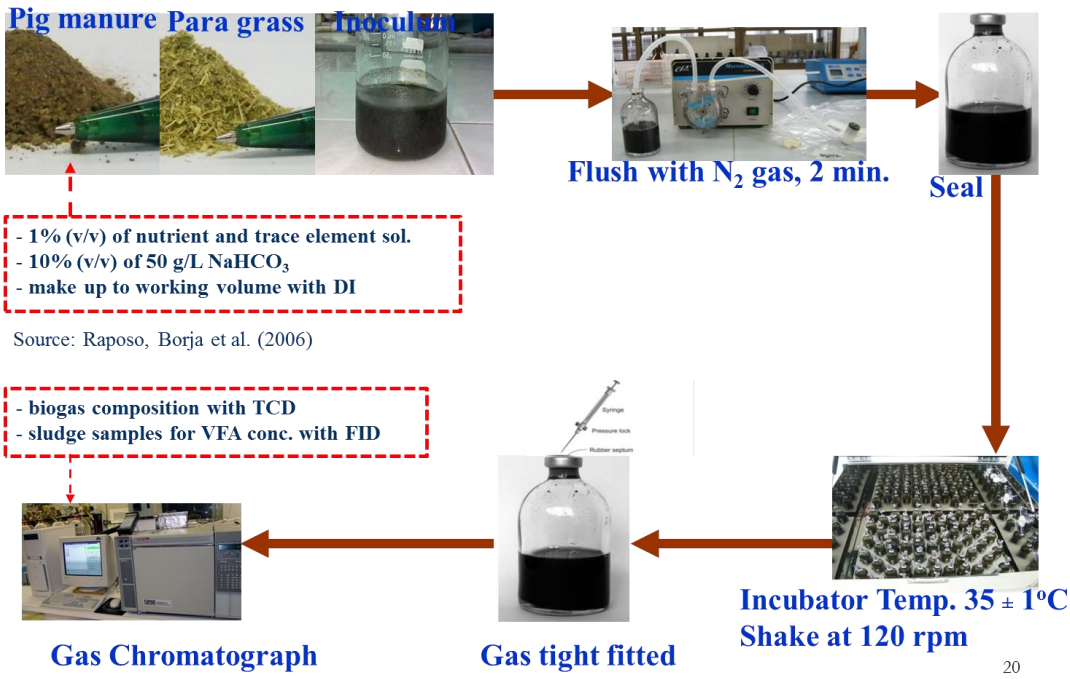

Figure 3.2 Diagram of Biochemical methane potential assay (BMP assay)

3.4 Experimental design and modeling

Inoculum samples from two sources were tested for methanogenic activity (SMA assay). Meanwhile, the microbial community of bacteria was analyzed using DGGE technique to identify the microbiological representation within each sludge for comparison.

In BMP experiment, two variables, inoculum to substrate ratio (ISR) and para-grass mix ratio (G), were investigated for each inoculum. Performance of the methane production was evaluated using a full factorial experimental design in triplicate at 4 levels of ISR (1, 2, 3, and 4), and 5 levels of G (0, 25, 50, 75, and 100 %).

The VFA species profiles and methane generation were taken at ISR=4 using pig farm digester (PFD) inoculum. This condition gave the highest methane yield from the preceding experiments. A set of this BMP assay was conducted at various substrate mix ratios for duration of 20 days where low or no change of the VFAs and methane production were detected. All tests were run in triplicate.

3.5 Analytical methods

3.5.1 Inocula and substrates

Inocula and substrates (para-grass and pig manure) were analyzed for TS and VS. The chemical compositions of the samples were analyzed using CHNS-O Analyzer, CE Instruments Flash EA 1112 Series, Thermo Quest, Italy with Dynamic Flash Combustion Technique.

3.5.2 Microbial community analysis by denaturing gradient gel electrophoresis (DGGE)

Sludge samples were taken from the rubber latex factory digester and the pig farm digester for microbiological analysis by using DGGE.

3.5.3 Individual volatile fatty acids (VFAs) and biogas composition

The supernatant from the serum bottles was collected and analyzed by gas chromatography (GC 7820A Agilent Technologies). Biogas composition was analyzed by gas chromatography (GC 7820A Agilent Technologies) equipped with thermal conductivity detector (TCD) where helium was used as carrier gas.

NOTE: The details can see in the chapter IV.

PART II. CO-DIGESTION OF SWINE MANURE AND GRASS IN SINGLE STAGE MESOPHILIC VERSUS TEMPERATURE-PHASED ANAEROBIC CONDITIONS

3.6 Reactor systems

In this study conducted 2 anaerobic systems for co-digestion of pig manure (PM) and para-grass (PG); single stage mesophilic reactor (Meso-Single) and two stage temperature-phased anaerobic digestion (TPAD) system which consisted of first stage thermophilic reactor (Thermo-1st) followed by second stage mesophilic reactor (Meso-2nd). All reactors were made of glass bottle. The water bath was used for temperature controlled to meet the target (35 ^{o}C or 55 ^{o}C).

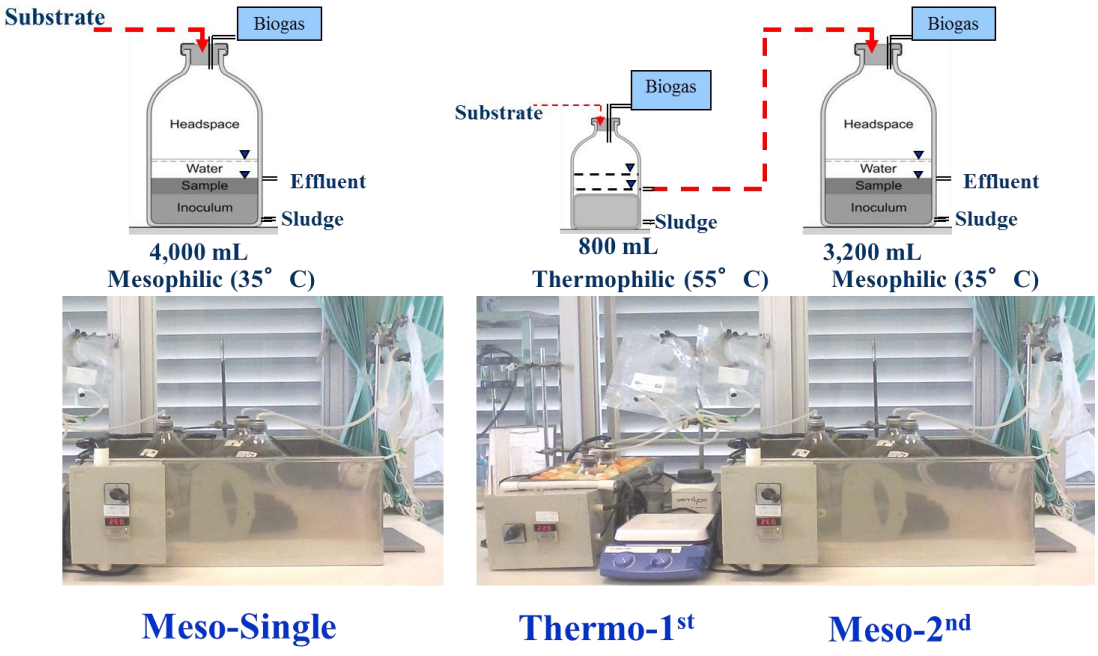

Figure 3.3 ASBR configuration: single stage of mesophilic (Meso-Single) and Temperature-phase anaerobic digester (TPAD); Thermo-1st and Meso-2nd)

3.7 Inoculum

The inoculum used in this laboratory scale experiment was collected from UASB (up-flow anaerobic sludge blanket) reactor treating pig waste slurry from the unit of finishing barn. Then it was measured for total solids (TS) and volatile solid (VS) concentration. Specific methanogenic activity (SMA) was also performed to test the methanogenic activity of sludge to be used to shorten the startup period.

3.8 Preparation of feed

Pig manure (PM) was obtained from the finishing unit in a pig farm. It was dried and ground in a mortar to small particles. The fresh green para-grass (PG) was randomly harvested from the commercial pig farm where PM was obtained. It was chopped with an agricultural cutting machine then dried and ground to the small particles and kept at 4 ^{o}C until use. The PM prepared was mixed with tap water before feeding.

3.9 System operation

The reactors were started up with an initial active sludge of 30% of the effective volume as inoculum and filled up with the prepared pig slurry wastewater (2.5 g/L) to an effective volume. Then reactors were rested for approximately 24 h before the scheduled feeding began. The organic loading (OLR) of the systems was increased from 0.10, 1.02, 1.93, and 3.76 gVS/L/d which corresponded to PG mixing of 0, 2, 4, and 8 % TS in feed. All conditions were run at 20 days HRT.

3.10 Analytical method

3.10.1 Inocula and substrates

Inoculum and substrates (PM and PG) were analyzed for TS and VS. The chemical compositions of the samples were analyzed using CHNS-O Analyzer. The methanogenic activity was tested before used as an initial sludge.

3.10.2 System performance analysis

Performance of the digesters was evaluated by the determination of pH, total COD (TCOD), soluble COD (SCOD), and volatile solid (VS) of the influent and effluent while alkalinity and VFA were determined by direct titration method. Individual volatile fatty acids (VFAs) were analyzed by gas chromatography (GC 7820A Agilent Technologies) equipped with a flame ionization detector (FID). A capillary column was used with helium as the carrier gas.

3.10.3 Biogas production and composition

The biogas produced was stored in gas bag and the volume was measured daily using a multi-chamber rotor wet gas meter (Ritter). Biogas composition was analyzed twice a week by gas chromatography (GC 7820A Agilent technologies) equipped with thermal conductivity detectors (TCD). Helium was used as carrier gas.

3.10.4 Microbial community analysis by denaturing gradient gel electrophoresis (DGGE)

Sludge samples for microbiological analyses were taken from the reactor after finished each condition. The DGGE was used for microbial community analysis.

3.11 Data analysis

The efficiency of acidification was quantified using the percentage of the initial substrate concentration converted to total VFA (TVFA). Therefore, the hydrolysis - acidification stage is sub-divided into two steps. Degree of hydrolysis can be expressed as the quotient between the effluent COD in filtered sample (SCOD) and influent COD in total sample (TCOD). And the acid phase digestion can be quantified using the percentage of the initial substrate concentration (influent TCOD) converted to VFAs.

The data at stable condition of each operating condition were analyzed using the data analysis toolbox in software Microsoft Excel. Mean and the standard deviation were calculated and used to compare the effect of each variable in the experiment. The comparison of means was carried out with SPSS software version 11.0 by one way analysis of variance (ANOVA) and Scheffe's multiple-range test.

NOTE: The details can see in the chapter V.

PART III. EFFECT OF FEEDING PATTERN ON CO-DIGESTION OF PIG MANURE AND GRASS UNDER MESOPHILIC ASBR

3.12 ASBR system design

The single stage mesophilic reactor was used in this part of an experiment. That compared the two feeding patterns; first pattern is regular feeding (RF) that fed continuously with the constant flow in every day and the other pattern is periodic feeding (PR) that fed for 11 days and then stop feeding until day 26. Both reactors were made from 5 L glass bottle. Temperature was maintained at 35±1 ^{o}C by using water bath.

3.13 Inoculum

The inoculum was collected from UASB (up-flow anaerobic sludge blanket) reactor treating pig waste slurry from finishing barn. Then it was measured for total solids (TS) and volatile solid (VS) concentration. Specific methanogenic activity (SMA) was also performed to test the methanogenic activity of sludge.

3.14 Preparation of feed

Pig manure (PM) was obtained from the finishing unit in a pig farm. It was dried and ground in a mortar to small particles. The fresh green para-grass (PG) was harvested from the commercial pig farm. It was chopped with an agricultural cutting machine then dried and ground to the small particle and kept at 4 ^{o}C until use. The PM and PG prepared was mixed with tap water before feeding.

3.15 Feeding pattern

Two feeding patterns applied were regular feeding (RF) and periodic feeding (PR). Both reactors were operated under 20 days hydraulic retention time (HRT). Meso-CF was continuously fed at a flow rate 200 mL/d. The PR was fed at a flow rate

364 mL/d for 11 day and unfed for 15 day period. The biogas was measured from day 1 until day 26. Both experiments were run in repeated for 3 cycles with constantly at 20 days HRT.

3.16 Experiment operation

The reactors were started up with initial active inoculum of 30% of an effective working volume (1,200 mL) and filled up with prepared wastewater to an effective volume. The reactors were then rested for approximately 24 h before the scheduled feeding began. After stable conditions, the feeding pattern was applied with the first experiment at OLR 0.10 gVS/L/d (0%PG). This condition was run and subsequently fed with the mixtures of PM and PG (2, 4 and 8 % TS or OLR 1.0016, 1.931, and 3.761 gVS/L/d).

3.17 Analytical method

3.17.1 Inocula and substrates

Inoculum and substrates (PM and PG) were analyzed for TS and VS. The chemical compositions of the samples were analyzed using CHNS-O Analyzer. The methanogenic activity was tested before used as an initial sludge.

3.17.2 System performance analysis

Performance of the digesters was evaluated by the determination of pH, total COD (TCOD), soluble COD (SCOD), and volatile solid (VS) of the influent and effluent while alkalinity and VFA were determined by direct titration method. Individual volatile fatty acids (VFAs) were analyzed by gas chromatography (GC 7820A Agilent Technologies) equipped with a flame ionization detector (FID). A capillary column was used with helium as the carrier gas.

3.17.3 Biogas and methane content

The biogas produced was stored in gas bag and the volume was measured daily using a multi-chamber rotor wet gas meter (Ritter). Biogas composition was analyzed twice a week by gas chromatography (GC 7820A Agilent technologies) equipped with thermal conductivity detectors (TCD). Helium was used as carrier gas.

3.17.4 Microbial community analysis by denaturing gradient gel electrophoresis (DGGE)

Sludge samples for microbiological analyses were taken from the reactor after finished each condition. The DGGE was used for microbial community analysis.

3.18 Data analysis

The efficiency of acidification was quantified using the percentage of the initial substrate concentration converted to total VFA (TVFA). Therefore, the hydrolysis - acidification stage is sub-divided into two steps. Degree of hydrolysis can be expressed as the quotient between the effluent COD in filtered sample (SCOD) and influent COD in total sample (TCOD). And the acid phase digestion can be quantified using the percentage of the initial substrate concentration (influent TCOD) converted to VFAs.

The data at stable condition of each operating condition were analyzed using the data analysis toolbox in software Microsoft Excel. Mean and the standard deviation were calculated and used to compare the effect of each variable in the experiment. The comparison of means was carried out with SPSS software version 11.0 by one way analysis of variance (ANOVA) and Scheffe's multiple-range test.

NOTE: The details can see in the chapter VI.

CHAPTER IV

EFFECTS OF INOCULUM TO SUBSTRATE RATIO, SUBSTRATE MIX RATIO AND INOCULUM SOURCE ON BATCH CO-DIGESTION OF GRASS AND PIG MANURE

Abstract

Biochemical methane potential (BMP) assay was conducted at 35^{o}C to evaluate the effects of inoculum to substrate ratio (ISR) and substrate mix ratio between para-grass and pig manure co-digesting using different inocula. Rubber latex digester (RLD) inoculum showed higher metanogenic activity (41.4 $mLCH_4/gVS$) than pig farm digester (PFD) inoculum (37.3 $mLCH_4/gVS$). However, the maximum methane yields, occurred at the highest para-grass mix ratio (G), were 369.6, 437.6, 465.9 and 442.6 $mLCH_4/gTS_{added}$ for RLD inoculum, versus 332.4, 475.0, 519.5 and 521.9 mL/gTS_{added} for PFD inoculum at ISR 1, 2, 3, and 4, respectively. HPr, HBu and HVa appeared at higher G, corresponding to substrate's higher biodegradability. Response surface indicated that higher ISR and G had a significantly positive impact on methane yield. It suggested the use of higher ISR, i.e. 3 or 4, for BMP assay of these co-substrates. Dominant species of fermentative bacteria in each inoculum was tested by DGGE.

Keywords: Biochemical methane potential (BMP); Inoculum to substrate ratio (ISR); Co-digestion; Pig manure; Grass

4.1 Introduction

Thailand raises an average of 9 million pigs a year, generating approximately 5 billion kilograms of manure annually (DLD, 2012). Natural degradation of pig manure is leading to emissions of methane gas during storage in open lagoons. This contributes to global warming resulted from the release of greenhouse gases, largely methane to the atmosphere (Moller *et al*., 2004; Pattanapongchai and Limmeechokchai, 2011). With a more stringent regulation on pig farms, treatment of wastewater from pig production operations is nowadays of utmost importance to farm's survivability. Anaerobic digester has been widely accepted in pig business as a way to treat their waste and produces biogas as a by-product. The biogas is generally converted to electricity and heat for use mainly in cooling and heating systems in the farm. These pig farms become energy intensive with modern animal growing technology.

Bioresource within the farm boundary that can be used to produce energy is of interest since bringing in the off-farm feedstock is against a farm disease control protocol. The possibility of co-digesting para-grass with pig manure in the farm's existing digester becomes a valid approach to enhance biogas production. Para-grass (*Branchiria mutica*) is the weed of no value and pervasive around the farm, particularly in tropical climate. It is a burden to the farm since it needs to be cut down and removed frequently for fire hazard, and disease and vector controls. Not only that addition of carbonaceous substrate such as grass to the low C:N pig waste (around 13:1) is deemed appropriate microbiologically as it helps raise C:N of the feedstock to be suitable for metabolic activities in anaerobic digestion system (Xie *et al*., 2011), but it can also provide incentive for weed management. Nevertheless, due to the different characteristics of both substrates, the effect of this para-grass mixing needs to be known.

Anaerobic digestion is a complex biological process used to treat organic wastes. There are four steps defined in anaerobic digestion; hydrolysis, acidogenesis,

acetogenesis and methanogenesis. At the first step, the complex molecules of carbohydrates, fats and proteins were broken into soluble organics by the enzymatic action of hydrolytic fermentative bacteria. These soluble organics were then converted to organic acids, alcohols, H_2 and CO_2 by acidogenic bacteria. The organic acids, so-called volatile fatty acids (VFAs), and alcohols were subsequently converted to acetic acid by the H_2-producing acetogenic bacteria, which are subsequently transformed into CH_4 by methanogens, a subgroup of archea (Angelidaki *et al*., 2009; Lozano *et al*., 2009). All processes take place in an oxygen free environment yielding methane, carbon dioxide and traces of other gases. The effectiveness of this digestion process is mostly based on the volume of methane produced. The methane production potential can be expressed per dry mass of the substrate (added or destroyed) for solid digestion, or per mass of substrate COD (added or destroyed) for liquid digestion. The last stage methanogenic conversion is inevitably dependent on the preceding steps.

Different kinds of substrate give different methane production, which can be evaluated using the biochemical methane potential (BMP) assay. The BMP assay is a useful tool to determine the ultimate biodegradability and methane conversion yield of organic substrates (Angelidaki *et al*., 2009). Numerous studies have been carried out in which the BMP assay of crop species, wastes and other forms of biomass were measured (Lopes *et al*., 2004; Xie *et al*., 2011; Ho and Sung, 2010; Labatut *et al*., 2011; Raposo *et al*., 2006; Raposo *et al*., 2008; Rincon *et al*., 2010). In this assay, the inoculum to substrate ratio (ISR) was identified as a key parameter affecting the efficiency of anaerobic degradation and more importantly the accuracy of the assay. Previous studies have shown that increasing ISR positively affected the ultimate practical methane yield. Batch digestion test on microalgae showed the ISR of 2, compared to 1 and 0.33, gave highest methane productivities ranging from 188 to 395 $mLCH_4/gVS_{added}$ over different types of micro algae (Alzate *et al*., 2012) while the digestion of sunflower oil cake (SuOC) at ISR 3, compared to 2, 1.5, 1, 0.8 and 0.5 gave the highest ultimate methane yield (Raposo *et al*., 2009). Although at low ISR, maximum specific methane production rate was higher (mL CH_4/gVSS/d), the methane production yield (mL $CH_4/gVSS_{added}$) was lower from the BMP test of maize

at ISR 3, 2, 1.5, and 1 (Raposo *et al*., 2006). This was associated with the accumulation of longer chain acids (HPr, HBu and HVa) within the system. The acetate produced during the digestion at high substrate concentration (low ISR) could inhibit methanogens within the consortia (Maya-Altamira *et al*., 2008). More substrate dilution, which is equivalent to raising ISR, could help improve the practical methane yield. These literatures, nonetheless, reported the influence of ISR on methane yield based on single substrate. The effects of ISR for co-digestion were very poorly documented. Moreover, the source of inoculum can play a vital role in the substrate degradation efficiency, especially for the complex mix of substrates, due to the different makeup of the microbial consortia within.

The objectives of this study were to evaluate the influence of ISR, substrate mix ratio and inoculum source on methane production potential in anaerobic digestion. Multiple regression was used to illustrate the relationship of these parameters, which elucidates the impacts of each parameter interactively. Information acquired from this study could provide a better understanding and the practicality on the batch test BMP assay for anaerobic co-digestion, employing the case study of pig manure and para-grass as co-substrates.

4.2 Methods

4.2.1 Inocula

Inocula used in this experiment were the anaerobic sludges taken from two different full scale anaerobic digesters; one from a commercial pig farm in Songkhla Province and another from a concentrated rubber latex factory in Songkhla Province, Thailand. The first digester is digesting pig waste slurry with high nitrogen and solids, while the latter is treating wastewater with high sulfate concentration from concentrated rubber latex processing. General characteristics of the wastewaters fed to these two digesters, the pig waste slurry and concentrated latex wastewater, were similar to those reported in Panichnumsin *et al*. (2010) and Saritpongteeraka and

Chaiprapat, (2008), respectively. This feed affected the makeup of microbial consortia in each sludge. These anaerobic sludges collected were tested for total solid (TS), volatile solid (VS) concentrations and chemical compositions, and used in the experiments within 24 hours after field collection.

4.2.2 Substrates

Pig manure and para-grass were used as co-substrate in this study. Pig manure (PM) was taken directly from excretions from the hog finishing (fattening) unit. It was dried at 60 ^{o}C and pulverized in a mortar. The fresh green para-grass (PG), *Branchiria mutica*, was randomly harvested from the same commercial pig farm the anaerobic sludge was taken. It was chopped with an agricultural cutting machine to approximately 2 cm, then dried at 60 ^{o}C and ground to the maximum length of less than 6 mm. Both substrates were stored at 4 ^{o}C until use in the experiments.

4.2.3 Specific methanogenic activity assay (SMA assay) and biochemical methane potential assay (BMP assay)

Inoculum activity test was performed using SMA assay to evaluate the activity of methanogens in the sludge from both sources. The assay was conducted in 120 mL serum glass bottles with 60 mL effective volume containing acetic acid as a substrate according to Ho and Sung (2010). The nutrients stock solution and trace elements solution were supplied to each bottle, which details were described in Raposo *et al*. (2006). Each serum bottle contained 10 gVS/L of inoculum with 0.75 mL of 1 M acetic acid. Three bottles of blank contained only 10 gVS/L of inoculum and filled up with DI water. Biogas production and its composition were measured every hour for 24 hours with a graduate glass syringe.

For BMP assay, the different mixtures of PM and PG were tested for the methane production potential per dry weight basis according to the procedures based on Angelidaki *et al*. (2009). In brief, 120-mL serum bottles were used as fermenting

reactor whose 60 mL was used as effective volume. There were 3 bottles of blank containing only 20 gVS/L of inoculum and filled with DI water to 60 mL. Biogas production was monitored daily during the experiment in a similar manner to SMA assay.

In both assays, each serum bottle was supplemented with 1% (v/v) of nutrient and trace element solution and buffered with 50 g/L $NaHCO_3$ at 10% (v/v). The final volume was adjusted to 60 mL by adding deionized water, and using small amount of 0.1 M NaOH to adjust pH to 7. They were purged with nitrogen gas for 2 minutes and sealed immediately to ensure anaerobic condition. The bottles were placed in an incubator shaker at temperature 35±1 ^{o}C with continuous shaking at 120 rpm.

The modified Gompertz equation (Equation 4.1) was used to calculate the methane production potential and maximum specific methane production rate (Ho and Sung, 2010).

$$H_{(t)} = H \times \exp\left\{-\exp\left[\frac{R_m \times e}{H}(\lambda - t) + 1\right]\right\} \qquad (4.1)$$

where H(t) is cumulative methane production (mL) at time t; e is exp (1) = 2.71828, R_m is maximum specific methane production rate (mL/day), H is methane production potential (mL) and λ is lag phase time (days). The parameters in this equation were estimated by least square method using Solver Function in Microsoft® Office Excel 2003. Note that the data used in methane production calculation was subtracted with blank. The ultimate methane yield was calculated by dividing the predicted cumulative methane production from the BMP assays (H value) by the initial loading based on TS of substrate. Gas measurement was reported at STP condition (standard temperature and pressure, 273 K, 1 atm).

4.2.4 Experimental design and modeling

In SMA assay, inoculum samples from different sources were tested for methanogenic activity. Meanwhile, the microbial community of bacteria was analyzed using DGGE technique to identify the microbiological representation within each sludge for comparison.

In BMP experiment, two variables, inoculum to substrate ratio (ISR) and para-grass mix ratio (G), were investigated for each inoculum. Performance of the methane production was evaluated using a full factorial experimental design in triplicate at 4 levels of ISR (1, 2, 3, and 4), and 5 levels of G (0, 25, 50, 75, and 100 %). Each of the ISRs was achieved by keeping a constant inoculum concentration at 20 gVS/L (Hansen *et al*., 2004), and varying the substrate concentration in the range of 5 to 20 gVS/L (Equation 4.2). All BMP tests lasted for 45 days.

$$ISR = \frac{gVS\ of\ Inoculum}{gVS\ of\ Substrate} \tag{4.2}$$

The relationships of ISR and G to the specific methane production response were explained by quadratic regression model (Equation 4.3). Three dimensional plots were used to visualize the effects and interactions of variables. Software package Essential Regression and Experimental Design for Chemists and Engineers was used to generate 3D response surfaces and contour plots from the derived equations.

$$y = \beta_o + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 + \ldots \tag{4.3}$$

where y is the corresponding response variable, β_o is the constant, β_1,β_2 are the linear coefficients, β_{11},β_{22} are the quadratic coefficients, β_{12} is the interactive coefficient, X_1,X_2 are the actual values of the independent variables.

In the last part of this work, observation of VFA species profiles and methane evolution were undertaken at the condition that gave the highest methane yield from the preceding experiments (ISR=4 using PFD inoculum). This was done to gain information on the balance of acidogenesis and methanogenesis with this co-substrate digestion. A set of batch digestion BMP assay was conducted at various substrate mix ratios for a duration of 20 days where low or no change of the VFAs and methane production were detected. An extra set of the serum bottles identical to those in the experiment was run in parallel in order to provide samples for VFA analysis. Destructive sampling was used where the serum bottles were discarded after sampled for VFA concentrations. All tests were run in triplicate.

4.2.5 Analytical methods

4.2.5.1 Inocula and substrates

Inocula and substrates (para-grass and pig manure) were analyzed for TS and VS according to Standard Methods (APHA, 1999). The characteristics of both inocula and substrates are shown in Table 4.1. The chemical compositions of the samples were analyzed using CHNS-O Analyzer, CE Instruments Flash EA 1112 Series, Thermo Quest, Italy with Dynamic Flash Combustion Technique.

Table 4.1 Characteristics of inocula, para-grass and pig manure

Characteristics	**Rubber latex digester inoculums ***	**Pig farm digester inoculums ***	**Pig manure**	**Para-grass**
Total solid, TS (g/kg wet)	49.9±1.3	82.6±0.9	292.0±3.8	193.0±1.7
Volatile solid, VS (g/kg dry)	39.8±1.1	50.1±0.5	730.8±10.9	883.1±1.3
Moisture (%)	95.0	91.7	72.48	77.25
Carbon (% dry wt.)	1.8	1.4	39.0	41.5
Nitrogen (% dry wt.)	0.34	0.16	3.01	1.29
C:N Ratio	5.2	8.5	13.0	32.2

* Unit of TS and VS are g/L

4.2.5.2 Microbial community analysis by denaturing gradient gel electrophoresis (DGGE)

Sludge samples for microbiological analyses were taken from the rubber latex factory digester and the pig farm digester in separate. Total genomic DNA was extracted from enrichment culture samples by using a slightly modified standard bacterial genomic DNA isolation method according to the procedures in Hniman *et al*. (2011). The bacterial 16S rDNA (w1400 base pair) was amplified by the first polymerase chain reaction (PCR) with universal primer 1492r and 27f. In the second PCR, primer K517r and L340f with CG clamp were used to amplify the fragment of V3 region of 16S rDNA product from the first PCR. Most of the bands were excised from the gel and re-amplified with primer 357f (without a GC clamp) and the reverse primer. After reamplification, PCR products were purified and sequenced using primer 518r (Bacteria) by the Macrogen sequencing facility (Macrogen Inc., Seoul, Korea). Closest matches for partial 16S rRNA gene sequences were identified by ribosomal database project with SeqMatch program and basic local alignment search tool (BLAST) with nucleotide database in National Center for Biotechnology Information (NCBI).

4.2.5.3 Individual volatile fatty acids (VFAs) and biogas composition

The supernatant from the serum bottles was collected and centrifuged at 9000 rpm for 20 minutes, then filtered with a nylon filter (0.22 mm.) before analyzed by gas chromatography (GC 7820A Agilent Technologies). A capillary column Agilent 19091N-133 HP- INNOWax polyethylene glycol was used with helium as carrier gas. Biogas composition was analyzed by gas chromatography (GC 7820A Agilent Technologies) equipped with thermal conductivity detector (TCD) where helium was used as carrier gas. The standard calibration curve was made with gas mixtures containing CH_4 at 3 levels covering the range of 20-99.999%, and verified with a standard gas mixture of 5% N_2, 60% CH_4, and 35% CO_2.

4.3 Results and discussion

4.3.1 Specific methanogenic activity of inoculum from concentrated rubber latex digester (RLD) and pig farm digester (PFD)

The SMA assay was studied with the same inoculum concentration of 10 gVS/L. Biogas and methane production data and the equation line for both inocula are shown in Figure 4.1. The methanogenic activity of inoculum from RLD and PFD were 41.4 $mLCH_4$/gVS and 37.3 $mLCH_4$/gVS, respectively. They were lower than those reported in Ho and Sung (2010) at 51.8 $mLCH_4$/gVS and Ince *et al*. (2001) at 67.0 $mLCH_4$/gVS in 24 hours which measured the sludge from anaerobic membrane bioreactors treating synthetic municipal wastewater and UASB reactor treating pharmaceutical wastewater, respectively. Results from the regression of modified Gompertz equation yielded methane production potential (H) at 25.7±0.2 and 21.8±0.5 mL CH_4 with R_{max} of 3.26±0.10 mL/h and 1.57±0.01 mL/h from RLD inoculum and PFD inoculum, respectively. Lag phase of PFD inoculum (2.89±0.03 h) was noticeably longer than that of RLD inoculum (0.55±0.05 h). Precision of the model was acceptable with R^2 of 0.986 for RLD inoculum and 0.992 for PFD inoculum.

The cumulative biogas productions at 24 hours were 32.67±1.42 mL from RLD inoculum and 28.00±1.73 mL from PFD inoculum. No biogas was produced from RLD inoculum after 24 hours but PFD inoculum still continuously produced up until 30 hours (28.95±0.78 mL). Over times, the total biogas production (at STP) of both inocula should be stoichiometrically close since the substrate (acetate) was all converted. Nevertheless each inoculum generated different methane contents which were measured at 10.7-38.4% (avg. 30.4%) and 2.6-32.6% (avg. 16.8%) from RLD inoculum and PFD inoculum, respectively, over the course of 24 hours.

Inoculum of RLD clearly showed higher methanogenic activity. These results signified the nature of both sources where the main feed was more soluble organics for RLD inoculum compared to the high particulate pig farm wastewater. The RLD inoculum had higher concentration of active microorganisms of both the acetogens and methanogens while PFD inoculum had lower concentration as exhibited by the longer lag phase on methane production. It corresponded well to the real methane composition in the full scale RLD at 79.8±2.5% (Charnnok *et al*., 2013) compared to 64.8±4.1% at PFD (survey data from 15 pig farms in Thailand).

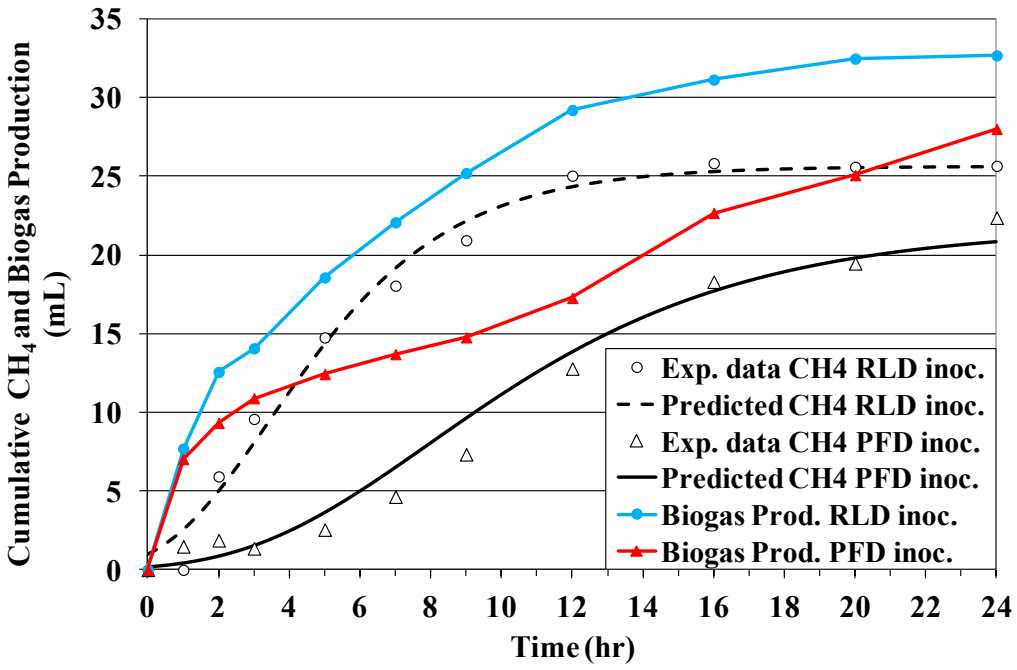

Figure 4.1 Cumulative biogas and methane production from pig farm digester (PFD) inoculum and concentrated rubber latex digester (RLD) inoculum

4.3.2 Microbial community analysis by DGGE

Bacterial communities from two different digesters treating different waste streams were analyzed using DGGE. The profile demonstrated several bands that represented various species of bacteria (Figure 4.2). The most dominant bands found in both sludges had a high sequence similarity to *Acinetobacter* sp., a propionic

producer, and *Halanaerobium* sp. that are known to produce acetate, H_2, and CO_2 from fermentation (Insam *et al*., 2010).

It was noticeable that the distinct bands in rubber latex digester included *Stapphylothermus* sp. that has special enzymes to work well in extreme environments. These species require sulfur for growth and can convert sulfur to hydrogen sulfide. Their appearance can be linked to the source of wastewater from concentrated rubber latex factory which contained high sulfate due to the heavy sulfuric acid use in the production. *Desulfotomaculum* sp. was also found. These sulfate reducing bacteria are highly competitive with methanogens in a digester with high sulfate environment. However, the alkalinity of RLD was rather low at 2,567±212 mg/L as $CaCO_3$ (from field measurement). At such level, methane content in the biogas could theoretically be up to 72-82% at pH 7.2-7.4 (Rittmann and McCarty, 2001) compared to the real value of 78±3% at the factory. Obviously, the sulfide produced was not at a prohibitory level to methanogens and the methanogens in this sludge were very active confirmed by the results of SMA assay. It was interesting to note that *Clostridium* sp. were only found in RLD sludge.

Among the other common bacteria found in the PFD sludge (Figure 4.2), two dominant bands were *Flavobacterium* sp. and uncultured bacterium clone. *Flavobacterium* sp. is able to use chitin as nitrogen and carbon source (Insam *et al*., 2010) and since chitin is similar to cellulose chemically, these bacteria species could have a certain capability to degrade cellulose in the grass. The synergistic reaction between *Flavobacterium* sp. and uncultured bacterium clone may have occurred that resulted in a higher degradation and inhibited the *Clostridium* sp. in PFD inoculum. The existence of this species would play a significant role in the subsequent co-digestion experiments which are discussed in the latter sections.

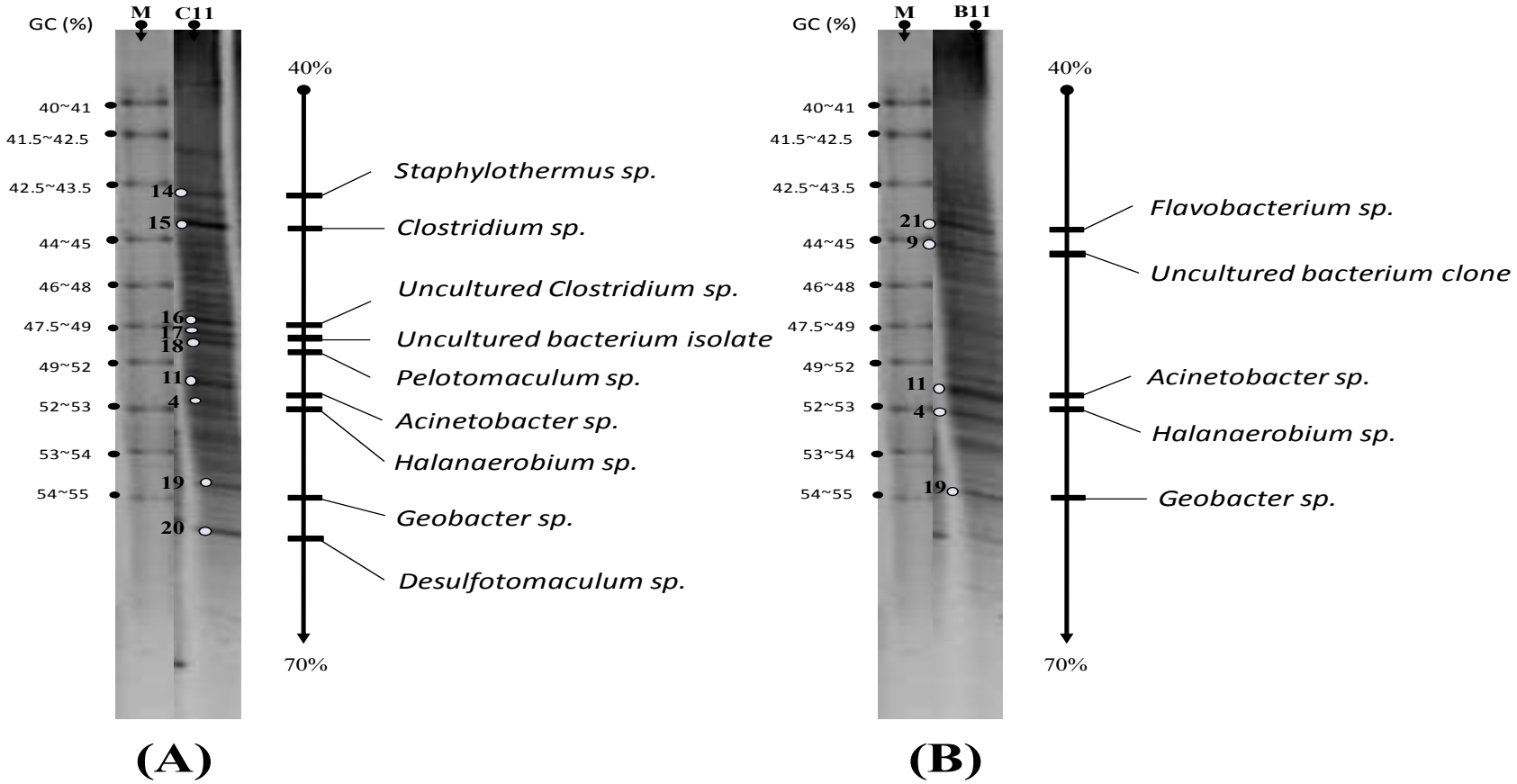

Figure 4.2 Bacterial community profile determined with PCR-DGGE of partial 16s rRNA genes fragments from the sludge of rubber latex digester (A), and pig farm digester (B)

4.3.3 Effects of inoculum to substrate ratio, substrate mix ratio and inoculum sources on methane conversion

The methane yield was calculated by dividing the methane production with dry weight (in TS) of substrate added at different para-grass mix ratios and ISR's (Figure 4.3). Two quadratic regression equations of methane yield were generated to evaluate the effect of ISR and para-grass mix ratio (G) when using the inoculum from RLD (Equation 4.4) and PFD (Equation 4.5). The PFD inoculum model showed slightly higher determination coefficient R^2 of 0.898 and the adjusted R^2 of 0.826 compared to the RLD inoculum R^2 of 0.883 and adjusted R^2 of 0.841. The ANOVA of both quadratic models were very low ($p<0.0002$ and $p<0.0004$) showing the models were highly significant. Therefore, these multiple regressions were used to construct surface and contour plots (Figure 4.3) to describe the responses of methane yield with ISR and G.

$$\text{Methane Yield} = 253.89 - 84.10*ISR + 1.825G + 3.388ISR^2 - 0.00749G^2 + 1.002*ISR*G \quad (4.4)$$

$$\text{Methane Yield} = 141.70 + 131.04*ISR + 0.343*G - 29.84ISR^2 - 0.01741*G^2 + 1.204*ISR*G \quad (4.5)$$

For the RLD inoculum (Figure 4.3A and 4.3B), the maximum methane yields were 369.6, 437.6, 465.9, and 442.6 mL/g TS_{added} at ISR 1, 2, 3, and 4, respectively which were noticeably occurred at the highest grass mix ratio (G). On the other hand, when the substrate had higher pig manure content, the methane yield faced greater variation across the ISR tested. This was due mainly to that the RLD inoculum was unfamiliar with the pig manure, and pig manure was virtually less biodegradable compared to grass. The pig feed was primarily digested in pig intestine that the easy-to-digest portion was assimilated. This is consistent with the results from PFD sludge (Figure 4.3C and 4.3D).

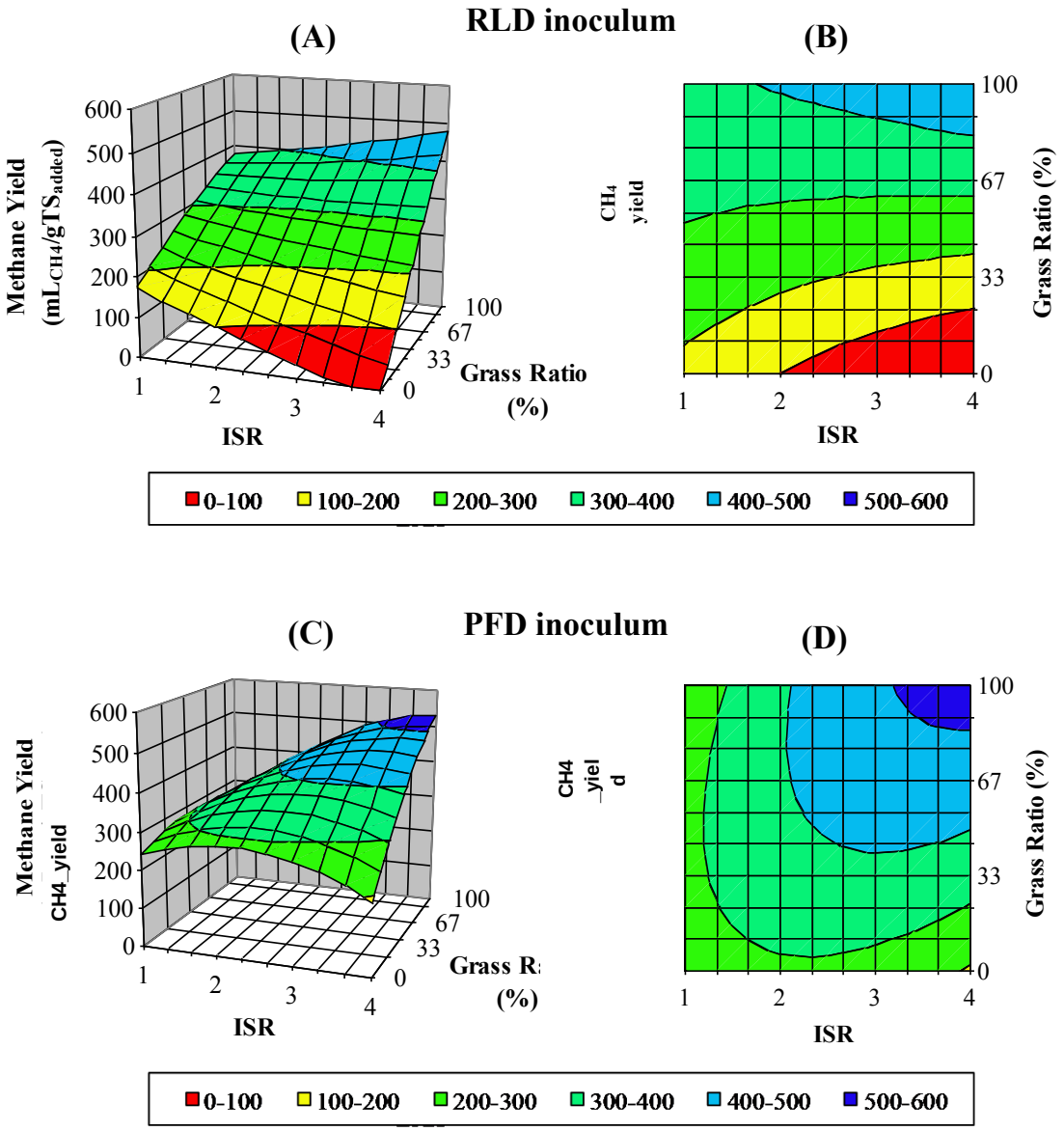

Figure 4.3 Response surface and contour plots for observed methane yield as a function of grass mix ratio and inoculum to substrate ratios (ISR) of batch digestion using the inoculum from concentrated rubber latex anaerobic digester (A, B) and pig farm anaerobic digesters (C, D)

The maximum methane yields were 332.4, 475.0, 519.5, and 521.9 mL/g TS_{added} at ISR 1, 2, 3, and 4, respectively, while the methane yields faced greater

variation at higher ISR. Results from both sludges confirmed that the para-grass, *Branchiria mutica*, was more biodegradable. It should be noted that we placed equal amount of substrate as volatile solids at the start of all treatments. It was interesting to note that the near optimum was able to be identified within the results of PFD inoculum. Higher ISR in combination with the higher grass mix ratio gave higher methane yield. The inoculum from the pig farm contained larger and rather dominant population of *Flavobacterium* sp and uncultured bacterium clone (Figure 4.2) that were capable of digesting lignocellulosic substrate. Use of the inoculum from a more soluble substrate digester is not advisable, and care must be taken seriously in the selection of inoculum source to conduct the batch type BMP assay of solid substrate.

The results also showed methane yields increased by increasing ISR ratio. The decrease in ISR could cause overload, the unfavorable situation where there was too much substrate than the microorganisms to convert it either in the hydrolysis, acidogenesis, or methanogenesis. Lack of methanogens typically leads to VFA accumulation and subsequent acidification of the reactor. Methanogenesis was typically inhibited when pH drops below 6.5 (Neves *et al*., 2004). Lack of hydrolytic microorganisms also brought about the inefficient hydrolysis of the substrate, particularly solid substrates. Thus, higher ISR could provide some kind of safety factor in the BMP assay since the sufficient inoculum is able to process a higher flow of metabolites such as hydrogen, acetate and VFA (Neves *et al*., 2004, Panichnumsin *et al*., 2006). This result was comparable to Raposo *et al*. (2008) that evaluated the methane production potential of sunflower oil cake at different ISRs of 3.0, 2.0, 1.5, 1.0, 0.8, and 0.5 by using inoculums from industrial anaerobic reactor treating brewery wastewater. The results showed the ultimate methane yield decreased from 227±23 to 107±11 mL CH_4/gVS_{added} when the ISR decreased from 3.0 to 0.5. In our study, the higher ISR was favorable for methane yield in the co-digestion. Methane potential in each ISR increased with rising ratio of para-grass. Results indicated BMP assay of solid co-substrate should be carried out at ISR higher than 3 and 4 to obtain a more representative result of methane production potential.

In order to verify the model, the specific (at 45 days) and ultimate methane yields of five para-grass mix ratios with two sources of inoculums were compared side by side at ISR=4 where the highest yields took place (Table 4.2). The results were consistent with the previous graphical displays that the heightened para-grass ratio would increase methane yield in both inocula, and the methane yields from PFD inoculum were superior in every para-grass mix ratio. Besides, PFD sludge also showed the higher maximum specific methane production rate (R_{max}) although the methanogens in RLD sludge were more active. Higher hydrolysis in PFD inoculum treatments enabled the release of soluble substrate from the lignocellulosic structure more effectively. As a result, higher R_{max} was evidenced at higher G but the increase was diminishing as G went above 75 % (75:25) in RLD sludge, while there was not much variation of R_{max} in PFD sludge across G values tested (Table 4.2).

To compare the biodegradability of the mix substrate, the ratio between the specific and ultimate methane yields were used. At only the pig manure substrate (G=0 or 0:100), biodegradability at 45 days, defined as the ratio between specific methane yield over ultimate methane yield, of only 0.15 was observed in RLD sludge as opposed to 0.68 in PFD sludge. This ratio went up to 0.81 and 0.80 in RLD and PFD inoculum, respectively. The more grass mix, the higher biodegradability of the mixture. It might be prudent to stress on the effect of the mix substrates with different characteristics which require a variety or a more diverse microbial culture able to consume a wide range of substances. Perhaps, this could lead to the idea of inoculum cocktail where various inocula should be brought in mix for use in the BMP assay. Such method could minimize or avoid the inconsistency results in the co-digestion assay.

4.3.4 Individual VFAs at different substrate mixtures

The distribution of VFAs species, which come from different pathways in digestion process, provided insight information on the health and status particularly the balance in acidogenesis and methanogenesis of the co-digestion. In the para-grass and pig manure co-digestion test at the selected setting of ISR=4 using PFD inoculum, the changes in VFA species and methane production were continuously monitored until VFA and methane generation were minimal, a total of 20 days. The VFAs were categorized into four species; acetate (C2), propionate (C3), isobutyrate+butyrate (C4), and isovalerate+valerate (C5). Changes in VFAs concentration and methane production rate during digestion are shown in Figure 4.4. The concentration of individual VFAs accumulated in all the grass added treatments (25, 50, 75, and 100%) was found to be in the following order: HPr > HAc > i-HVa+HVa> i-HBu+HBu. In the pig manure only treatment, the order followed the size of the VFA molecules: HAc (C2) > HPr (C3) > i-HBu+HBu (C4) > i-HVa+HVa (C5).

The easily degradable compounds in the grass had been hydrolyzed and acidified in the first few days. The peaks of TVFA occurred (at day 1 or 2) before the methane formation peaks in all para-grass mix ratios at day 3 (Figure 4.4). This trend showed that the rate of hydrolysis for easy degradable compounds in grass and acidogenesis were higher than methanogenesis, and that methanogens required 1-2 days longer to process the peak TVFA to reach their peak. During such period if the buffer was not sufficiently be in place, pH drop may occur within the system. This emphasized the need for buffering especially in high substrate concentration or low microbial cells mass, i.e. low ISR. Methane generation increased drastically during the first 3 days and reached the maximum value at day 3 as similar pattern in all para-grass mix ratios (G) (Figure 4.4).

Table 4.2 Comparison of the methane yields from anaerobic co-digestion with different inocula and para-grass mix ratio at ISR = 4

Mix ratio PG:PM	R_{max} ($STPmLCH_4/day$)		Specific methane yield @45d ($STPmLCH_4/gTS_{added}$)		Ultimate methane yield ($STPmLCH_4/gTS_{added}$)	
	RLD	**PFD**	**RLD**	**PFD**	**RLD**	**PFD**
0:100	0.98	11.62	12.57	257.34	80.67	378.82
25:75	1.12	13.21	65.93	314.05	168.93	436.73
50:50	3.24	14.70	292.77	382.99	482.92	510.96
75:25	10.40	15.62	442.59	452.50	516.93	582.84
100:0	10.33	16.16	417.82	521.93	513.39	655.04

The maximum methane generation on G = 0, 25, 50, 75, and 100 were 431.1, 560.5, 598.5, 608.4, and 612.3 mL/L/day, respectively. The TVFA concentration and biogas production correlated to the heightened G values.

The longer chain fatty acids (HPr, HBu and HVa) occurred at higher G but disappeared within only 3-4 days in the digestion. HPr, HBu and HVa could be converted to HAc thru acetogenesis but it was not thermodynamically favorable: $\Delta G^{o'}$= +76.1, +48.1, +25.1 kJ for HPr, HBu, and HVa, respectively. When coupled with the H_2 consuming reaction, the reactions became feasible thermodynamically giving $\Delta G^{o'}$ = -102.4 for HPr and -39.4 kJ for HBu (Wang *et al*., 1999). Acetogenesis, hydrogenotrophic and methanogenesis were intense during the first 3-4 days period. Subsequently, HPr still persisted as the highest species in the middle time range while HAc concentration lasted the longest as it was the product of HPr, HBu and HVa cleaving. Appearance of these longer chain fatty acids pointed to the higher soluble substrate concentration relative to the inoculum concentration in acidogenesis (Rapaso *et al*., 2006). This fact also designated a more hydrolysable and/or more biodegradable organics in the system, which was, therefore, an indicator of an appropriate substrate for anaerobic digestion. Care must be taken in referring the biodegradability of the grass substrate since age and growing condition of the grass can greatly affect the fiber and lignin contents. If tough fiber was to be degraded, HPr, HBu and HVa should not accumulate to such high level because the hydrolysis rate would be slower while the soluble components could be converted through the then faster acidification and methanogenation.

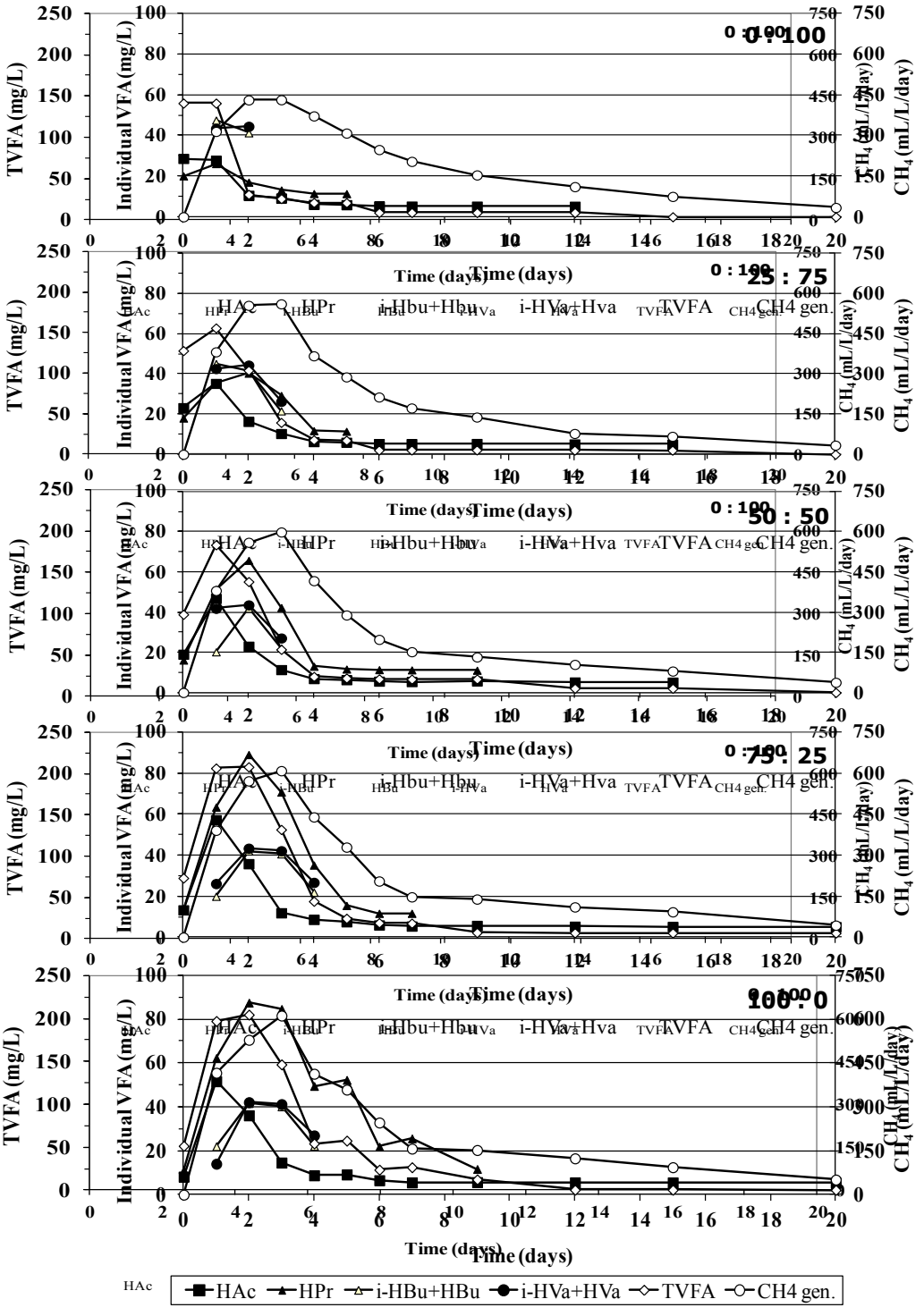

Figure 4.4 Change of VFAs concentration and methane generation from batch digestion with different mix ratios of para-grass and pig manure at ISR 4 using pig farm digester (PFD) inoculum

CHAPTER V

CO-DIGESTION OF SWINE MANURE AND GRASS IN SINGLE STAGE MESOPHILIC VERSUS TEMPERATURE-PHASED ANAEROBIC CONDITIONS

Abstract:

Anaerobic co-digestion of pig manure with para-grass can improve the potential of methane production in pig farm. Anaerobic single-stage mesophilic 35°C, and temperature-phased anaerobic digestion (TPAD) system (thermophilic 55°C – mesophilic 35°C) were evaluated at different solid loadings from 0.10-3.76 gVS/L/d (0-8% dry para-grass mixing ratios, PG). Results showed that methane yield from TPAD system was slightly higher than the mesophilic reactor but was not statistically significant. The highest methane yield of 158.6 $mLCH_4/gVS_{added}$ was obtained from TPAD at 4 %PG with a methane content of around 55%. The highest biogas yield resulted from grass addition was 66.3 m^3_{biogas}/ton_{fresh} at 4 %PG. Accumulation of volatile fatty acids was found in the first stage thermophilic reactor with the domination of acetate. Analysis of microbial communities by DGGE indicated that an addition of para-grass had shifted the domination of bacteria while achaea were rather stable. TPAD system possessed higher microbial diversity.

Keywords: Co-digestion, Pig manure, Para-grass, Mesophilic, Thermophilic

5.1 Introduction

Nowadays energy and environment have been a world crisis since humans are consuming large amount of non-renewable energy and resources. The amount of waste produced is growing each year. Producing renewable energy from waste therefore has

various advantages. Animal manures have been used as a source of excellent material for anaerobic digestion with clear environmental benefit. In Thailand, swine farms generate an estimate of 5 billion kilograms of manure annually (DLD 2012). The application of anaerobic digestion technology is widely used as it can repay the investment from the energy produced. While the principles of anaerobic digesters remain biological destruction of organic content (Gerardi, 2003), the newer generation of anaerobic digesters are capable of accepting various kinds of feedstocks either solid or liquid with high organic content. The main products from anaerobic digestion are the biogas that consists mostly of methane which is a good fuel for heat and power generation, and valuable digested residue that can be used as organic soil improvement substance rich in plant nutrients.

Para-grass (*Branchiria mutica*) is a tropical weed that widely grows on wet soils in Thailand (Hare *et al*., 1999), especially in animal raising farms. Thus, it needs to be cut down frequently for the farm biosecurity, disease and vector controls. It has received some attention as a potential biogas feedstock in the farms. Para-grass has a great potential as a co-digestion substrate in anaerobic digestion with pig manure to enhance the biogas production. Co-digestion of manure with this cellulosic biomass can increase biogas production because it is a good source of organic carbon for biogas production. And it can improve C:N of the feedstock to be suitable for metabolic activities in anaerobic digestion system (Xie *et al*., 2011), and can indirectly decrease ammonia inhibition.

For the conventional anaerobic digestion, the acid-forming and methane forming microorganisms are kept together in a single reactor. Both two groups are different in terms of physiology, nutritional, growth kinetic and environmental condition (Elbeshbishy and Nakhla, 2011). The two groups of organisms need a delicate balance. Therefore, the two stage of reactor are the physical separation of acid-formers and methane-formers where optimum environmental conditions for each group of microorganisms can be attained. The two-stage configuration could provide the optimum condition to enhance the overall process stability (Nasr *et al*., 2012).

Many factors are important in successful anaerobic digester (AD) operation such as pH, mixing, pretreatments, temperature, and loading. The operating parameters of the digester must be controlled to enhance microbial activities and increase the anaerobic degradation efficiency of the substrate. Among others, temperature has a great effect on the performance of AD. The substrate degradation rate and biogas production rate would be higher at higher temperature (Yu *et al*., 2002). However, operating AD at high temperature requires more extensive control since the system becomes quite sensitive at higher temperatures. The organic loading rate (OLR) is another important factor as it indicates the amount of waste that can be treated per unit reactor volume (Speece, 1996). Whilst increasing the OLR could increase the volumetric methane production but it could increase the risk of system failure due to an accumulation of VFAs (Xie *et al*., 2012).

Anaerobic sequencing batch reactors (ASBR) are currently used for the treatment of wastewaters with particulate organic matter such as waste activated sludge, swine manure, leachate and dairy (Dugba and Zhang, 1999). There are five steps in ASBR operation; fill, react, settle, draw and idle (Sarti *et al*., 2007). ASBR is able to attain a high solid retention time because of its settling phase, and can apply for the treatment of various wastewaters. Therefore, it can retain high concentration of slow-growing anaerobic bacteria in the reactor (Dugba and Zhang, 1999). Zupancic, Straziscar *et al*. (2007) reported that ASBR experiments were conducted for the treatment of brewery slurry under different organic loading rates (OLR) from 3.23 to 8.57 kgCOD/m^3/day. It achieved with COD removal efficiency varied from 79.6% to 88.9%. For the VSS removal efficiency was from 78.5% to 90.5%.

The temperature-phased anaerobic digestion (TPAD) is one of anaerobic digestion processes that biomethane can hold great potential by enhancing the efficiency in the effluent quality, good performance of the organic matter removal, high digestion rate, methane yield, volatile solid reduction, process stability, and pathogen control (Riau *et al*., 2010, De La Rubia *et al.,* 2009). It combines thermophilic and mesophilic process in one treatment system with the advantages of

two individual processes. First, it has high digestion rate and pathogen destruction with the thermophilic (55 ^{o}C) process. Second, it requires low energy and gives high quality of effluent with the mesophilic 35 ^{o}C process (Dugba and Zhang, 1999, Song *et al*., 2004). The TPAD process could be operated at higher loading rates compared to single-stage processes. Mesophilic digester in a TPAD system has a longer HRT than the thermophilic digester so that sufficient microbial biomass of acetogens and methanogents can stay in for a long time (Lv *et al*., 2010). This system is operated by feeding the feedstock into thermophilic digester and then transferring the digestate into mesophilic digester. The four steps of biomethanation process can be divided in to hydrolysis and acidogenesis (or fermentation) primarily occurring in the thermophilic digester, while acetogenesis and methanogenesis take place mainly in the mesophilic digester (Riau *et al*., 2010).

Anaerobic co-digestion is applied widely for many waste treatments, especially pig and cattle manure. Because manure has high buffering capacity and it is rich in a variety of nutrients that necessary for optimum bacterial growth (Panichnumsin *et al*., 2010). Therefore, co-digestion with manure would give the balance of nutrients, at an appropriate C:N ratio to improve methane production (Callaghan *et al*., 2002). Wu Yao, *et al*.(2010) indicate that significant increases in volumetric biogas production can be achieved by adding carbon rich agricultural residues to the co-digestion process with swine manure. The main reason for co-digestion of feedstock is the adjustment of the carbon-to-nitrogen (C: N) ratio. Microorganisms generally utilize carbon and nitrogen in the ratio of 25–30:1 *(*Ward *et al*., 2008). Lansing, *et al*. (2010) proved that co-digesting used cooking grease with swine manure in low-cost digesters is a simple way to double energy production. A small volume of grease (2.5%), which corresponded to a 113% increase in organic matter, increased methane production by 124%. Pig manure can co-digest with wheat straw to get a 10% increase in methane production when the straw was added for 4.6 kg to 1 t of manure (Wang *et al.,* 2009).

The objective of the study was to evaluate the performance of single stage mesophilic ASBR in comparison with TPAD system for co-digesting pig manure and para-grass at different solid loading of para-grass.

5.2 Methods

5.2.1 Reactor systems

There were 2 anaerobic systems evaluated in this study; single stage mesophilic reactor (Meso-Single) and two stage temperature-phased anaerobic digestions (TPAD) system which consisted of first stage thermophilic reactor (Thermo-1st) followed by second stage mesophilic reactor (Meso-2nd). All mesophilic reactors were made of glass with 5 L volume and a diameter of 182 mm. For Meso-Single reactor, the effective volume was 4 L and a temperature was maintained at 35±1 °C. In TPAD configuration, the 1-L front reactor (Thermo-1st) had an effective volume of 0.8 L operated at 55±1 °C while the second stage reactor (Meso-2nd) possessed an effective volume of 3.2 L to make up a total effective volume of 4 L equal to Meso-Single reactor. The reactor was capped with a rubber stopper that had one port for biogas collection. Three ports on the side of bottles were assigned to influent feeding, effluent withdrawal, and sludge sampling. It was kept in a temperature controlled water bath. The reactor content was mixed manually twice a day each for approximately 2-5 min to ensure homogeneity in the reactors. The mixed liquor from Thermo-1st was withdrawn immediately after mixing and used as feed to Meso-2nd. In Meso-Single and Meso-2nd, the reactors were left to settle for 30 min after mixing before effluent withdrawal at mid depth of the liquid level.

5.2.2 Inoculum

The inoculum used in this lab scale experiment was collected from UASB (up-flow anaerobic sludge blanket) reactor treating pig waste slurry from the unit of finishing (fattening) barn, in Songkhla Province, Thailand. The sludge, which was

dispersive with no granules, was sieved to remove large particles. It was then measured for total solids (TS) and volatile solid (VS) concentration. Specific methanogenic activity (SMA) was also performed to measure the methanogenic activity of sludge to be used. The sludge was inoculated to the systems within 48 h after field collection.

5.2.3 Preparation of feed

Pig manure (PM) was obtained from excretions from the finishing unit in a pig farm in Pattalung Province, Thailand. It was dried at 60 ^{o}C and ground in a mortar to small particles. The fresh green para-grass (PG), *Branchiria mutica*, was randomly harvested from the same commercial pig farm where PM was obtained. It was chopped with an agricultural cutting machine to approximately 2 cm, then dried at 60 ^{o}C and ground to the maximum length of less than 6 mm. Both substrates were kept at 4 ^{o}C until use. The PM prepared was brought to mix with tap water to imitate the pig slurry wastewater that had a COD approximately 3,000-4,000 mg/L. The mixtures of para-grass (PG) at 4 levels; 0%, 2%, 4% and 8%TS as co-substrate were tested. The substrate was homogenized with tap water before feeding to the reactors.

5.2.4 System operation

The reactors were started up with an initial active sludge of 30% of the effective volume as inoculum and filled up with the prepared pig slurry wastewater (2.5 gTS/L or 2.015 gVS/L) to an effective volume. The reactors were then rested for approximately 24 h before the scheduled feeding began. Meso-Single and Thermo-1st reactors were fed with pure PM wastewater (0% PG) equivalent to OLR 0.10 gVS/L/d, and the Meso-2nd reactor was fed with the mixed liquor discharged from Thermo-1st reactor until both systems entered stable condition, i.e. variation of biogas production less than 10% and stable pH which took around 65 days. Performance data at 0% PG were recorded. The organic loading of the systems was increased stepwise from 0.10, 1.02, 1.93, and 3.76 gVS/L/d which corresponded to PG mixing of 0, 2, 4,

and 8 % TS in feed. The load increase was performed after the systems reached stable condition. All conditions were run at 20 days HRT. Experimental parameters of two systems are shown in Table 5.1.

Table 5.1 Experimental parameters of two ASBR anaerobic treatment systems

Operational parameters	**Meso-Single**	**TPAD**		
		Thermo-1st	**Meso-2nd**	**Overall**
Reactor volume (L)	4	0.8	3.2	4
Temperature (^{o}C)	35	55	35	
HRT (days)	20	4	16	20
VS loading rate	0.101	0.504	*	0.101
(gVS/L/day)	1.016	5.079	*	1.016
	1.931	9.654	*	1.931
	3.761	18.804	*	3.761

* Depends on VS removal by the first stage reactor

5.2.5 Analytical method

5.2.5.1 Inocula and substrates

Inoculum and substrates (PM and PG) were analyzed for TS and VS according to the Standard Methods (APHA, 1999). The chemical compositions of the samples were analyzed using CHNS-O Analyzer. TS and VS of the inoculum were 78.0 and 46.0 g/L, respectively. The methanogenic activity was 32.99±0.18 $mLCH_4$/gVS which showed high concentration of active microorganisms of both the acetogens and methanogens.

5.2.5.2 System performance analysis

Performance of the digesters was evaluated by the determination of pH, total COD (TCOD), soluble COD (SCOD), and volatile solid (VS) of the influent

and effluent according to Standard Methods (APHA, 1999) while available alkalinity and VFA were determined by direct titration method (Anderson and Yang, 1992). Individual volatile fatty acids (VFAs) were analyzed by gas chromatography (GC 7820A Agilent Technologies) equipped with a flame ionization detector (FID). A capillary column Agilent 19091N-133 HP- INNOWax polyethylene glycol was used with helium as the carrier gas.

5.2.5.3 Biogas production and composition

The biogas produced was stored in gas bag and the volume was measured daily using a multi-chamber rotor wet gas meter (Ritter). Biogas composition was analyzed twice a week by gas chromatography (GC 7820A Agilent technologies) equipped with thermal conductivity detectors (TCD). Helium was used as carrier gas.

5.2.5.4 Microbial community analysis by denaturing gradient gel electrophoresis (DGGE)

Sludge samples for microbiological analyses were taken from the reactor after finishing each condition (stable condition). Total genomic DNA was extracted from enrichment culture samples by using a slightly modified standard bacterial genomic DNA isolation method according to the procedures in Hniman *et al*. (2011). The bacterial 16S rDNA was amplified by the first polymerase chain reaction (PCR) with universal primer 1492r and 27f as shown in Table 5.2. The primer K517r and L340f with CG clamp were used to amplify the fragment of V3 region of 16S rDNA product from the first PCR. The bands were excised from the gel and re-amplified with primer 357f (without a GC clamp) and the reverse primer. PCR products were purified and sequenced using primer 518r for bacteria by the Macrogen sequencing facility (Macrogen Inc., Seoul, Korea). Closest matches for partial 16S rRNA gene sequences were identified by ribosomal database project with SeqMatch

program and basic local alignment search tool (BLAST) with nucleotide database in National Center for Biotechnology Information (NCBI).

The PCR amplification targeting archaea was carried out with the forward primer PRA46F and the reverse primer PREA1100R as shown in Table 5.2 to generate a product of 1072 bp. This PCR product was then used as a template for the PCR amplification of 179 bp using the forward primer PARCH340F containing a GC clamp and the reverse primer PARCH519R. PCR products were purified and sequenced using the primer PARCH340F and PARCH519R (Ovreas *et al*., 1997).

Table 5.2 Primers used for PCR-DGGE in this study.

Group	**Primer name**	**Nucleotide sequences (5'- 3')**
Bacteria	1492r	GAAAGGAGGTGATCCAGCC
	27f	GAGTTTGATCCTTGGCTCAG
	K517r	ATTACCGCGCTGCTGG
	L340f	CCTACGGGAGGCAGCAG
	L340f-GC	GC clamp-CCTACGGGAGGCAGCAG
Archaea	PRA46F	C/TTAAGCCATGCG/AAGT
	PREA1100R	T/CGGGTCTCGCTCGTTG/ACC
	PARCH340F	CCCTACGGGGC/TGCAG/CCAG
	PARCH519R	TTACCGCGGCG/TGCTG
	GC clamp	CGCCCGCCGCGCCCCGCGCCCGTCCCG CCGCCCCCGCCCG

5.2.6 Data analysis

5.2.6.1 Hydrolysis and acidogenesis evaluation

First step of anaerobic digestion pathway, hydrolysis yield is defined as the solubilization of organic matter to hydrophilic soluble compounds by using hydrolytic bacteria. Degree of hydrolysis can be expressed as the quotient between the effluent COD in filtered sample (SCOD) and influent COD in total sample (TCOD). In the second step, the performance of the acid phase digestion can be quantified using the percentage of the initial substrate concentration (influent TCOD) converted to VFAs. The quantity of each VFA species was converted to gCOD/g by using the COD equivalents of each VFA. The COD equivalents for volatile acids for acetic, propionic, butyric, valeric and caproic are 1.066, 1.512, 1,816, 2,036 and 2.204, respectively (Demirel and Yenigun, 2004). It is noted that COD equivalence of methane was added to the nominator of both hydrolysis and acidgenesis yield calculations because it was an end product from both reactions.

Therefore, the percentages of hydrolysis (H) and acidogenesis (A) were calculated according to the following equations (5.1) and (5.2), respectively (Hamed *et al*, 2004).

$$H\ (\%) = \left[\frac{CH_4\ as\ COD + Effluent\ CODdis}{Influent\ CODt}\right] \times 100 \qquad (5.1)$$

$$A\ (\%) = \left[\frac{CH_4\ as\ COD + Effluent\ VFA\ as\ COD}{Influent\ CODt}\right] \times 100 \qquad (5.2)$$

where $H(\%)$ is the percentages of hydrolysis, $A(\%)$ is the percentages of acidification, $CH_4\ as\ COD$ is gCOD equivalence of methane at STP, $Effluent\ COD_{dis}$ is SCOD of effluent, *Effluent VFA as COD* is gCOD calculated from VFA species, *Influent CODt* is TCOD of influent.

Biodegradability of the substrate is defined as the ratio of the measured methane yield over the theoretical methane yield.

Biodegradability = (Specific methane yield / Theoretical methane yield) ×100 (5.3)

5.2.6.2 Statistical analysis

The values reported were calculated from data at stable condition of each operating condition. The data were analyzed using the data analysis toolbox in software Microsoft Excel. Mean and the standard deviation were calculated and used to compare the effect of each variable in the experiment. The comparison of means was carried out with SPSS software version 11.0 by one way analysis of variance (ANOVA) and Scheffe's multiple-range test.

5.3 Results and discussion

5.3.1 Feedstock characterization

The fiber content of pig manure (PM) and para-grass (PG) comprised mostly of hemi-cellulose and cellulose, in order. PM had higher lignin composition than PG as shown in Table 5.3. Higher lignin composition makes it more difficult to degrade in anaerobic condition because its structural complexity consisting of interconnected aromatic group (Rittmann and McCarty, 2001). The carbon in PG was higher than PM whereas the nitrogen content was lower. C/N ratios were 32.21 and 12.65 in PG and PM, respectively and had high moisture suitable for anaerobic digestion.

Table 5.3 Chemical and elemental composition of pig manure and para-grass

Composition	Unit	Pig manure	Para-grass
Moisture	%	72.48 ± 0.78	77.25 ± 1.96
Total solid, TS	g/kg wet	248.14 ± 4.25	190.63 ± 6.39
Volatile solid, VS	g/kg dry	806.32 ± 2.98	914.91 ± 6.19
Cellulose	% dry wt.	12.65 ± 0.18	38.84 ± 2.35
Hemi-cellulose	% dry wt.	25.72 ± 3.92	29.53 ± 1.51
Lignin	% dry wt.	8.96 ± 5.00	7.95 ± 2.02
Carbon, C	% dry wt.	38.09	41.55
Hydrogen, H	% dry wt.	5.42	5.32
Oxygen, O	% dry wt.	22.25	27.28
Nitrogen, N	% dry wt.	3.01	1.29
Sulfur, S	% dry wt.	0.32	0.30
C:N ratio		12.65	32.21

The initial COD of the solid substrate calculated from the stoichiometric equations using C, H, O, N, S (%) at different solid loadings (0, 2, 4, and 8% PG). The COD equivalents of the mixture of PM and PG (0%, 2%, 4%, and 8%) were 1.689, 1.638, 1.628, and 1.631 $gCOD/g_{substrate\ dry,}$ which calculated from equations as shown in equation 5.4-5.7, respectively.

$$C_{318}H_{538}O_{139}N_{22}S + 366O_2 = 318CO_2 + 236H_2O + 22NH_3 + H_2S \quad (5.4)$$

$$C_{364}H_{560}O_{177}N_{11}S + 406.75O_2 = 364CO_2 + 262.5H_2O + 11NH_3 + H_2S \quad (5.5)$$

$$C_{367}H_{561}O_{180}N_{11}S + 408.5O_2 = 367CO_2 + 263H_2O + 11NH_3 + H_2S \quad (5.6)$$

$$C_{368}H_{562}O_{181}N_{10}S + 410O_2 = 368CO_2 + 265H_2O + 10NH_3 + H_2S \quad (5.7)$$

5.3.2 pH, VFA and alkalinity

The role of pH of the reactor is related to the concentration of VFA and alkalinity. These parameters are important in anaerobic digester as they reveal a

balance in the system. pH of influent and effluent from the single stage mesophilic reactor (Meso-single) and TPAD reactor are shown in Figure 5.1. The average pH of influent was 6.8, 6.5, 5.8 and 5.5 at 0%, 2%, 4% and 8% PG mixture, respectively. pH change is a result of the acid or alkaline accumulated in the feedstock. The pretreated solid substrate such as ensilaged biomass or alkaline treated biomass will certainly cause changes in pH of the feed. However, no pretreatment of the grass was undertaken, only drying was carried out for preservation prior to use. It appeared that the para-grass naturally possessed some acidity within the cells. This kind of inherited organically acidity would be biodegraded in the digester.

In the first stage thermophilic (Thermo-1st) of TPAD, pH decreased with increasing OLR (Figure 5.1), which corresponded to the increased VFA production (Figure 5.2). The average pH values at stable condition were 6.3, 5.8, 5.6 and 5.4 with 0%, 2%, 4% and 8% PG mixtures. These pH values were in the optimum pH range for hydrolysis under anaerobic condition at 4-6 (Orozco, *et al*., 2013). Both VFA and alkalinity of Thermo-1st increased with higher loadings yielding VFA/Alkalinity in the range of 1.0-1.1. This range is not suitable for methanogenic acitivity as evidenced by the minimal biogas production which is discussed later in this paper. The higher PG mixture resulted in a lower pH in the influent and, due to the high OLR, in the effluent from Thermo-1st as well.

In Meso-Single and Meso-2nd, the pH values varied only slightly between 6.5-6.8. This pH range remained within the optimum range of methanogenesis (pH 6.8-7.4) (Appels *et al*., 2008). pH of reactor, represented by the effluent pH, was occasionally controlled with 0.1 N NaOH when it went below 6.8. The alkalinity increased with the increasing PG (Figure 5.2) which made VFA/Alkalinity ratio stay in the range of 0.15-0.18. This result stated that both reactor systems had high buffering capacity at below 0.4 as suggested in Song, *et al*. (2004).

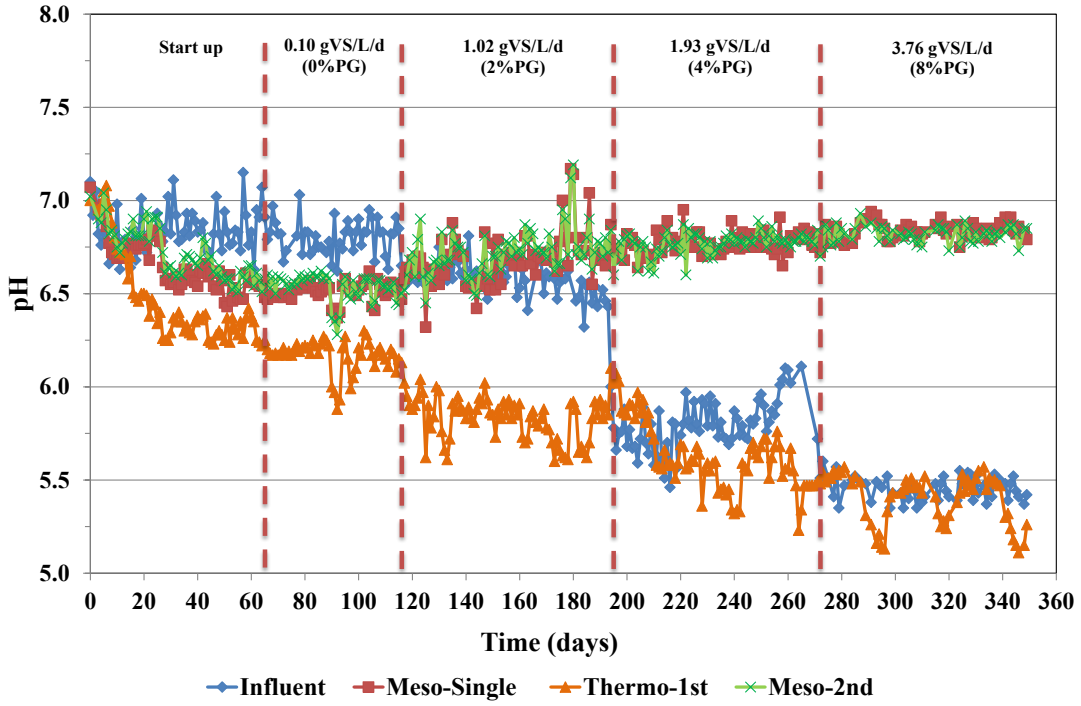

Figure 5.1 pH of influent and effluent when operating with different loadings in Meso-Single and TPAD system

5.3.3 VS reduction

The reduction in VS in anaerobic digestion was the sum of residual VFAs and methane gas produced (Song, *et al*., 2004). In this study, the increasing VS of influent at higher PG mixing caused an increase in VS in effluent. There was quite low solid removal in the Thermo-1st reactor (Figure 5.3) only 19.0%, 26.0%, 24.5% and 18.7% with 0%, 2%, 4% and 8% PG, respectively. The highest overall VS removal percentage achieved was at OLR 1.0 gVS/L/d and the lowest VS removal was at OLR 3.76 gVS/L/d. It was observed that effluent from Meso-2nd had slightly lower VS concentration than Meso-Single (Figure 5.3) but they were not statistically different. VS in effluent of Thermo-1st also increased with increasing PG but slightly lower than VS of influent as it was converted to gaseous end product.

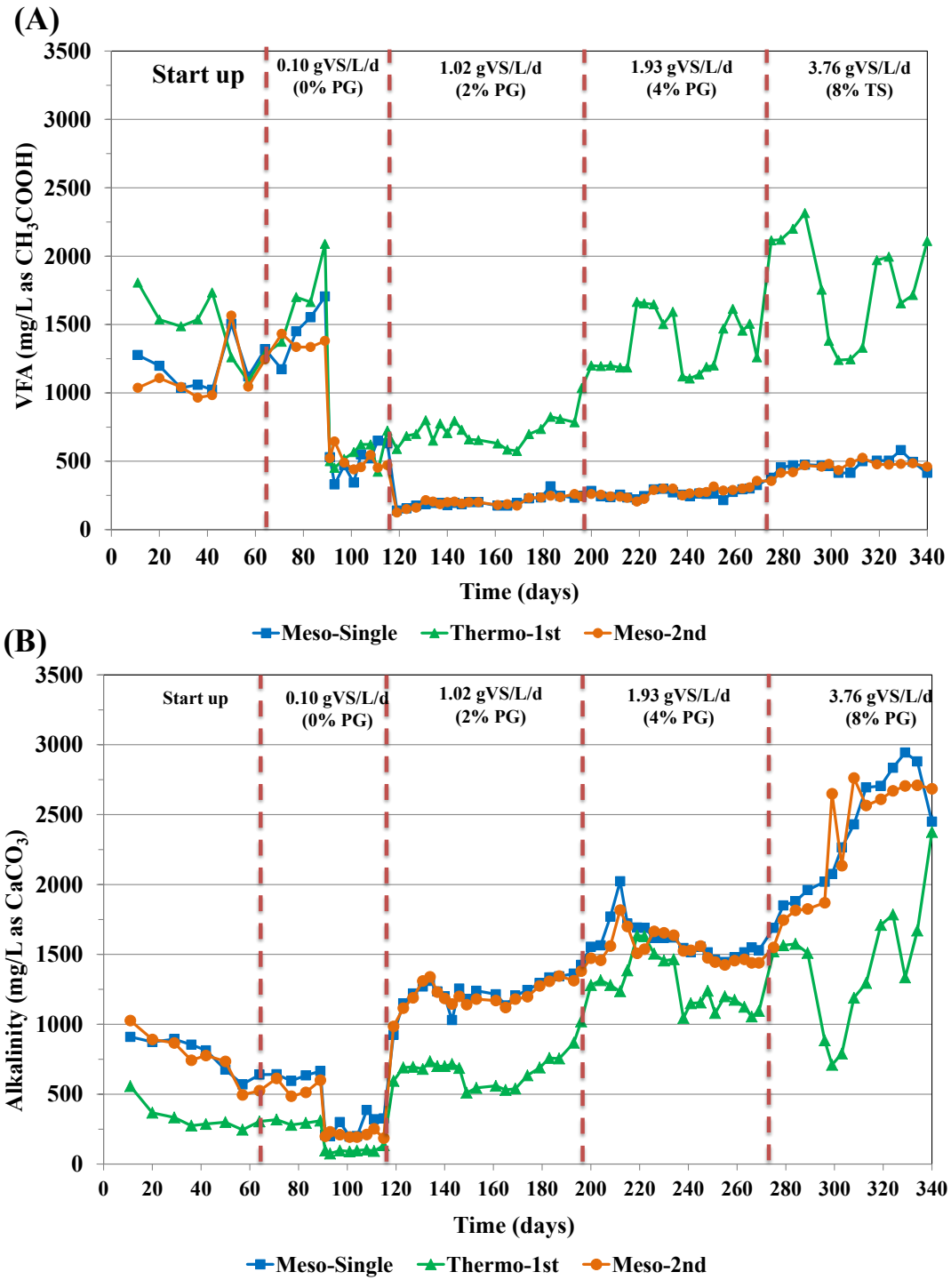

Figure 5.2 VFA (A) and alkalinity (B) of effluent when operating with different organic loadings in Meso-Single and TPAD system

The other observation in the experiment was the accumulation of solid in the reactor particularly at higher solid loadings. It was apparent that at 8% PG mixture there was an obvious floating layer of biomass towards the end of the experiment. The loading at this level, 3.76 gVS/L/d, was considered high load and not recommended for operating the typical digesters for this substrate. Mixing mechanism that can continuously provide rigorous stirring action in the digester is needed in combination with the strict control of acidity within the system.

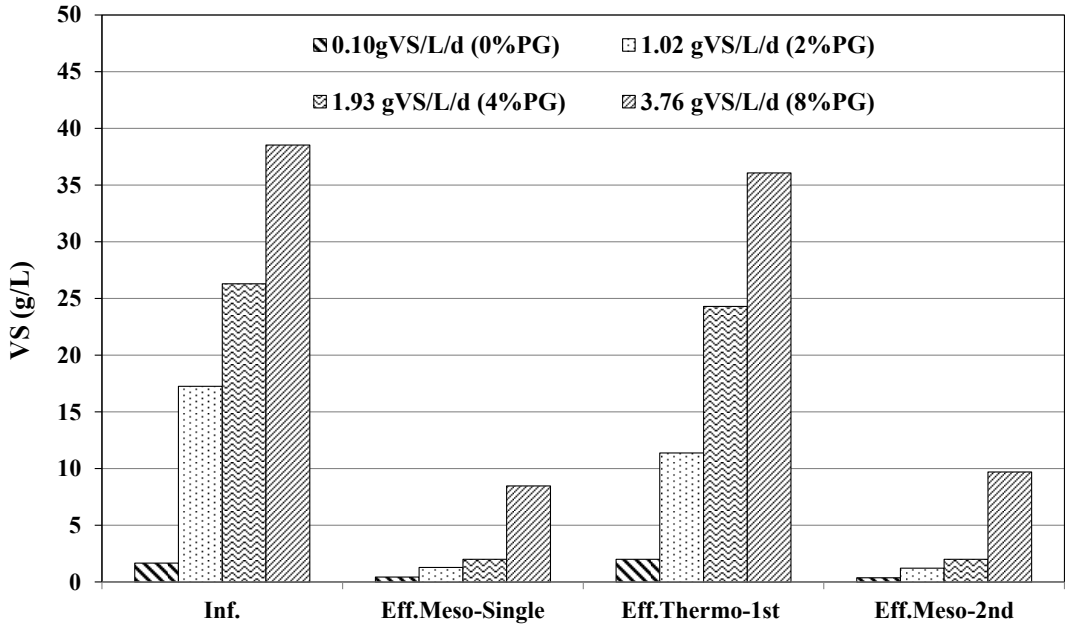

Figure 5.3 Volatile solids (VS) of influent and effluent from single stage of mesophilic and TPAD reactor at different organic loading rates

5.3.4 Individual VFA

Composition of volatile fatty acid in the reactors varied greatly due to the reactor staging and OLR. Distribution of VFAs was measured in the effluents of each reactor over the experimental period, as shown in Table 5.4. VFA (C_2-C_5) were not detected at OLR 0.10 gVS/L/d in Meso-Single hypothetically because the VFA were converted all to biogas end product. The dominant VFA species was iso-valeric and

valeric acid (i-HVa+HVa) as it composed of approximately 35.5% and 49.9% of total VFA (TVFA) at OLR 1.016 gVS/L/d and 1.931 gVS/L/d, respectively. The lessor groups in order were iso-butyric and butyric acid (i-Hbu+HBu), acetic acid (HAc), and propionic acid (HPr) at all OLR. But i-HVa+HVa species were not found in Meso-Single at OLR 3.8 acid gVS/L/d.

In Thermo-1st reactor, the dominant VFA species was HAc followed by i-Hbu+HBu, i-HVa+HVa, and HPr in all OLRs. HAc composed of approximately 54-62% of TVFA and increased with the increasing OLR. The level of VFA was high in the Thermo-1st which was accompanied by a low pH. Therefore, vary minimal methane gas was produced in this reactor. The VFA products in Meso-2nd were found only at OLR 1.93 and 3.76 gVS/L/d. It was interesting that Meso-2nd was evidently quite effective in utilizing VFAs particularly at OLR lower than 1.02 gVS/L/d. VFAs concentration in Meso-2nd were lower than Meso-Single. The substrate already was pre-hydrolyzed in the thermophilic first stage to an easier digestible and smaller acid molecule, HAc in particular, which was easy to be converted to methane gas by methanogens in the ensuing reactor. Acetoclastic methanization were very active in Meso-2nd.

Table 5.4 Volatile fatty acids composition for all reactors at different solid loading rate; 0%, 2%, 4% and 8%

Reactor	OLR (gVS/L/d)	HAc (mg/L)	HPr (mg/L)	i-Hbu+HBu (mg/L)	i-Hva+Hva (mg/L)	TVFA (mg/L)
Meso-Single	0.101 (0%)	N.D.	N.D.	N.D.	N.D.	N.D.
	1.016 (2%)	17.37	13.58	23.59	30.00	84.54
	1.931 (4%)	8.84	12.90	19.73	41.27	82.73
	3.761 (8%)	14.83	12.45	20.29	N.D.	47.57
Thermo-1st	0.101 (0%)	27.51	13.30	24.96	29.94	95.73
	1.016 (2%)	139.72	20.01	51.57	44.34	255.63
	1.931 (4%)	239.14	26.14	77.60	46.78	389.66
	3.761 (8%)	234.29	20.28	110.77	44.52	409.87
Meso-2nd	0.101 (0%)	N.D.	N.D.	N.D.	N.D.	N.D.
	1.016 (2%)	N.D.	N.D.	N.D.	N.D.	N.D.
	1.931 (4%)	7.75	12.78	18.58	N.D.	39.11
	3.761 (8%)	10.40	13.61	18.94	N.D.	42.94

Note N.D. = not detectable

5.3.5 Hydrolysis and acidogenesis

The level of hydrolysis and acidogenesis can be presented in percentage. The performance of these two crucial steps in anaerobic digester was affected by temperature in our phase separation to different degrees of various OLRs.

5.3.5.1 Hydrolysis yield

Hydrolysis represents solid break down from particulate form to soluble form in the reactors. The hydrolysis yield in Thermo-1st reactor improved from 8.3% to 10.7% when PG mix increased from 0-8% in feed (Table 5.5). It was also found that the hydrolysis yield at 8% PG did not improve compared to a condition 4% PG. Hydrolysis rate in thermophilic reactor would less likely to further rise beyond the feeding of 4% PG. This coincided with the expanding scum layer in the reactor at 8% PG that separation of floating substrate from the rich microbial culture in liquid phase. Continuous mixing could help create the contact of the hydrolytic enzymes with the substrate. Hydrolysis yields at each OLR in mesophilic reactors, both Meso-2nd and Meso-Single, were quite comparable (Table 5.5). The last OLR at 8% PG, hydrolysis yield dropped with the same reasoning. The hydrolysis yields obtained from this study are comparable to that from a two-stage anaerobic digestion of sunflower oil cake at OLRs higher than 7 gVS/L/d at 20.5-30.1% (Rubia *et al*., 2009), and higher than grass silage in leaching bed reactors (LBRs) at 16.5-22.4% (Xie *et al*, 2012). However, the value of around 81% at OLR 7.5 gCOD/L/d in two-phase anaerobic digestion of a mixture of fruit and vegetable wastes at OLR 7.5 gCOD/L/d is much higher than our reported range. This is due to the nature of feedstock as the fruits and vegetables are higher degradable. Rittmann and McCarty (2001) reported that hydrolysis rate for Napier grass was very low (k_{hyd} = 0.090 d^{-1}; represent the first order rate constant for such hydrolysis), so it needs longer time to hydrolyze. They suggested that the rate of hydrolysis was dependent on temperature. In this present study, Thermo-1st had provided a pre-digestion at 55^{o}C for 4 days that could give a clear benefit that a more complete utilization of fatty acids in Meso-2nd

(Table 5.4). Several pretreatment methods such as ultrasonic, thermal, chemical and thermo-chemical can improve poor liquefaction of grass, however, the cost and complexity of technology for large scale of such pre-treatment may have limited their feasibility compared to a simple temperature phased digestion.

5.3.5.2 Acidogenesis yield

Acidogenesis yield is one way to measure the degree of success in fermentation. Acidogenesis yield of Meso-Single and Meso-2nd reactors were higher when PG increased until OLR 1.931 gVS/L/d at 4%PG (Table 5.5). When hydrolysis is high, acidogenic bacteria could function in a faster rate to convert solubilized substrate to VFAs. In this case, acidogenesis was likely limited by the preceding hydrolysis reaction. The best acidogenesis yield in Meso-Single and overall TPAD system were 26.63% and 27.42%, respectively, which were achieved at OLR 1.93 gVS/L/d (4% PG). These values are lower than those from the study for grass silage fermentation in LBRs under OLRs of 0.5, 0.8, and 1.0 kgVS/m^3/d that gave the highest acidogenesis yield of 57-60% (Xie *et al.*, 2012). Acidogenesis yield in Thermo-1st decreased when hydrolysis yield increased at higher OLR. This was deemed as an effect of product inhibition from heightened TVFA concentration in Thermo-1st (Table 5.4). Pretreatment of substrate and type of reactor could play an important role to contribute to the success of hydrolysis-acidogenesis of solid substrates.

Table 5.5 Performance at stable condition of single stage mesophilic reactor (Meso-Single), and two stage temperature-phased anaerobic digestion (TPAD) system receiving pig manure wastewater co-digesting with para grass at 0, 2, 4, and 8 %TS

	Organic loading rate (gVS/L/d)			
	0.101 (0%PG)	**1.016 (2%PG)**	**1.931 (4%PG)**	**3.761 (8%PG)**
Hydrolysis yields (%)				
Meso-Single	25.23±2.60	28.28±4.64	29.98±0.77	16.55±1.09
TPAD	25.86±1.65	26.59±5.26	29.79±1.16	18.57±1.26
-Thermo-1st	8.27±4.54	9.31±1.27	10.70±1.21	10.69±0.86
-Meso-2nd	17.59±3.50	17.28±4.13	18.44±0.38	7.88±0.44
Acidification yields (%)				
Meso-Single	18.87±1.08	23.43±3.31	26.63±1.60	15.27±3.68
TPAD	18.37±1.52	23.34±3.95	27.42±1.12	16.36±0.09
-Thermo-1st	6.40±0.37	1.4±0.09	0.43±0.02	0.54±0.09
-Meso-2nd	11.97±1.26	21.94±3.87	26.99±1.14	15.82±0.15

Table 5.5 Performance at stable condition of single stage mesophilic reactor (Meso-Single), and two stage temperature-phased anaerobic digestion (TPAD) system receiving pig manure wastewater co-digesting with para grass at 0, 2, 4, and 8 %TS (continued)

	Organic loading rate (gVS/L/d)			
	0.101 (0%PG)	**1.016 (2% PG)**	**1.931 (4% PG)**	**3.761 (8% PG)**
Theoretical methane yields ($mLCH_4/gVS$)	524.98	541.01	541.79	542.39
Specific methane yields ($mLCH_4/gVS_{added}$)				
Meso-Single	119.65 ± 3.33^{b}	138.23 ± 23.78^{b}	158.29 ± 5.77^{c}	83.31 ± 4.72^{a}
TPAD	$117.11\pm9.43^{a,b}$	$142.52\pm24.01^{b,c}$	158.55 ± 3.31^{c}	94.12 ± 7.82^{a}
- Thermo-1st	6.86 ± 3.84^{c}	$1.82\pm0.11^{a,b}$	$2.69\pm0.14^{a,b}$	0.49 ± 0.44^{a}
- Meso-2nd	$110.24\pm5.88^{a,b}$	$140.70\pm24.1^{b,c}$	155.86 ± 3.18^{c}	93.64 ± 8.25^{a}
Biodegradability (%)				
Meso-Single	22.79±0.63	25.55±4.40	29.22±1.06	15.36±0.87
TPAD	22.31±1.80	26.34±4.44	29.26±0.61	17.35±1.44
- Thermo-1st	1.31±0.73	0.34±0.02	0.50±0.03	0.09±0.08
- Meso-2nd	21.00±1.12	26.01±4.45	28.77±0.59	17.26±1.52

Note: Means in each row followed by a different letter are significantly different using Scheffe's multiple-range test ($p<0.05$).

5.3.6 Biogas production

One of the most important parameters in anaerobic digestion is the amount and composition of the produced biogas. These parameters were influenced by substrate, OLR and reactor type applied as observed in our results. Biogas production and methane content from the single stage mesophilic reactor (Meso-Single) and TPAD system at different OLR are shown in Figure 5.4. The startup of 65 days was taken to ensure stable condition of both systems. Biogas production increased in both reactors with increasing PG at 103.0, 1,003.7, 2,396.6 and 2,371.1 mL/d for Meso-Single, and 119.7, 1,024.1, 2,324.9 and 2,682.4 mL for TPAD at 0-8% PG, respectively. It was noticeable that at solid loading 8%PG, the biogas production rose sharply (day 272) and dropped (day 282) immediately in Meso-Single. Then it returned to the production of about the same level with 4% PG condition. There were occasional scums forming caused by the grass floated to the liquid surface in the reactor giving less contact of substrates with microorganisms. Koch *et al*. (2009) showed that the TS concentration should not exceed 12% feed in order to guarantee satisfying gas yield. It is noteworthy that the mixing was very important for high solid digestion.

Moreover, the methane content of all reactors was fluctuated during the first 65 days. Because of the microorganisms took time for adaptation to be fully functional. Methane contents varied between 43-55% in Meso-Single and Meso-2nd reactors while there was low in methane content in Thermo-1st from 2 to 30 %. It was slightly elevated to 43.1% and 45.2% in Meso-Single and Meso-2nd under 0%PG at stable condition, respectively. Increasing of grass mix ratio promoted an increase in the methane content in both mesophilic reactors to reach the stable level of 53.3±3.2 and 54.7±3.3% at 2%PG for Meso-Single and Meso-2nd reactors, respectively. The first stage in TPAD (Thermo-1st) clearly gave lower methane content compared to mesophilic reactors. Thermo-1st reactor spent approximately 100 days to reach stable condition that gave about 25% methane in the biogas and stayed in such level until system OLR reached 1.02 gVS/L/d (2% PG), Thermo-1st OLR of 5.079 gVS/L/d (Table 5.1). Methane content had declined to below 20% at system OLR 1.93

gVS/L/d (4% PG) and severely dropped to around 2% at system OLR 3.76 gVS/L/d (8% PG) which was equivalent to Thermo-1st OLR of 18.804 gVS/L/d. In this stage, the biomass was pre-hydrolyzed with high enzymatic activity and the derived soluble organic was subsequently acidified at higher rate. This was seen in the high VFA concentration in the Thermo-1st effluent. The VFA accumulation also caused low pH environment, which was not favorable for methanogenic archaea to function effectively. Therefore, this Thermo-1st reactor produced little biogas that composed of low methane content.

The yield of biogas per fresh mass can be calculated from the data in Figure 5.4 with the moisture content of 77.25% (Table 5.3). It was found that the highest biogas yields in this experiment were achieved at 4 %PG mixture at 66.3 and 61.8 $m^3/ton_{fresh\ added}$ from Meso-Single and TPAD system, respectively, with the methane yield of 32.6 and 31.7 $m^3/ton_{fresh\ added}$ accordingly. These values can be useful for project design and financial analysis since the cost of grass harvesting and preparation must be balanced with the additional benefit from it.

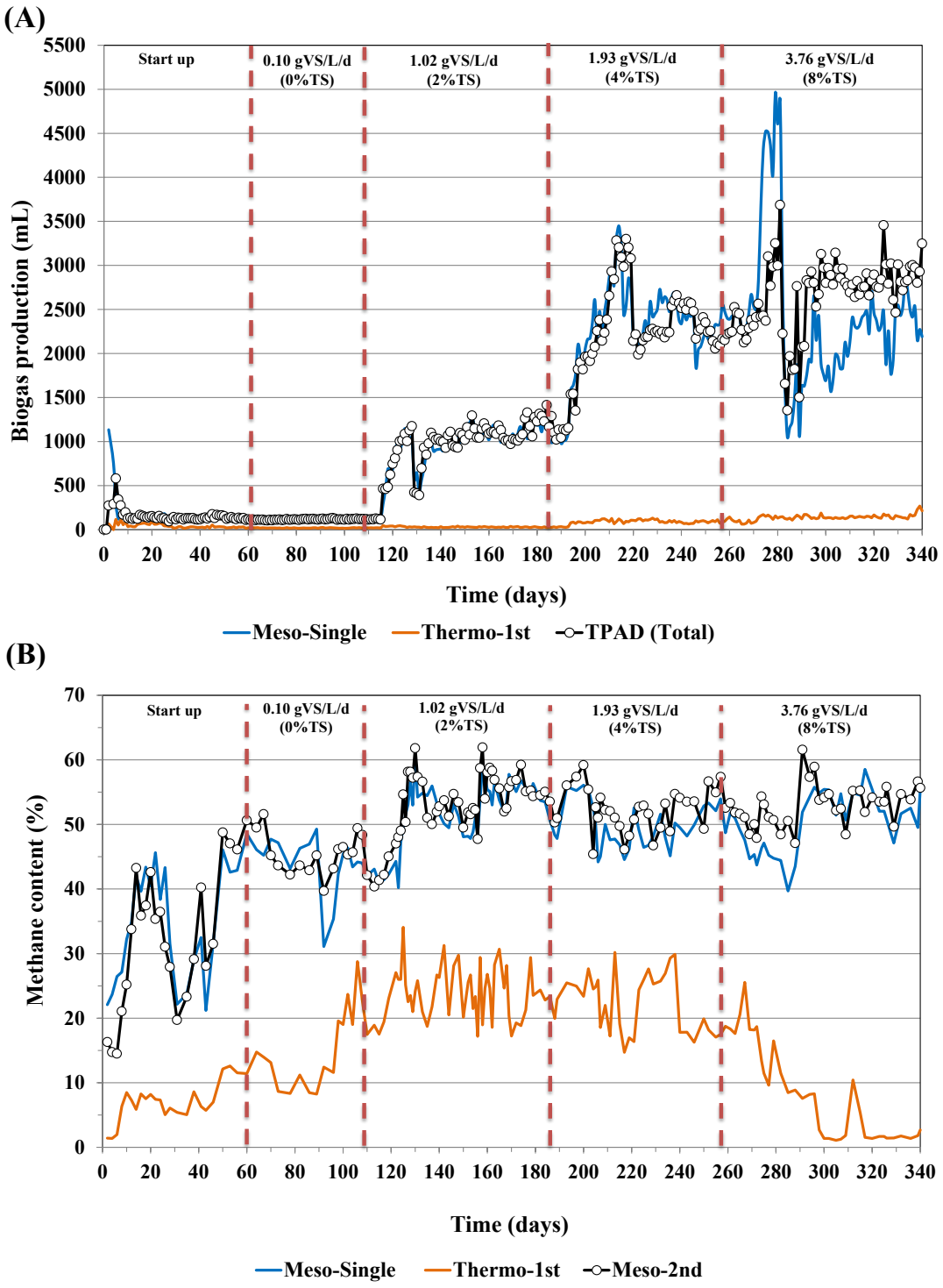

Figure 5.4 Biogas production (A) and methane content (B) from single stage of mesophilic reactors and TPAD reactors at difference organic loading rates

5.3.7 Methane yield and biodegradability

The characteristics of pig manure and para-grass (Table 5.3) were used to calculate the theoretical methane yields from Bushwell's formula in VS units. The theoretical methane yield was based on the substrate's atomic composition. It is also presented for comparison with the specific methane yield in Table 5.5. In both Meso-Single and TPAD system, the specific methane yield was higher with increasing percentage of grass from 0% to 4% mixture and decreased at 8%PG. In comparison, Meso-Single had statistically (p<0.05) higher methane yield at 4 %PG while TPAD system could also gave higher methane yield at 4 %PG as well but not exclusive from 2 %PG. Thermo-1st reactor gave low specific methane yield because of the low biogas production and methane content as it produced more acids. Theoretical methane yield was higher than specific methane yield since a fraction of substrate was used to synthesize the mass of microbial cells and a big portion of it was lost in the effluent un-degraded due to the high lignin content that made it difficult to degrade (Moller *et* al., 2004; Hansen *et al.,* 1998) although it was measured as volatile solids. The digestibility of the solid materials thus depends largely only on a portion of volatile solid, which is biodegradable. In addition, the actual methane yield is always lower than the potential yield because the biological process in the system is inhibited by inhibitors such as volatile fatty acids and ammonia, etc. (Panichnumsin *et al.,* 2006) compared to the ideal calculation. This ration, biodegradability in Table 5.5 was used to represent such degradable fraction. From our data, higher biodegradability was achieved when PG mix ratio was increased from 0-4%. At 8% PG, both systems exhibited the drip as the grass was not degraded well due to the solid accumulation problem mentioned. In contrast, it was also found that biodegradability in Thermo-1st was decreased when PG mix ratio went up. This was due to that biodegradability calculation was based on data of methane of the substrate over the theoretical yield. At higher OLR, Thermo-1st had become a pre-acidified reactor rather than the methane producing one while higher substrate feed rate was introduced.

The co-substrate at 4%PG improved specific methane yield by 36.7% and 23.9% from Meso-Single and TPAD, respectively, when compared to pig manure only (0%PG). The highest methane yield and biodegradability of Meso-Single and TPAD were obtained at 4% PG. Biodegradability of the substrate in Thermo-1st was minimal since most of it was transformed from solid to soluble compounds as the total organic content was still theoretically unchanged. It can be confirmed with the result that study for the BMP in different substrate mixed ratio, PG is more biodegradable (Dechrugsa *et al*., 2013). Lansing *et al*. (2010) suggested that manure co-digestion with substrates that have higher VS content than manure itself is beneficial.

The results from this study are summarized and compared with the other reports as shown in Table 5.6. VS removals increased when PG increased to 2 and 4% and the percentage almost the same in both systems (Meso-Single and TPAD). The result showed low efficiency of VS removal when used PM only as feed. Pig feed was already digested in the intestine, thus, pig manure contained solid that was less degradable (Dechrugsa *et al*., 2013). The VS removal of our PM and PG co-digestion was greater than other studies. The maximum VS reduction (91.0%) was achieved from both Meso-Single and TPAD at 4% PG, OLR 1.9 gVS/L/d. It is noted that both Meso-Single and Meso-2nd were operated in the mode of anaerobic sequencing batch reactor. In the ASBR, the loss of biomass to the effluent was minimal because of the settling phase prior to effluent withdrawn. Therefore, ASBR system would induce a longer sludge retention time (SRT) than CSTR. This led to a higher VS removal at higher organic loading rates (Agler *et al*., 2008). However, Lee *et al*. (2011) reported that longer HRT can improve the efficiency of VS destruction, although the CSTR cannot be operated as long SRT as the ASBR. It was found that VS removal in both studied systems were not efficient at high organic loading (3.8 gVS/L/d). This was associated with solid accumulation and scum formation in the reactors. The mixing apparatus and intermittent frequency were crucial for homogenizing the substrate to the liquid phase. Property of feed used also played an important role in this problem. Small air pockets in our dried para-grass could be broken down if proper pretreatment of the feed is applied. Microorganism in the system cannot well degrade the grass,

since the grass floated and tap gas bubbles making the scum layer to expand through the depth of the reactor and did not settle down before decant. Both mesophilic reactors (Meso-Single and Meso-2st were operated in sequencing batch mode. It appeared that the feed at 8% TS to the long interval mixing scheme approached its operational limit. Lowering solid content in feed or modifying operation such as more frequent mixing or sludge level control must be performed to take advantage of the long solid retention in ASBR operation mode.

In comparison to other studies (Table 5.6), the methane yield from pig manure alone was close to Kaparaju and Rintala (2005) and Ndegwa, *et al*. (2008). Mixing and reactor type were not relevant in this low TS operation. It is noted that most published studies were conducted on CSTR system. Mixing scheme may be the reason of the lower methane yield in this present study.

Table 5.6 A summaries of comparisons with other studies

Substrates	Temperature (^{o}C)	Digester	HRT (days)	VS reduction (%)	Methane Yield (L/gVS_{added})	Organic loading rate (gVS/L/d)	References
Pig manure	35	ASBR	20	73	0.12	0.101	This study
Pig manure	35, 55	TPAD	20	68	0.12	0.101	This study
PM and PG 2%TS	35	ASBR	20	92	0.14	1.016	This study
PM and PG 2%TS	35, 55	TPAD	20	89	0.14	1.016	This study
PM and PG 4%TS	35	ASBR	20	91	0.16	1.931	This study
PM and PG 4%TS	35, 55	TPAD	20	91	0.16	1.931	This study
PM and PG 8%TS	35	ASBR	20	77	0.08	3.761	This study
PM and PG 8%TS	35, 55	TPAD	20	65	0.09	3.761	This study

Table 5.6 A summaries of comparisons with other studies (continued)

Substrates	Temperature (^{o}C)	Digester	HRT (days)	VS reduction (%)	Methane Yield (L/gVS$_{added}$)	Organic loading rate (gVS/L/d)	References
Grass silage	38	Loop reactor	50	60	0.26	1-3.5	Koch *et al*., 2009
Grass waste	Ambient	AF	NA	67	0.17	NA	Yu *et al*.,2002
Swine waste	35	ASBR	12	-	0.12[a]	1.2[b]	Ndegwa *et al*., 2008
Pig manure	35	CSTR	44	-	0.15	2.0	Kaparaju and Rintala, 2005
PM : PP (80:20)	35	CSTR	26	-	0.33	2.0	Kaparaju and Rintala, 2005
PM : PT (80:20)	35	CSTR	39	-	0.28	3.0	Kaparaju and Rintala, 2005
Swith grass: PM	55	Batch	30	58	0.23	15%TS	Ahn and Smith, 2008
Swith grass: DM	55	Batch	30	24	0.01	15%TS	Ahn and Smith, 2008

Table 5.6 A summaries of comparisons with other studies (continued)

Substrates	Temperature (^{o}C)	Digester	HRT (days)	VS reduction (%)	Methane Yield (L/gVS_{added})	Organic loading rate (gVS/L/d)	References
PM:DGS	35	CSTR	30	68	0.27	1.0	Xie *et al*,2012
PM:DGS	35	CSTR	30	63	0.27	1.5	Xie *et al*,2012
PM:DGS	35	CSTR	30	56	0.22	2.0	Xie *et al*,2012
PM:DGS	35	CSTR	30	44	0.17	3.0	Xie *et al*,2012
PM:Maize	39	CSTR	45	83	≤0.25	2.1	Bulkowska *et al*, 2012
PM:Maize	39	CSTR	45	75	≤0.40	2.1	Bulkowska *et al*, 2012

ASBR: anaerobic sequencing bath reactor
CSTR: continuous stirred tank reactor
TPAD: temperature-phased anaerobic digestion
AF: anaerobic filter
[a:] L/gCOD
[b:] gCOD/L/d
PM: pig manure
DM: dairy manure
PT: potato tuber
PP: potato peel
DGS: dried grass silage
NA: not available

5.3.8 Microbial communities

Hydrolysis, acidification, and methanation reactions in anaerobic digestion pathways are performed by different groups of microorganisms within the microbial community. The yield of both reactions is, thus, inevitably influenced by microbial communities (Lin *et al*, 2013). The microbial communities in each reactor were surveyed at 0% and 4% solid loading of PG (OLR 0.10 and 1.93 gVS/L/d) using DGGE techniques. The diversity of bacteria and archaea communities were shown in Figure 5.5 and Figure 5.6, respectively. The results showed differences in the microbial makeup of both of bacterial and archaea.

It was found that *Clostridium* sp. were detected in all reactors. *Clostridium* sp. are common bacteria in anaerobic environment that have been frequently reported for their capability of converting variety of organic substances to sugars, ethanol, acetate, lactate and hydrogen (Rincon *et al*., 2006). They are categorized as acetate producing microorganisms and found in animal manure since they reside in animal rumens (Lin *et al*, 2013). In the thermophilic reactor of TPAD system (Thermo-1st), higher diversity of bacteria species was found when grass was added to the feed (4% PG) compared to pig manure only (0% PG). Two species of *Clostridium*; *Clostridium* sp. MF18_Ns and *Clostridium stercorarium* strain DSM 8532 were detected in Thermo-1st reactor with 4% PG feed. *Clostridium stercorarium* strain DSM 8532 is a thermophilic bacterium capable of producing thermoactive cellulase efficiently degrading polysaccharides in plant biomass and converting the derived sugars to ethanol and acetate (Poehlein, *et al*., 2013). Acetic acid is an essential product for methane production by acetoclastic methanogenic archaea. Due to the fact that only hydrogenotriphic methanogenic archaea was found in our thermophilic reactors both 0% PG and 4% PG feed (Figure 5.6), the accumulation of acetate was resulted in this reactor (Table 5.4). The higher diversity of bacteria with grass mixed feed was also hold true for *Thermoanaerobacterium* sp. and the uncultured bacteria that could synergistically work to degrade lignocellulosic grass substrate. At 4% PG feed, separated bands of *Thermoanaerobacterium* sp. enrichment culture clone D5 and

Thermophilic anaerobic bacterium K1L1 were added to the community, while there were 4 uncultured bacterium groups were present instead of only 1 at pure PM feed. The existence of diverse species in Thermo-1st had played a significant role in the hydrolysis and subsequent production of VFA, which allowed a more stable operation of the following Meso-2nd to produce biogas.

In the single stage meshophilic reactor (Meso-Single), the dominant species were different between 0% and 4% PG feed. *Rhodobacteraceae* sp., *Acinetobacter* sp., *Halanaerobium* sp. and *Ruminococcaceae* sp. were dominant at manure fed Meso-Single reactor while the groups of *Pseudomonas* sp., *Roseburia* sp., and *Lachnospiraceae bacterium* were dominant at 4% PG feed condition. *Halanaerobium* sp. and *Roseburia* sp. are known as the producer of acetate and butyrate, respectively, from fermentation (Insam *et al*., 2010). Some *Pseudomonas* sp. was reported for their ability to produce endoglucanase, exoglucanase and β-glucosidase and xylanase which were very effective in hydrolyzing agricultural wastes such as bagasse (Cheng and Chang, 2011). *Lachnospiraceae bacterium* is a common of rumen flora, especially the animals that fed with legume hay and it can produce acetate, lactate, H_2 and CO_2 (Madigan, *et al*., 1997). There was an obvious shift of dominance in the culture when the substrate was changed although many groups were still present but at less number as seen in light bands in the gels in Figure 5.5.

It was observed that there was almost identical microbial community makeup between the Meso-Single and Meso-2nd at 0% PG feed. This might be associated with the common substrate of pure pig manure. Compositions of the liquid exposed to the bacteria were rather similar as confirmed by VFAs concentration in Table 5.4. At this relatively low organic loading, VFAs were processed so effectively that very low levels of each VFA species (shown as non-detectable level) were present within the mesophilic digesters. However, when compared the dominant bacterial species found in the second stage mesophilic digesters (Meso-2nd) at 0% and 4% PG, there was a drastic shift of microbial dominance in the reactor (Figure 5.5). The presence of *Pseudomonas* sp. R-45822, *SRB bacterium* enrichment culture clone SRtB-otu1-52,

Flavobacterium sp. 01WB03.1-18, *Microbacterium aerolatum* strain KUDC1073 were detected when fed with grass. The composition of the substrate has great influence on the microbial community makeup. The addition of PG had changed the characteristics of liquid that the microbes exposed as evidenced in Table 5.4. The mentioned groups of dominant bacteria were those capable of hydrolyzing the lignocellulose and bearing the higher organic acid concentrations.

The archaea found in mesophilic reactor in this study consisted of 4 groups. The diversity of archaea was not much changed between 0% PG and 4% PG in each reactor. *Methanobacterium formicicum* and *Methanocella conradii* were predominant methanogen species in thermophilic reactors. These two species are the hydrogenotrophic methanogens that utilize hydrogen to produce methane and more able to withstand the low pH environment. The absence of acetoclastic methanogens in the thermophilic reactors corresponded well with the results of high acetate concentration at this stage shown in Table 5.4 and the low biogas and methane production (Figure 5.4A). It is widely known that in methanogenesis, 72% of the methane is produced thru acetoclastic pathway and approximately 28% is by hydrogenotrophic one (Khanal, 2008). Methane in this reactor was produced mostly from H_2 and CO_2. Instead, the acetoclastic methanogens; *Methanomethylovorans* sp., uncultured *Methanosaeta* sp. and uncultured *Methanolinea* sp., were found dominant in mesophilic reactors at both 0% and 4% PG. And there also found the hydrogenotrophic *Methanobacterium formicicum* in the mesophilic reactor as well. This explains the effective utilization of the intermediates in the second stage in the staged configuration as well as the versatility in the one tank model.

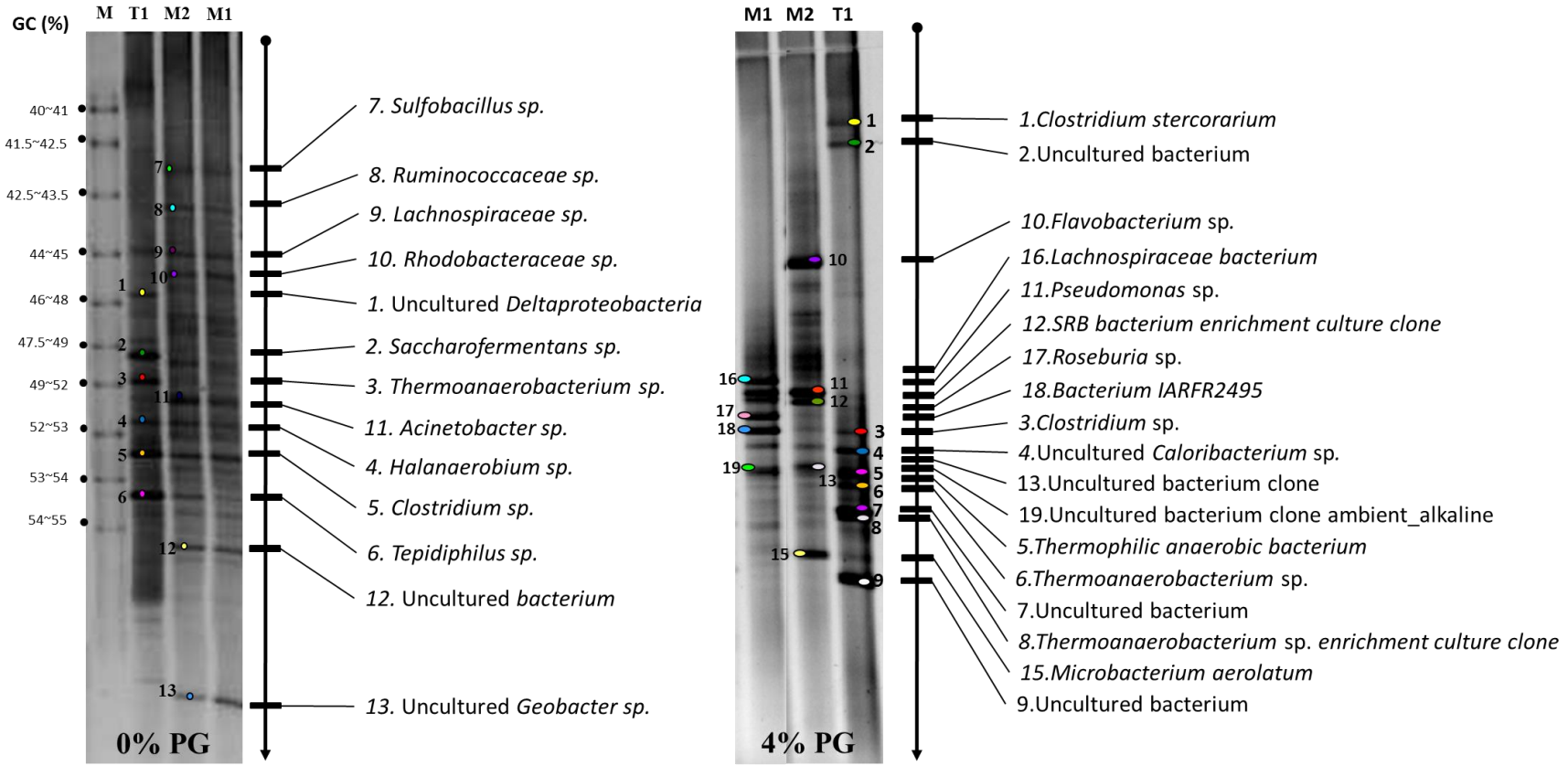

Figure 5.5 Bacterial community profile determined with PCR-DGGE of partial 16s rRNA genes fragments at different solid loading (0 and 4%TS of PG) from three reactors; (M1) Mesophilic, (T1) first stage thermophilic reactor of TPAD and (M2) second stage mesophilic of TPAD

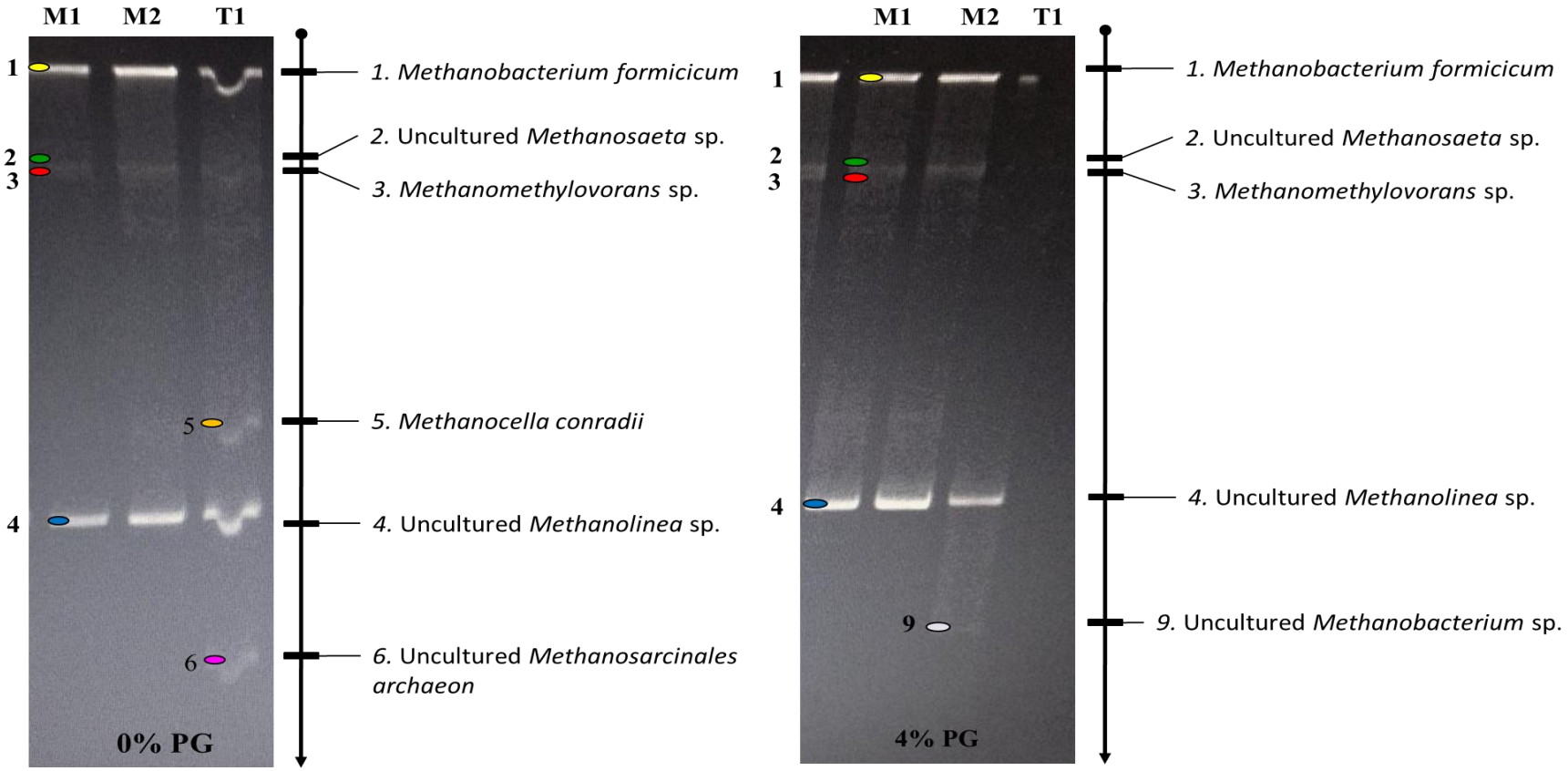

Figure 5.6 Archaeal community profile determined with PCR-DGGE of partial 16s rRNA genes fragments at different solid loading (0 and 4%TS of PG) from three reactors; (M1) Mesophilic, (T1) first stage thermophilic reactor of TPAD and (M2) second stage mesophilic of TPAD

CHAPTER VI

EFFECT OF FEEDING PATTERN ON CO-DIGESTION OF PIG MANURE AND GRASS UNDER MESOPHILIC ASBR

Abstract:

The goal of this study was to examine the effect of feeding pattern on the performance for anaerobic co-digestion of pig manure and para-grass. Anaerobic sequencing batch reactor (ASBR) was operated at 35°C under increasing solid loadings in a regular ASBR feeding (RF) and a periodic feeding (PF) that loaded solids to the digester only the first 11 days of a 26-day round. Experiments were conducted at different solid loadings from 0.10-3.76 gVS/L/d (0-8% dry para-grass mixing ratios, PG) with 20 d hydraulic retention time (HRT). Results showed that methane yield from PF reactor was slightly higher than the RF reactor at all solid loadings. The highest methane yield of 169.8 $mLCH_4/gVS_{added}$ was obtained from periodic feeding at 2 %PG while 158.3 $mLCH_4/gVS_{added}$ was obtained from continuous feeding at 4 %PG. The methane contents were 53.3% and 52.0% from RF and PF, respectively. Longer chain fatty acids were found in PF reactor at 8 %PG and their concentration oscillated corresponding to the feeding period. PF pattern could also be advantageous since it could save time and labor to operate. Analysis of microbial communities by DGGE indicated that RF and PF pattern had no effect to the dominance of bacteria and archaea in ASBR.

Keywords: Anaerobic sequencing batch reactor, Co-digestion, Pig manure, Para-grass, DGGE

6.1 Introduction

In recent years, the number of small pig farms in Thailand has decreased considerably owned to the economy of scale in animal production business and the stringent environmental regulation. Therefore, large scale pig farm operations are currently dominating the majority of pig production. Nevertheless, environmental issues of pig farms still exist such as air, soil, and water pollution. In addition, the disease control must be strictly applied as the outbreak of disease into a farm would cause massive losses of animal lives and money. Limit access in and from the farms is practiced to minimize the chances that the diseases coming with the incoming vehicles could break into the farm perimeter. Pig waste must therefore be handled within the farms. Large amount of pig barn wastewater is produced daily and it became widely accepted that biogas plant be installed in standard pig farm since it is capable of treating it with economic return. Anaerobic digestion is a workable solution to reduce pollution from pig waste and produces biogas as a by-product. The biogas is converted to electricity for use mainly in cooling and heating systems in the farm.

Para-grass is a kind of plant that grows well in tropical areas. No exception in pig farms, its fast growing has caused troubles since it becomes a fire hazard and a source of disease and vector hatching. On the other hand, this grass can be a biomass used in co-digestion with pig manure to enhance biogas production. This co-digestion can provide a better C:N ratio in the digester feedstock (Xie *et al*., 2011). Moreover, co-digestion of manure with energy crop residues helped decrease ammonia inhibition (Kaparaju and Rintala, 2005; Xie *et al*., 2011).

There are several types of anaerobic digesters being used such as the continuously stirred reactors and plug flow that virtually have the same solids retention time (SRT) and hydraulic retention time (HRT) (Ndewa *et al*., 2008) due to the complete mixed nature. But many anaerobic reactors can separate HRT and SRT such as up-flow anaerobic sludge blanket, anaerobic biofilter, etc. For wastewater with higher solid content, anaerobic sequencing batch reactor (ASBR) is another type

that effectively separating SRT from HRT. The operating principles of the ASBR follow four stages: feed, react, settle and decant in a cycle. Since only the supernatant is discharged, the solids have longer retention time than liquid (Zhang and Dugba, 2000; Ndewa *et al*., 2008; Luo *et al*., 2009). Application of ASBR on the high solid digestion, i.e. above 2 % total solid (TS) content, has not been studied. Moreover, the pattern of the feeding to the ASBR by shifting and concentrating the feeding into a shorter period and let the digester run without feeding until the end of a round could hypothetically give more time for the microbes to degrade the biomass substrate more completely. However, this feeding regime could become a challenge since the organic load is concentrated at the start of each round.

The objective of the study is to evaluate the performance of single stage mesophilic ASBR at different feeding patterns between a typical regular feeding versus a periodic feeding. The experiment was conducted in co-digestion of pig manure and para-grass at different total solid of para-grass. Microbial community analysis in each feeding pattern was analyzed using DGGE technique.

6.2 Methods

6.2.1 ASBR system

Reactors were made of glass with an volume of 5 L. The effective volume was set at 4 L. The reactor had four ports; one port assigned for biogas collection located on the top of reactor and other three ports on the side of reactor assigned for influent feeding, effluent withdrawal, and sludge sampling. Temperature was maintained at 35±1 ^{o}C by a temperature controlled chamber (water bath). The reactor was capped with a rubber stopper. One operation cycle consisted of fill, react, settle and decant. The reactor content was let to settle 30 min. before decanting which was done in the last 10 min. of each cycle. To begin the cycle, feeding was done in the first 10 min. and react period was 22 hr 40 min. to make up a 24-hr cycle. The reactor content was

mixed manually for 1 min two times a day. This mixing provided sufficient distribution of sludge contact to the wastewater and biomass in the reactor.

6.2.2 Inoculum

The sludge used in this experiment was collected from UASB (up-flow anaerobic sludge blanket) reactor treating pig waste slurry from the unit of finishing barn, in a pig farm in Songkhla Province, Thailand. The sludge was measured for specific methanogenic activity (SMA), total solids (TS) and volatile solid (VS) before used.

6.2.3 Preparation of feed

The mixture of pig manure (PM) and para-grass (PG) as co-substrate were used in this experiment. PM was obtained from excretions of the finishing unit. It was dried at 60 ^{o}C and ground in a mortar to small particles. The fresh green PG, *Branchiria mutica*, was randomly harvested from the commercial pig farm where obtaining pig manure, in Pattalung Province, Thailand. It was chopped with an agricultural cutting machine to approximately 2 cm, and then dried at 60 ^{o}C and shredded to the maximum length of less than 6 mm. Both substrates were kept at 4 ^{o}C until use throughout the experiment. In a preparation of feed before use, the dried PM was diluted with tap water to 2,500 mgTS/L to imitate the pig farm wastewater. The mixtures of para-grass (PG) was done at 4 levels; 0%, 2%, 4% and 8%TS to the prepared wastewater as co-substrate. The substrate mixture was homogenized with tap water before feeding to the reactors as slurry.

6.2.4 Reactor operation

Two reactors were started up with an initial active sludge of 30% of the effective volume (1,200 mL) as inoculum and filled up with the prepared pig slurry wastewater (2.50 gTS/L or 2.015 gVS/L) to an effective volume. The reactors were

then rested for approximately 24 h before the scheduled feeding began. During start-up, both mesophilic reactors were fed with PM at OLR of 0.10 gVS/L/d until they went to the stable condition which took 65 days.

This experiment consisted of two feeding patterns; regular feeding (RF) and periodic feeding (PF). In regular feeding, the feed was introduced into the reactor at the beginning of each ASBR cycle (feed-react-settle-decant). The system was operated at 20 days hydraulic retention time (HRT) with an influent flow rate of 200 mL/d. Both reactors were operated under this condition for the start-up until reaching stable condition, signified by stable biogas production and pH.

After the stable condition of RF pattern, one of the two reactors was switched to PF pattern. In PF pattern, the higher amount of feed was introduced to the reactor only in the first 11 days out of a round of 26 days (Table 6.1). To begin the round, the effluent was decanted until a total volume of 800 mL of mixed liquor was left. Then, 364 mL of waste was fed daily until day 11 without effluent withdrawal. The reactor was left unfed until day 26, a 15-day period. It is noted that the amount of substrate input to the system and the averaged HRT during a round of 26 days was intentionally designed to be equal between RF and PF reactors, as shown in Table 6.1. Hydraulic retention time (HRT) was calculated by dividing the summation of day old of each feed with the feeding day. At day 26, HRT of 20 days was achieved which was calculated from the summation of day old of all 11 day feeding divided by 11 days. Both feeding patterns were repeated for 3 rounds or until the systems performance was stable. The corresponding organic loadings of PF reactor is illustrated in Figure 6.1. After stable condition was reached, organic loading was raised by increasing the concentration of solid in feed with para-grass mixture to 2, 4 and 8 % of total solids which corresponded to OLR 1.016, 1.931, and 3.761 gVS/L/d, respectively. During the experiment, pH was maintained at 6.8±0.2 with 0.1 N NaOH as needed.

Table 6.1 Feeding pattern of periodic feeding (PF) reactor

Day	Feed (mL/d)	Substrate retention time in PF reactor (d)											Substrate retention (d)	Avg. retention time(d)
1	364	0											0	0.0
2	364	1	0										1	0.5
3	364	2	1	0									3	1.0
4	364	3	2	1	0								6	1.5
5	364	4	3	2	1	0							10	2.0
6	364	5	4	3	2	1	0						15	2.5
7	364	6	5	4	3	2	1	0					21	3.0
8	364	7	6	5	4	3	2	1	0				28	3.5
9	364	8	7	6	5	4	3	2	1	0			36	4.0
10	364	9	8	7	6	5	4	3	2	1	0		45	4.5
11	364	10	9	8	7	6	5	4	3	2	1	0	55	5.0
12	-	11	10	9	8	7	6	5	4	3	2	1	66	6.0
13	-	12	11	10	9	8	7	6	5	4	3	2	77	7.0
14	-	13	12	11	10	9	8	7	6	5	4	3	88	8.0
15	-	14	13	12	11	10	9	8	7	6	5	4	99	9.0
16	-	15	14	13	12	11	10	9	8	7	6	5	110	10.0
17	-	16	15	14	13	12	11	10	9	8	7	6	121	11.0
18	-	17	16	15	14	13	12	11	10	9	8	7	132	12.0
19	-	18	17	16	15	14	13	12	11	10	9	8	143	13.0
20	-	19	18	17	16	15	14	13	12	11	10	9	154	14.0
21	-	20	19	18	17	16	15	14	13	12	11	10	165	15.0
22	-	21	20	19	18	17	16	15	14	13	12	11	176	16.0
23	-	22	21	20	19	18	17	16	15	14	13	12	187	17.0
24	-	23	22	21	20	19	18	17	16	15	14	13	198	18.0
25	-	24	23	22	21	20	19	18	17	16	15	14	209	19.0
26		25	24	23	22	21	20	19	18	17	16	15	220	20.0

Note: Line at day 11 indicates the last day of feeding in PF in each cycle

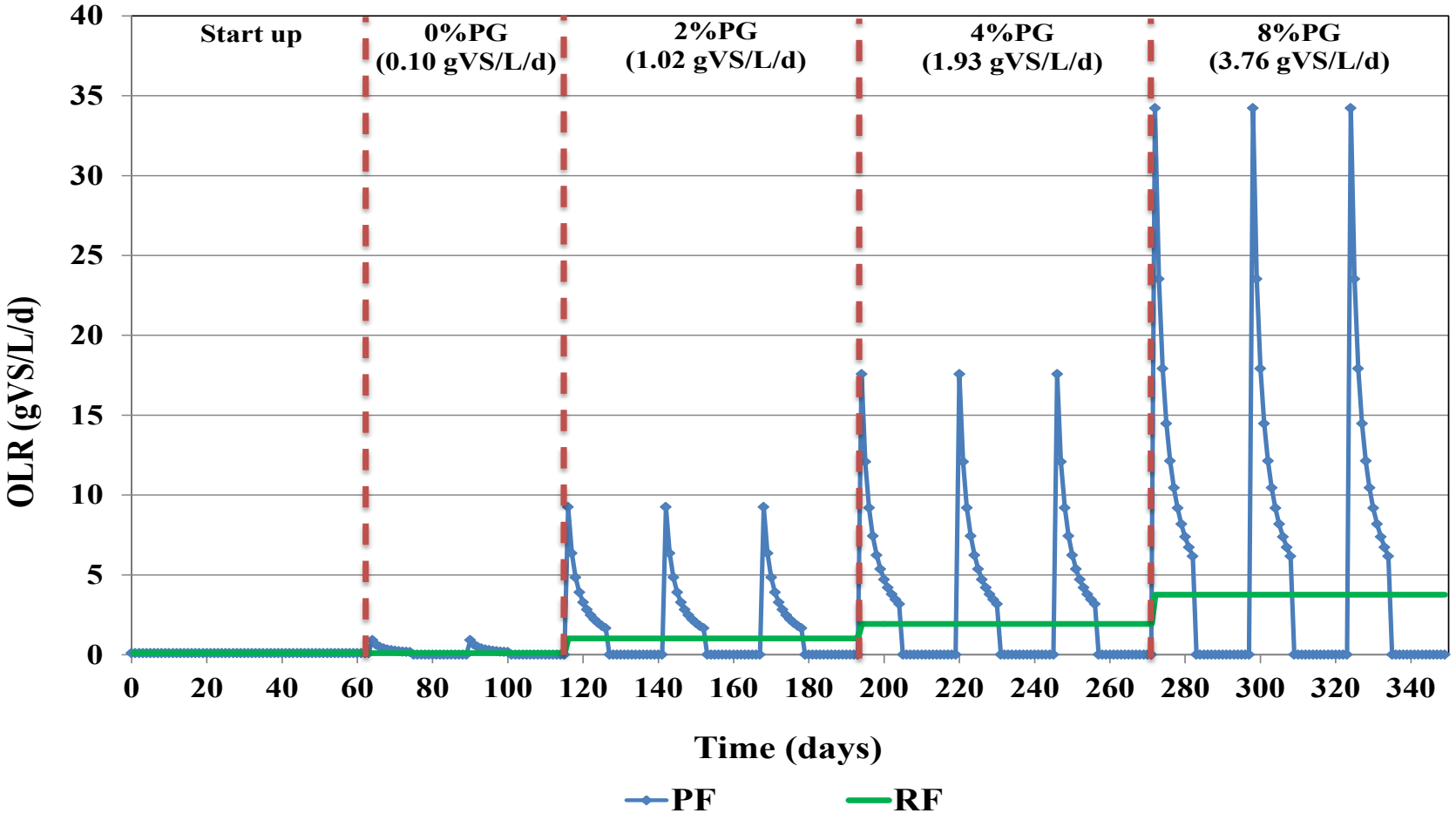

Figure 6.1 Feeding patterns of regular feeding (RF) and periodic feeding (PF) reactor throughout the experiment

6.2.5 Analytical method

6.2.5.1 Inocula and substrates

Inoculum and substrates (PM and PG) were analyzed for TS and VS according to the Standard Methods (APHA, 1999). The chemical compositions of the samples were analyzed using CHNS-O Analyzer. The specific methanogenic activity assay (SMA) was performed to confirm the activeness of methanogens in the inoculum before use.

6.2.5.2 System performance analysis

Performance of the ASBR was evaluated by the determination of pH, total and soluble COD (TCOD and SCOD), and volatile solid (VS) of the influent and effluent according to the Standard Methods (APHA, 1999) while available alkalinity and volatile fatty acid (VFA) concentration were determined by direct titration method (Anderson and Yang, 1992). Individual volatile fatty acids (VFAs) were analyzed by gas chromatography (GC 7820A Agilent Technologies) equipped with a flame ionization detector (FID). A capillary column Agilent 19091N-133 HP-INNOWax polyethylene glycol was used with helium as the carrier gas.

6.2.5.3 Biogas and methane content

The biogas produced was measured daily using a multi-chamber rotor gas meter (Ritter). Biogas composition was analyzed twice a week by gas chromatography (GC 7820A Agilent technologies) equipped with thermal conductivity detectors (TCD). Helium was used as carrier gas.

6.2.5.4 Microbial community analysis by denaturing gradient gel electrophoresis (DGGE)

Sludge samples for microbiological analyses were taken from the reactor after finished the condition of OLR 0.10 gVS/L/d (0%PG) and 1.93 gVS/L/d (4%PG). The method was used for microbial community analysis according to the procedures in Hniman *et al.* (2011). The bacterial 16S rDNA was amplified by the first polymerase chain reaction (PCR) with universal primer 1492r and 27f. The primer K517r and L340f with CG clamp were used to amplify the fragment of V3 region of 16S rDNA product from the first PCR. The bands were excised from the gel and re-amplified with primer 357f (without a GC clamp) and the reverse primer. PCR products were purified and sequenced using primer 518r for bacteria by the Macrogen sequencing facility (Macrogen Inc., Seoul, Korea). Closest matches for partial 16S rRNA gene sequences were identified by ribosomal database project with SeqMatch program and basic local alignment search tool (BLAST) with nucleotide database in National Center for Biotechnology Information (NCBI).

The PCR amplification targeting archaea was carried out with the forward primer PRA46F and the reverse primer PREA1100R. This PCR product was then used as a template for the PCR amplification of 179 bp using the forward primer PARCH340F containing a GC clamp and the reverse primer PARCH519R. PCR products were purified and sequenced using the primer PARCH340F and PARCH519R (Ovreas *et al*., 1997).

6.2.6 Degree of hydrolysis and acidogenic calculations

First step of anaerobic digestion pathway, degree of hydrolysis at any point in time is defined as the solubilization of organic matter to hydrophilic soluble compounds by using hydrolytic bacteria. Degree of hydrolysis can be expressed as the quotient between the effluent COD in filtered sample (SCOD) and influent COD in total sample (TCOD). In the second step, the performance of the acid phase digestion

can be quantified using the percentage of the initial substrate concentration (influent TCOD) converted to VFAs. The quantity of each VFA species was converted to gCOD/g by using the COD equivalents of each VFA. The COD equivalents for volatile acids for acetic, propionic, butyric, valeric and caproic are 1.066, 1.512, 1,816, 2,036 and 2.204, respectively (Demirel and Yenigun, 2004). It is noted that COD equivalence of methane was added to the nominator of both hydrolysis and acidgenesis yield calculations because it was an end product from both reactions.

Therefore, the degrees of hydrolysis (H) and acidogenesis (A) were calculated according to the following equations (6.1) and (6.2), respectively (Hamed *et al*, 2004).

$$H\ (\%) = \left[\frac{CH_4\ as\ COD + Effluent\ CODdis}{Influent\ CODt}\right] \times 100 \qquad (6.1)$$

$$A\ (\%) = \left[\frac{CH_4\ as\ COD + Effluent\ VFA\ as\ COD}{Influent\ CODt}\right] \times 100 \qquad (6.2)$$

where $H(\%)$ is the percentages of hydrolysis, $A(\%)$ is the percentages of acidification, $CH_4\ as\ COD$ is gCOD equivalence of methane at STP, $Effluent\ COD_{dis}$ is SCOD of effluent, *Effluent VFA as COD* is gCOD calculated from VFA species, *Influent CODt* is TCOD of influent.

Biodegradability of the substrate is defined as the ratio of the measured methane yield over the theoretical methane yield.

Biodegradability = (Specific methane yield / Theoretical methane yield) ×100 (6.3)

6.3 Results and discussion

The characteristics of pig manure (PM) and para-grass (PG) used in this experiment are shown in chapter V. TS and VS of inoculum were 78.03 and 46.02 g/L, respectively. The methanogenic activity was 32.99±0.18 $mLCH_4/gVS$ which

showed high concentration of active acetoclastic methanogens in the sludge (Dechrugsa *et al.,* 2013).

6.3.1 pH, VFA and alkalinity

The pH of influent slightly decreased from start-up to day 194 (2 %PG) and it decreased further with the increasing PG to 4% and 8% from 6.82, 6.57, 5.80 to 5.46, respectively. This influent pH decreased with increasing PG mixing was due to the specific characteristics of para-grass. The pH of effluent from RF and PF were quite steady in a range of 6.5-6.8 and 6.6-6.8, respectively over all OLRs. Both reactors gave similar pH values especially after day 116. During the start-up period, VFA was fluctuating around 1,000-1,500 mg/L as $CaCO_3$ while the alkalinity level declined from around 900 to 600 mg/L as $CaCO_3$. VFA/Alkalinity ratios were 1.8-2.0 and 1.5-2.1 in RF and PF reactors, respectively. This high VFA/Alkalinity signified the unstable buffering capacity within the systems, that pH control measure was undertaken. NaOH solution 0.1 N was administered to the reactor to maintain pH at 6.8±0.2 from day 82 onwards. It was observed in both reactors that alkalinity had elevated with the increasing OLR while VFA was under control to below 400 mg/L as $CaCO_3$ until OLR 1.93 gVS/L/d (4 %PG). However, at 8 %PG VFA in RF touched 500 mg/L as $CaCO_3$ level and in PF operation it obviously swung corresponding to the peak loading of PG at the beginning of each round (26 day period). VFA to alkalinity ratio stayed in the range of 0.16-0.19 at 2 and 4 %PG. It went up to 0.20-0.28 at 8 %PG of feed. It was still below 0.4 which was suggested as a threshold of buffer adequacy in anaerobic digesters (Song, *et al*., 2004). It appeared that the systems were capable to operate under these applied conditions with sufficient buffering capacity. However, a more frequent pH adjustment particularly at 8 %PG was not permissible for a large scale operation.

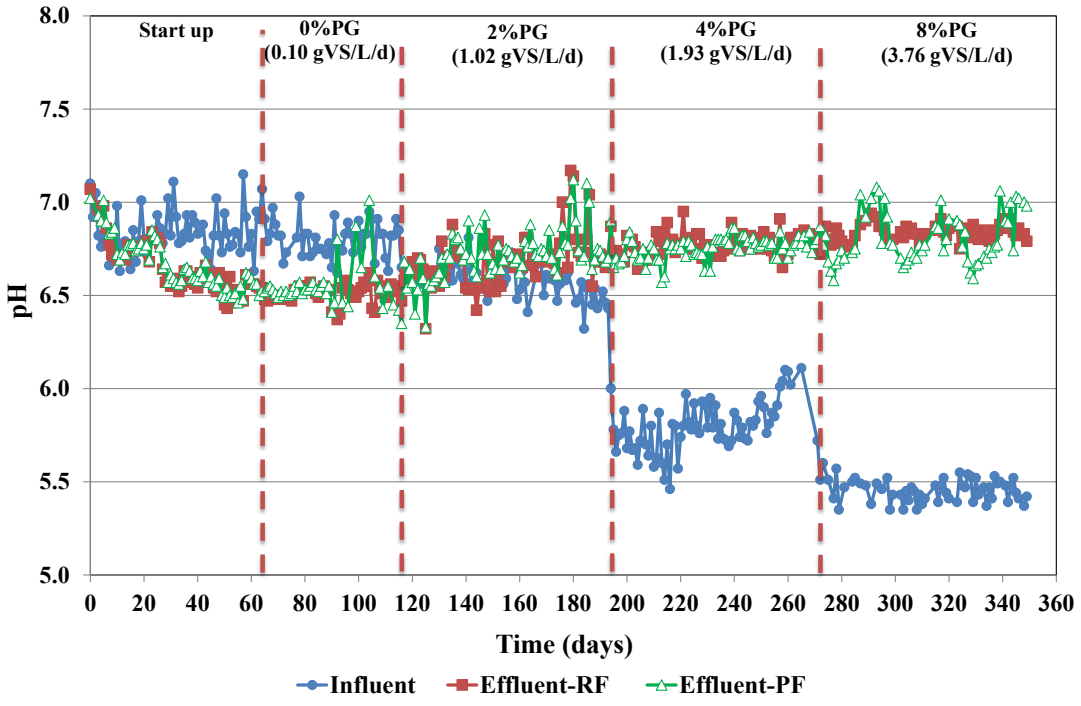

Figure 6.2 pH of influent and effluent when operating with different para-grass mixture from mesophilic ASBR with regular feeding (RF) and periodic feeding (PF)

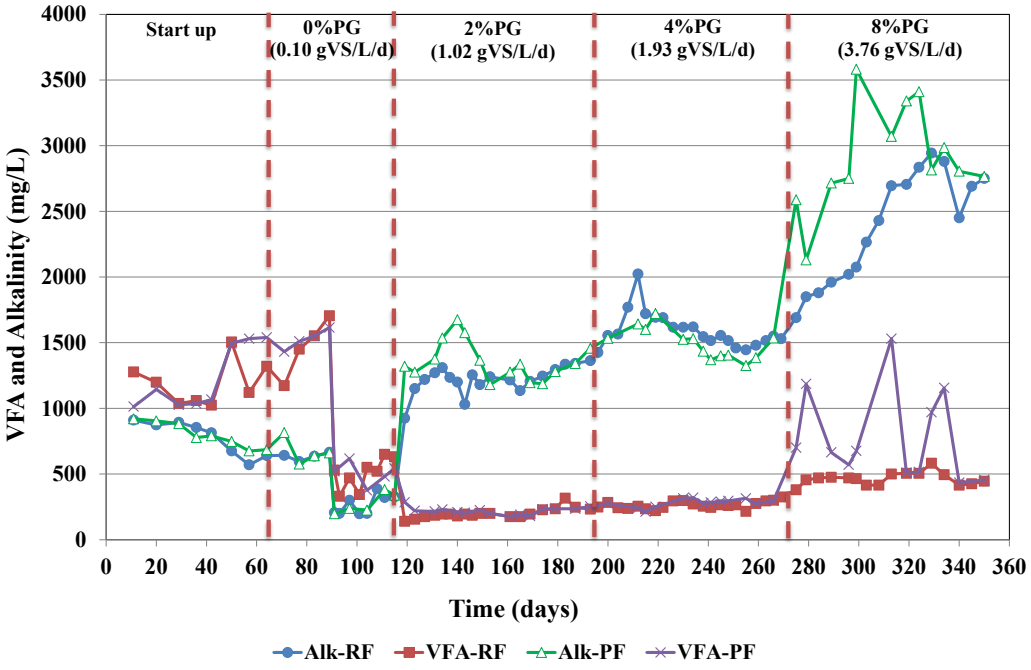

Figure 6.3 VFA and alkalinity of effluent when operating with different loadings from mesophilic ASBR with regular feeding (RF) and periodic feeding (PF)

6.3.2 VS reduction

The volatile solid (VS) concentrations of influent and effluent are shown in Figure 6.4. The average VS values from RF and PF reactor were 0.45 and 0.44, 1.29 and 1.40, 2.0 and 1.84 and 8.48 and 7.79 when added PG at 0, 2, 4 and 8 %TS, respectively. Both reactors gave close value of VS removals. Result showed that VS in the effluent increased with the higher para-grass loadings. There was quite clear that at 8 %PG (OLR 3.76 gVS/L/d) both systems had reached the limit of ASBR operation. A large portion of solid was accumulated in the reactor with the expanding scum layer (grass trapped with gas bubbles) while there was still a lot leaving the reactor undegraded. VS removal efficiencies were gradually increased until 4 %PG (OLR 1.93 gVS/L/d), and failed at 8 %PG. It was evidenced that at 8 %PG, ASBRs could convert less solid substrate to VFA and methane.

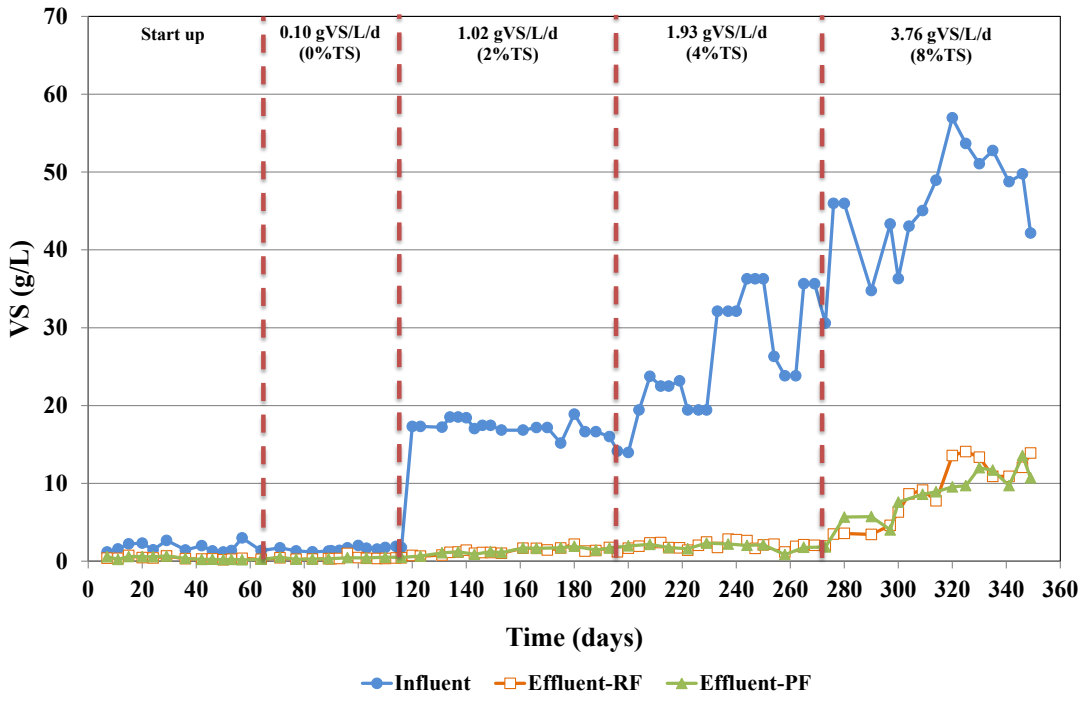

Figure 6.4 VS of effluent from the regular (RF) and periodic feeding (PF) reactors at different solid loadings

6.3.3 Individual VFA

The VFA composition from regular feeding (RF) reactor and periodic feeding (PF) reactor at different solid loadings is shown in Figure 6.5. It is noted that the y-scale in Figure 6.5A and 6.5B are different. No VFA species were detected at 0 %PG (OLR 0.10 gVS/L/d) because they were efficiently converted end products such as biogas, alcohols, cell tissues and other chemicals. The VFA species were found at higher loads at 2, 4, and 8 %PG. The majority of VFA in RF and PF reactors was acetic acid (HAc) that was found at all OLRs. In RF reactor, VFAs increased sharply after the first day of 2 %PG feeding. Then, it decreased suddenly to an undetectable level 10 days afterwards. It showed that the system went to a stable condition and able to cope with this loading. The VFAs was found again when PG was increased to 4 % and 8 %PG, most of the times the total VFA (TVFA) was under 50 mg/L as $CaCO_3$. This level is considered low in anaerobic digesters. Iso-butyric and butyric acids (iHBu+HBu) were the dominant species followed by the propionic (HPr) acid and acetic (HAc) at 4 %PG. HPr had disappeared during the highest OLR of 8 %PG leaving only iHBu+HBu around 20 mg/L with the increasing trend of HAc. This might be due to the shift in microbial community where the activity of acetoclastic methanogens that utilized acetate was diminishing at higher loading (Supaphol *et al*., 2011).

In PF reactor, it was found that VFAs concentration was under similar level to RF reactor up to 4 %PG. At 2 %PG mixture, only HAc and HPr were found at first 10 days of the first round. There exhibited the swing or loop of VFAs concentration but still under 50 mg/L as $CaCO_3$ at 4 %PG with the composition of HAc, HPr and iHBu+HBu (iso-butyric and butyric acid). AT 8 %PG, there was a great fluctuation of TVFA in cyclic curve and all individual species, HAc, HPr, iHBu+HBu and iHVa+HVa, were detected. In each round, VFAs concentration increased for 12 days, 1 day longer than the feeding period, and decreased after stop feeding. Higher PG added is linked to the emergence of longer chain VFAs, especially during feeding period in PF reactor. Therefore, the periodic feeding pattern may not be suitable for

ASBR at high loading (>1.93 gVS/L/d), but it is a good feeding pattern when applied at lower OLR, i.e. under 1.93 gVS/L/d. It also saves energy, labor and time to operate. This feeding strategy could be designed to accommodate the operators to minimize the task of feeding to each digester daily. Instead, they can feed to multiple feeding digesters but do one at a time for only a certain period before switching to the next digester.

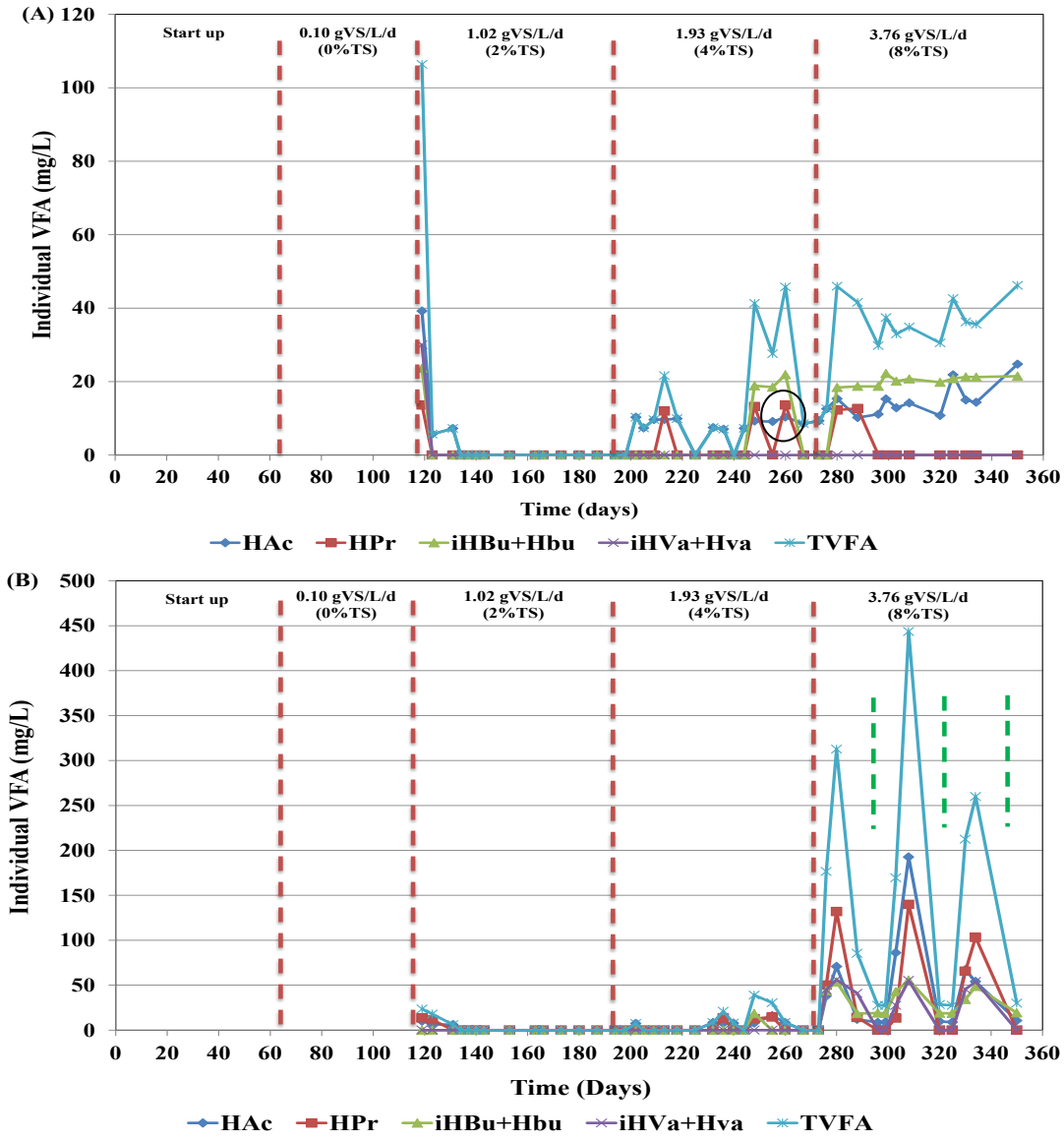

Figure 6.5 Change of individual VFAs concentration from different feeding pattern; (A) regular feeding, and (B) periodic feeding with different solid loadings

6.3.4 Hydrolysis and acidification

Hydrolysis and acidification rates were shown in terms of the percentages in Figure 6.6.

6.3.4.1 Hydrolysis yield

RF reactor was fed with PM (OLR 0.101 gVS/L/d) from the start that gave the hydrolysis yield in the range of 25% at this OLR. After PG was added to the system at 2 %PG at the first round, hydrolysis yield decreased which may be a result of the adaptation of microorganism for new feedstock. Then hydrolysis yield increased to the range of 26-33 % at second and third cycle of 2 %PG. For the 4 %PG the yield increased to the range of 30-35 %. In contrast, hydrolysis yield dropped suddenly at 8 %PG because too high loading and limited conversion efficiency of the system. Also, in PF reactor, the hydrolysis yield was affected by the feeding period (Figure 6.6(A)). The yield increased in the feeding period and declined after stopped feeding similar in each cycle. Compared to Xie *et al*. (2012) that studied the fermentation of the grass silage in leaching bed reactors (LBRs) with OLRs 0.5, 0.8, 1.0 kgVS/m^3/d, our hydrolysis values are higher than theirs (16.5-22.4%).

6.3.4.2 Acidification yield

Acidification yield from this study both RF and PF reactors were inevitably related to the hydrolysis yield since the hydrolyzed product from hydrolysis is the starting materials for acidogenesis. In RF reactor, acidification yield was in the range of 18-22% and slightly decreased with PG addition up to 4 %PG in first cycle. Then the yield increased at second and third cycle to the constant. The acidification yield increased with increasing PG to 4 % (1.93 gVS/L/d) and then decreased sharply with 8 %PG (3.761 gVS/L/d). The highest yield was found at 4 %PG mixture. The yield decreased when OLR was up to 3.761 gVS/L/d because of incapability to deal with scum. Therefore, mixing was very important on solid digestion to protect the

scum in the reactor. Microbial can be in contact with the substrate which promotes higher potential to convert solubilized matter to VFAs.

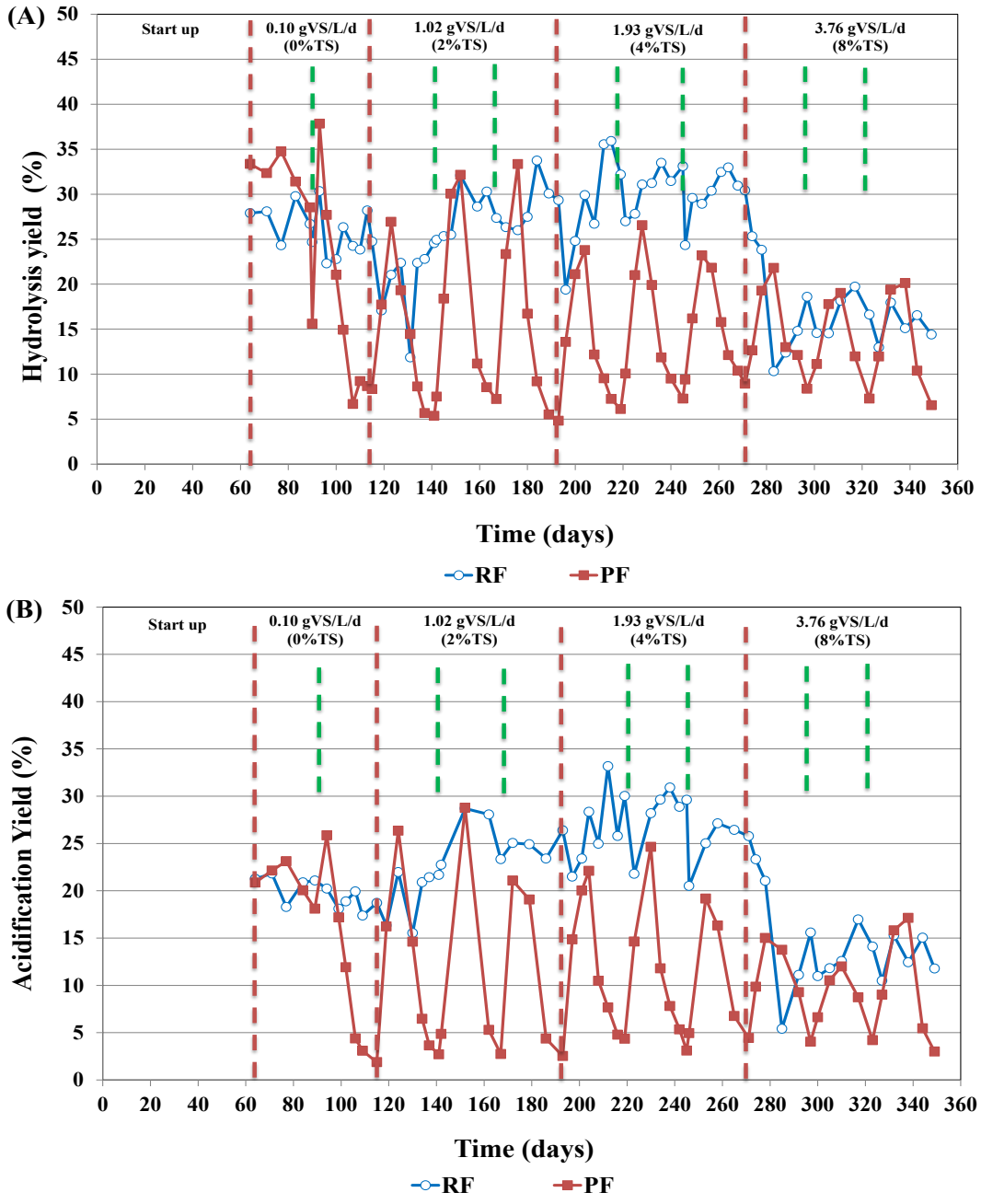

Figure 6.6 Hydrolysis yield (A) and acidification yield (B) at 0, 2, 4 and 8 %PG from regular feeding (RF) and periodic feeding (PF) reactors

6.3.5 Biogas production

Biogas production and methane content from RF and PF are shown in Figure 6.7. The experiment was run for 65 days before entering stable condition. Since PF reactor was fed for 11 days but biogas was recorded until day 26, the cyclic fluctuation in biogas production was clearly observed. After started adding the grass at 2 %P, biogas production slightly increased in the first day and went up and peaked around day 12, one day after stop feeding. After that, the biogas production started to drop until day 26. This cyclic character was repeated consistently. The biogas production obviously increased with the increasing PG and OLR. On average, biogas productions from RF reactor were 103, 1,004, 2,397 and 2,371 mL/d, while PF reactor could deliver at 102, 1,027, 1,985 and 3,153 mL/d on average at OLR 0.10, 1.01, 1.93 and 3.76 gVS/L/d, respectively. It showed that PF performance was comparable to RF's at low OLR, i.e. up to 1.01 gVS/L/d, and at higher OLR the stress built upon the microorganisms in the system had shown negative impact on the solid digestion.

Also, the methane content was fluctuated during the first 65 days of system start-up. Due to the active methanogenic inoculum, it took only 45 days until methane content in biogas went to a stable level between 40 and 50%. Methane was produced from the prepared pig farm wastewater only during this period. At 0 %PG, the methane content in the biogas from both reactors was similarly in a range of 43-53%. The highest methane content occurred at 2 %PG at 53.3±3.2% in RF and 52.0±4.8% in PF operation, although the two were not statistically different. Nevertheless, the swing in methane content was also observed as a result of the periodic feeding. A drip in methane content is more likely to correspond to the concentrated feeding in the first 11 days of each round.

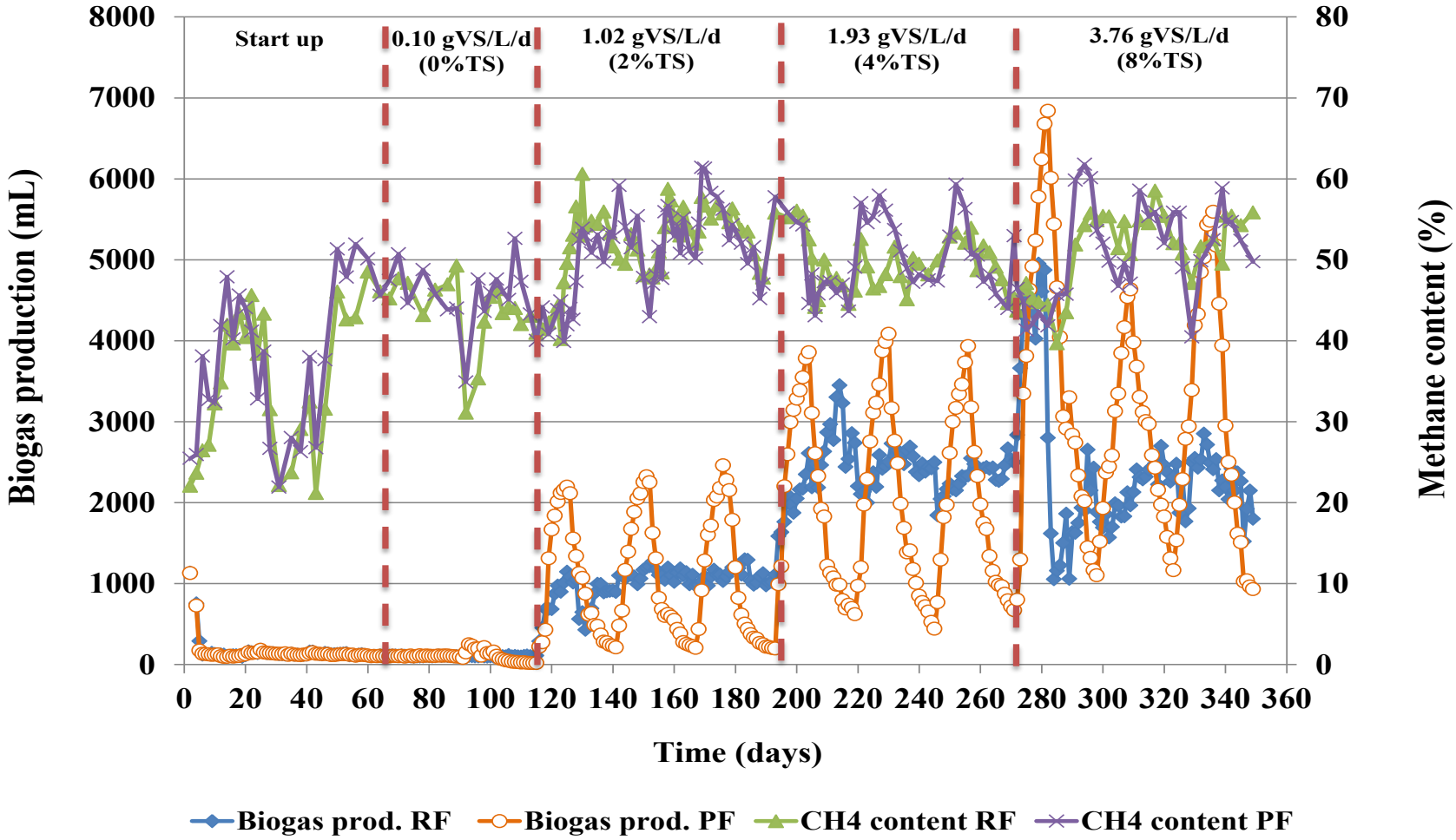

Figure 6.7 Biogas production and methane content from Meso-CF and Meso-PF reactors at difference PG mixed

6.3.6 Methane yield and biodegradability

Bushwell's formula was used for the theoretical methane yields calculation according to the Chapter V. This yield showed the potential of substrate that can be converted to methane gas based on the substrate's atomic composition. Noted that there are always non-digestible or hard to degrade portion in the substrate of which the chemical equation balance was not accounted. Meanwhile, the specific methane yield showed the potential from an experimental anaerobic digestion testing in a controlled environment, which gives a more realistic biogas value. Thus, the specific methane yield is always lower than the theoretical yield.

Table 6.2 Theoretical yield, specific methane yield and biodegradability at 0, 2, 4 and 8%PG from RF and PF reactor

OLR (gVS/L/d)	Theoretical methane yield (mL_{CH4}/gVS)	Specific methane yield (mL_{CH4}/gVS_{added})		Biodegradability (%)	
		RF	PF	RF	PF
0.101	525.0	119.7±3.3	131.2±14.2	22.8	25
1.016	541.0	138.2±23.8	169.8±6.0	25.6	31.4
1.931	541.8	158.3±5.8	162.4±4.0	29.2	30.0
3.761	542.39	83.3±4.7	146.1±14.3	15.4	26.9

The ratio of specific methane yield to theoretical methane yield is termed biodegradability. Higher biodegradability could represent how easy the substrate could be degraded or how efficient the system is capable of converting the substrate. The latter is of interest in our experiment. For RF reactor, the highest methane production per kg of VS was found at OLR 1.931 gVS/L/d (158.3±5.8

mL_{CH4}/gVS_{added}). The theoretical methane yield ($Y_{th,CH4}$) was 541.8 $mL_{CH4}/gVS_{added.}$ The highest methane yield from PF reactor was found at the same OLR with 162.4±4.0 $mL_{CH4}/gVS_{added.}$ The methane yields from both patterns were not statistically different (p<0.05) at 0 to 4 %PG (0.10-1.93 gVS/L/d. Biodegradability of RF and PF reactors reached the top level at 29.2% and 30.0%, respectively, at 4 %PG. The methane yield and biodegradability increased with increasing OLR up to 1.931 gVS/L/d. But at the OLR 3.761 gVS/L/d the specific methane yield and biodegradability declined to 83.31±4.7 mL_{CH4}/gVS_{added} and 15.4 % in RF, respectively, and to 146.1±14.3 mL_{CH4}/gVS_{added} and 27.0 % in PF mode, respectively.

6.3.7 Microbial communities

Bacterial dominance profile determined by PCR-DGGE of partial 16s rRNA genes fragments with and without solid feeding (0 and 4 %PG) in RF and PF is shown in Figure 6.8. The species of microbial community could affect the hydrolysis and acidification (Lin *et al.*, 2013). Dominant species found in RF and PF at 0 %PG were quite similar with most dominant species represented by *Clostridium* sp. that is common bacteria in anaerobic process (Rincon *et al*., 2006). They are acetate producing bacteria (acetogen) that can produce acetic acid, the essential VFA for producing methane. Other dominant species found were *Rhodobacteraceae* sp., *Acinetobacter* sp. *Geobacter* sp. and *Ruminococcaceae* sp. which could be found in the digestive system (Supaphol *et al*., 2011; Leven *et al*., 2007).

The dominant species found at 4 %PG in both reactors were; *Pseudomonas* sp. which is the hydrolytic and acidogenic bacterium (Salwan *et al*., 2010). Sulfate reducing bacteria (SRB) were also found due to the existent of sulfur in the substrate that sulfate is to be used as final electron acceptor. *Flavobacterium* sp. found in PF but not in RF. This species was found in the co-digestion of grass and pig manure (Dechrugsa, *et al*., 2013). While RF found the different species; *Lachnospiraceae bacterium* is a common rumen flora and especially the animal feed that component of legume. And *Roseburia* sp. is butyrate producing bacteria (Madigan *et al*., 1997).

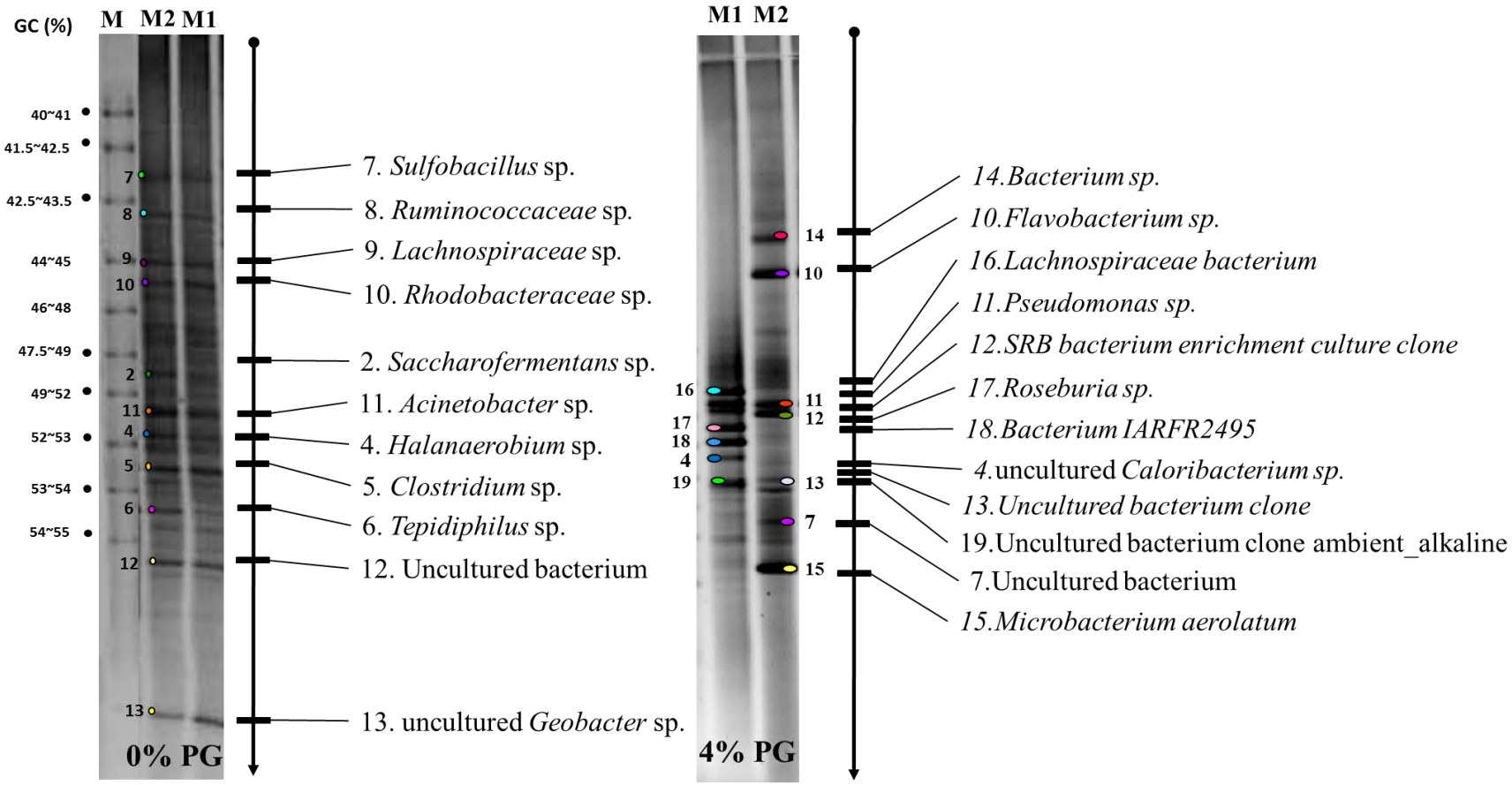

Figure 6.8 Bacterial community profile determined with PCR-DGGE of partial 16s rRNA genes fragments at different solid loading (0 and 4%TS of PG) from regular feeding (RF) and periodic feeding (PF) ASBRs

The archaeal communities found from two reactors at 0 %PG and 4 %PG were the same species. Table 6.3 shows the range of similarity at 99-100% which is considered high. It consisted of 4 species. *Methanobacterium formicicum* is hydrogenotrophic methanogen that produced methane from hydrogen. *Methanomethylovorans* sp., uncultured *Methanosaeta* sp. and uncultured *Methanolinea* sp. are acetoclastic methanogens. These species may provide synergistic reaction to work with other groups to produce more methane. The result indicated that feeding pattern and high percentage of grass did not affect to archaeal communities in the system as long as key operating condition of the system; pH, substrate type and etc., is maintained. This may be due to the substrate of methanogens was still the same VFA which could be produced in either case. Feed pattern did not affect the methanogenic community in the ASBR system.

Periodic feeding caused high organic loading in a short period that may cause the acid accumulation in a certain period. Microbial activities would be affected. Therefore, the regular feeding scheme gave a more even feed over time. The reactor with lower loading is deemed to protect microbes from exposing to high substrate concentration that causes osmotic pressure towards the substrate. If the substrate concentration is too high, it will inhibit to microorganism growth by osmotic pressure that cells will lose water to outside. But the result from this study revealed that both feeding patterns did not affect to the performance greatly because the consortium can still hydrolyze the substrate in the system but at a lesser efficiency. VFA produced was still continually be used by the archaea. At this VFA level, feedback inhibition, the inhibitory effect caused by the end production accumulation, did not occur in the systems.

Table 6.3 Archaeal community profile determined with PCR-DGGE of partial 16s rRNA genes fragments at different solid loading (0 and 4 %PG)

DNA band	Bacterial strains	Accession number	Similarity (%)
1	*Methanobacterium formicicum*	JX042445	99
2	uncultured *Methanosaeta* sp.	JX301662	100
3	*Methanomethylovorans* sp.	JN836398	99
4	uncultured *Methanolinea* sp.	JN394651	99

CHAPTER VII

CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

7.1.1 Conclusion of Part I

This experiment evaluated the influence of ISR, substrate mix ratio and inoculum source on methane production potential in anaerobic digestion. The study demonstrated higher ISR and para-grass mix ratio had positive effect on the methane yield in co-digestion of para-grass and pig manure. BMP assay of solid substrate should be carried out at ISR higher than 3 and 4 to gain consistent results. The inoculum from a digester treating a specific substrate may not be suitable for use in BMP assay of the mixture of solid substrates although it may have superior methanogenic activity. Dominant species of fermentative bacteria could be tested and be used as an indicator for the fitness of the inoculum for a batch type digestion test.

7.1.2 Conclusion of Part II

This experiment evaluated the performance of single stage mesophilic ASBR in comparison with TPAD system for co-digesting pig manure and para-grass at different solid loading of para-grass. The present study demonstrated the performance of co-digestion of pig manure and para-grass under different reactor systems and solid loadings. The methane yield was improved with the increased para-grass mixture in both systems until reaching the system OLR of 1.931 gVS/L/d (4 %PG). Overall, the TPAD system gave only slightly higher methane content (%) and yield (L/gVS_{added}) than the single stage mesophilic reactor in this study. Biogas yield from grass addition was highest at 66.3 m^3_{biogas}/ton_{fresh} at 4 %PG. Addition of para-grass to the systems had shifted the domination of bacterial species in the mesophilic reactors while achaea species were rather consistent in both systems. However, only hydrogenotropic

archaea was found dominant in the thermophilic reactor but played unimportant role in methane production. Higher diversity of microorganisms was found in TPAD system compared to the single stage mesophilic reactor.

7.1.3 Conclusion of Part III

This experiment evaluated the performance of single stage mesophilic ASBR at different feeding patterns between a typical regular feeding versus a periodic feeding. This study demonstrated the performance of ASBR in co-digestion of pig manure and para-grass with different feeding patterns and solid loadings under mesophilic reactor. The methane yield from continuous and periodic feeding pattern were improved with the increased para-grass mixture until reaching the system OLR of 1.931 gVS/L/d (4 %PG). The reactor that operated with the periodic feeding gave higher methane yield than regular ASBR feeding. Addition of para-grass to the systems had shifted the domination of bacterial species in the mesophilic reactors. Microbial communities from different feeding patterns were rather consistent in both reactors.

7.2 Recommendations

The following studies are recommended for further studies.

7.2.1 The inoculum from a digester treating a specific substrate may not be suitable for use in the biochemical methane potential (BMP) assay of the mixture of solid substrates although it may have superior methanogenic activity. Therefore, the BMP test should be conducted with the inoculum from source that treating a similar substrate for more accurate results.

7.2.2 Co-digestion with the solid substrate at high solid loading (>1.93 gVS/L/d) caused a scum floated to the surface. Microorganisms in the system cannot be in contact with the feedstock thoroughly, thus they cannot effectively degrade the grass. This problem from the experiment indicated that the role of mixing should be

considered for this level of solid substrate digestion to get more methane yield and prevent the scum formation.

7.2.3 Periodic feeding pattern gave the results close the regular feeding pattern in ASBR especially at the range of solid loading 0.10-1.93 gVS/L/d. But at the solid loading higher than 1.93 gVS/L/d, pH in the periodic feeding reactor had higher fluctuation that it may negatively affect the methanogenic activity. The advantages of this periodic feeding pattern include a longer of sludge age and solid substrate detention, saving of labor cost, energy and time in reactor operation. Nevertheless, more research on periodic feeding by fine-tuning the time period for feeding to enhance digestibility of various solid feedstock.

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APPENDICES

APPENDIX A

Experimental set-up

A.1 Feed stock and inoculun preparation

Figure A.1 Inoculum from concentrated rubber latex digester (RLD)

Figure A.2 Inoculum from pig farm digester (PFD)

(a)

(b)

(C)

Figure A.3 Pig manure (PM) preparation for an experiment (a) fattening pig farm, (b) fresh PM (c) dried PM and ground before used.

(a)

(b)

(c)

Figure A.4 Para-grass (PG) preparation for an experiment was harvested from pig farm (a) PG field, (b) chopped PG by cutting machine and (c) dried PG and ground before used.

A.2 Laboratory scale; SMA, BMP

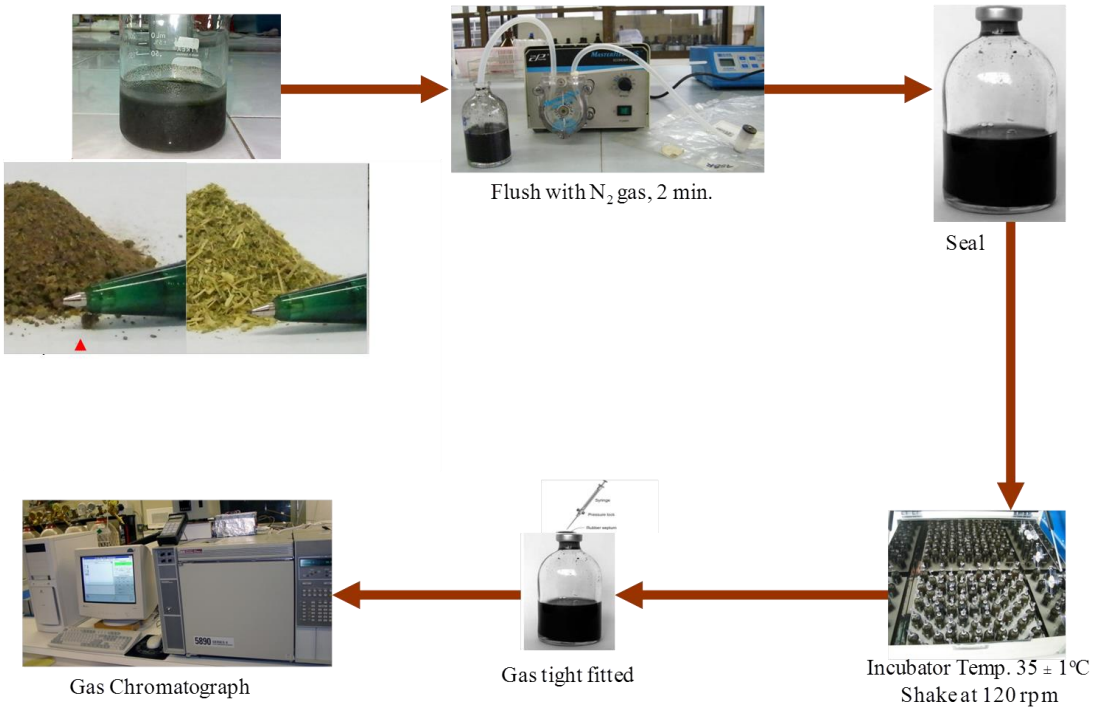


Figure A.5 Diagram of Specific methanogenic activities assay (SMA) and Biochemical methane potential assay (BMP assay)

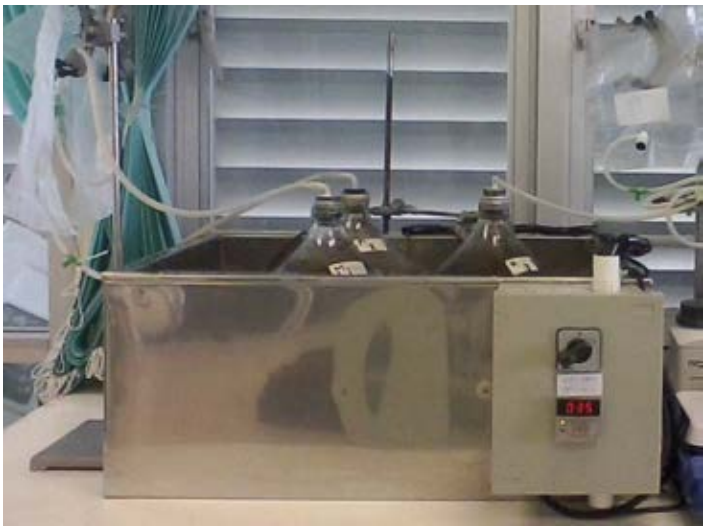
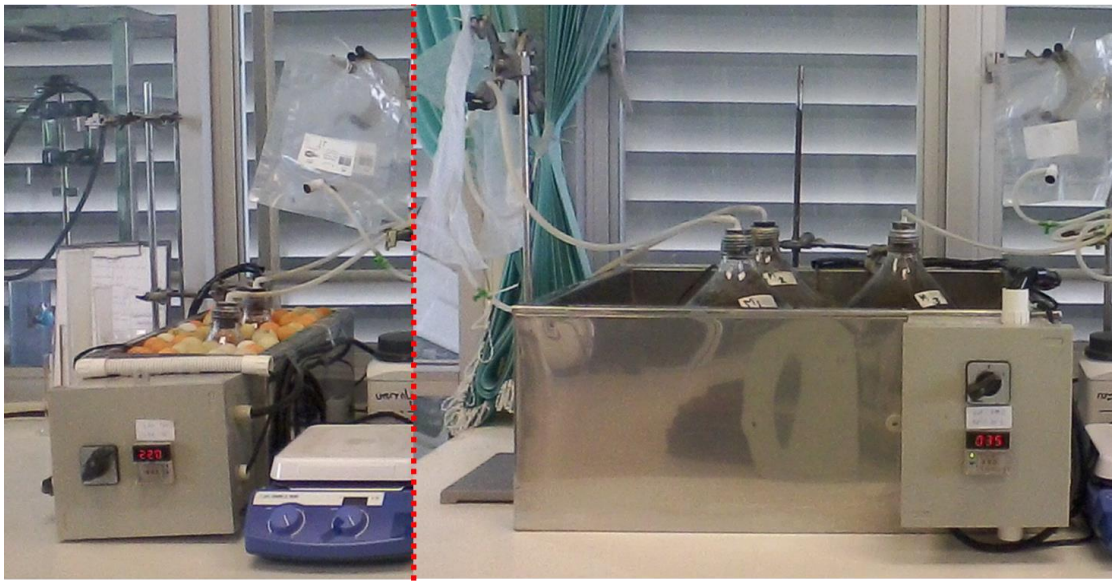


Figure A.6 Single stage of mesophilic reacters (35 ^{0}C)



(a) (b)

Figure A.7 Temperature-phase anaerobic digester (TPAD); (a) first stage of thermophilic (55 ^{0}C) and second stage of mesophilic reacter (35 ^{0}C)

APPENDIX B

SMA and BMP assay

B1. SMA assay

Table B.1 Cumulative biogas and methane production from pig farm digester (PFD) inoculum and concentrated rubber latex digester (RLD) inoculum.

Time (h)	Cumulative biogas production (mL)		Cumulative methane production (mL)			
	RLD inoc.	PFD inoc.	RLD inoc.		PFD inoc.	
			Exp. data	Predicted data	Exp. data	Predicted data
0	0.000	0.00	0.00	0.96	0.00	0.18
1	7.700	7.03	0.00	2.51	1.49	0.42
2	12.57	9.33	5.93	4.94	1.88	0.86
3	14.07	10.90	9.58	7.99	1.37	1.31
5	18.57	12.43	14.76	14.30	2.56	2.55
7	22.07	13.70	18.04	19.13	4.65	4.99
9	25.20	14.77	20.93	22.14	7.34	8.05
12	29.20	17.27	25.03	24.34	12.78	12.54
16	31.13	22.67	25.83	25.32	18.30	17.71
20	32.46	25.07	25.59	25.57	19.47	19.82
24	32.67	28.00	25.66	25.63	22.37	20.86

B2. BMP assay

Table B.2 Methane yield from difference inoculum source

ISR	Grass Ratio (%)	Methane Yield (mL_{CH4}/gTS_{added})	
		PFD inoculum	**RLD inoculum**
1	100	87.24	369.63
	75	301.57	313.20
	50	332.43	256.14
	25	286.49	233.56
	0	244.23	204.01
2	100	474.89	437.60
	75	437.24	385.90
	50	375.04	350.54
	25	309.66	254.12
	0	255.50	51.68
3	100	519.45	465.94
	75	453.55	299.28
	50	384.27	168.68
	25	320.60	101.04
	0	247.29	25.68
4	100	521.93	417.82
	75	452.50	442.59
	50	382.99	292.77
	25	314.05	65.93
	0	257.34	12.57

B3. Individual VFA

Table B.3 Changing of individual and TVFA concentration and methane generation versus digestion time from anaerobic co-digestion with difference mix ratio of para-grass and pig manure at ISR 4

Time (d)	HAc	HPr	i-HBu	HBu	i-HVa	HVa	TVFA	CH_4 generation (ml/L/day)
Ratio 0:100								
0	28.65	20.09	22.75	24.72	28.15	15.26	139.62	0.00
1	27.74	26.12	23.71	17.54	30.10	14.35	139.55	314.51
2	10.55	17.00					27.55	430.48
3	9.20	13.16					22.36	431.13
4	6.10	11.42					17.52	371.51
5	5.93	11.38					17.30	308.28
6	5.29						5.29	247.59
7	5.24						5.24	203.97
9	5.22						5.22	154.12
12	5.17						5.17	110.95
15							0.00	74.98
20							0.00	34.27
Ratio 25:75								
0	23.18	17.99	22.32	22.74	27.95	14.62	128.79	0.00
1	35.21	35.68	23.46	18.26	29.86	14.46	156.92	381.74
2	16.31	40.28	21.55		26.26		104.40	554.98
3	10.31	29.24					39.55	560.54
4	6.28	11.72					18.00	366.86
5	5.99	11.42					17.40	288.27
6	5.34						5.34	213.44
7	5.31						5.31	172.56
9	5.38						5.38	138.73
12	5.15						5.15	77.55
15	5.06						5.06	66.97
20							0.00	34.04
Ratio 50:50								
0	19.02	16.10		20.20	27.41	14.40	97.13	0.00
1	46.76	51.33	23.38	18.51	29.05	14.50	183.53	380.22
2	23.00	65.69	22.08		27.08		137.86	559.99
3	11.36	42.21					53.57	598.50
4	6.90	13.02					19.91	416.91
5	6.34	11.64					17.97	289.00
6	5.61	11.34					16.95	197.98
7	5.39	11.22					16.61	152.00
9	5.72	11.05					16.77	132.44
12	5.22						5.22	104.66
15	5.15						5.15	79.87
20							0.00	37.88

Table B.3 Changing of individual and TVFA concentration and methane generation versus digestion time from anaerobic co-digestion with difference mix ratio of para-grass and pig manure at ISR 4 (continued)

Time (d)	HAc	HPr	i-HBu	HBu	i-HVa	HVa	TVFA	CH_4 generation (ml/L/day)
Ratio 75:25								
0	13.29	12.83		19.92	26.11		72.14	0.00
1	57.22	63.37	23.41	18.62	28.82	14.50	205.94	390.65
2	35.80	97.99	22.65	17.94	28.05	14.04	216.47	571.18
3	11.87	70.72	21.90		26.68		131.17	608.42
4	8.64	35.14					43.78	439.35
5	7.49	15.43					22.93	329.08
6	5.93	11.61					17.55	205.08
7	5.39	11.63					17.02	147.29
9	5.70						5.70	139.26
12	5.31						5.31	110.49
15	5.18						5.18	92.48
20	5.10						5.10	43.75
Ratio 100:0								
0	8.21	11.26	22.06			14.09	55.61	0.00
1	51.70	62.30	23.06	18.68	27.81	14.33	197.88	416.71
2	36.15	87.52	22.25	17.97	27.30	13.96	205.15	529.40
3	14.62	84.66	22.20		26.98		148.46	612.27
4	8.82	49.53					58.35	413.31
5	9.20	52.31					61.51	359.11
6	6.56	21.88					28.44	246.57
7	5.57	25.62					31.20	157.37
9	5.69	11.69					17.37	152.53
12	5.58						5.58	124.02
15	5.55						5.55	93.72
20	5.41						5.41	51.40

APPENDIX C

Lab scale results

C1. ASBR performance

Table C.1 The average biogas production from single stage mesophilic and temperature-phased anaerobic digestion

OLR (gVS/L/d)	Biogas production (mL/d)			
	Meso-Single	**Thermo-1st**	**Meso-2nd**	**Overall TPAD**
0.101	104.00±1.41	116.96±3.92	19.12±0.71	97.85±3.21
1.016	1005.65±167.09	1024.05±164.57	29.79±1.70	994.26±166.27
1.931	2396.56±62.60	2324.86±59.86	93.72±1.13	2231.14±59.53
3.761	2371.14±319.22	2682.41±185.48	146.73±9.47	2535.68±184.02

Table C.2 Methane content from single stage mesophilic and temperature-phased anaerobic digestion

OLR (gVS/L/d)	Methane content (%)		
	Meso-Single	**Thermo-1st**	**Meso-2nd**
0.101	43.08	45.21	21.72
1.016	53.29	54.7	24.09
1.931	49.17	51.94	21.54
3.761	50.88	53.42	5.95

Table C.3 The pH of influent and effluent from single stage mesophilic and temperature-phased anaerobic digestion

OLR (gVS/L/d)		Influent	pH of effluent		
			Meso-Single	Thermo-1st	Meso-2nd
0.101	Max	7.15	7.07	7.08	7.04
	Min	5.83	6.37	5.88	6.28
	Mean	6.82	6.59	6.33	6.64
1.016	Max	6.81	7.17	6.13	7.19
	Min	5.81	6.32	5.60	6.45
	Mean	6.57	6.68	5.83	6.72
1.931	Max	6.11	6.95	6.10	6.89
	Min	5.46	6.64	5.23	6.60
	Mean	5.80	6.77	5.63	6.77
3.761	Max	5.60	6.94	5.57	6.93
	Min	5.35	6.72	5.11	6.70
	Mean	5.46	6.83	5.40	6.82

Table C.4 Total and soluble chemical oxygen demand (TCOD and SCOD), total solid (TS), volatile solid (VS) and removal efficiency at steady state of anaerobic treatment

OLR (gVS/L/d)	Reactor type	TCOD (g/L)	Removal (%)	SCOD (g/L)	Removal (%)	TS (g/L)	Removal (%)	VS (g/L)	Removal (%)
0.101	RF	0.42	89.97	0.23	66.14	0.89	58.90	0.45	73.22
	PF	0.51	87.82	0.21	68.16	0.90	76.30	0.44	85.26
	Thermo-1st	2.68	-	0.77	-	2.74	-	2.02	-
	Meso-2nd	0.37	85.97	0.24	68.98	0.84	61.06	0.38	68.40
1.016	RF	1.20	82.93	0.88	64.05	2.89	85.33	1.29	92.49
	PF	1.26	82.06	0.95	60.71	3.21	83.71	1.40	91.84
	Thermo-1st	7.74	-	2.96	-	12.94	-	11.38	-
	Meso-2nd	1.21	84.46	0.86	71.30	2.82	77.67	1.23	89.28

:NOTE: RF is Meso-single

Table C.4 Total and soluble chemical oxygen demand (TCOD and SCOD), total solid (TS), volatile solid (VS) and removal efficiency at steady state of anaerobic treatment (continued)

OLR (gVS/L/d)	Reactor type	TCOD (g/L)	Removal (%)	SCOD (g/L)	Removal (%)	TS (g/L)	Removal (%)	VS (g/L)	Removal (%)
1.931	RF	2.58	75.19	2.00	64.30	4.89	83.52	2.00	91.98
	PF	2.55	75.39	2.01	63.95	4.95	83.13	1.84	92.44
	Thermo-1st	12.53	-	6.46	-	28.67	-	24.30	-
	Meso-2nd	2.56	79.74	1.91	70.63	4.89	82.42	2.01	91.19
3.761	RF	5.40	82.49	3.30	66.52	9.67	68.34	5.77	76.65
	PF	7.80	75.41	4.81	51.34	13.48	71.59	7.79	78.99
	Thermo-1st	34.42	-	12.94	-	43.61	-	36.23	-
	Meso-2nd	4.63	86.16	3.15	75.15	12.62	61.36	7.67	64.83

NOTE: RF is Meso-single

Table C.5 Individual volatile fatty acid (VFAs) from regular feeding (RF) reactor and periodic feeding (PF) reactor at different solid loading

Day	Regular feeding (RF)					Periodic feeding (PF)				
	HAc	HPr	iHBu+ Hbu	iHVa+ Hva	TVFA	HAc	HPr	iHBu+ Hbu	iHVa+ Hva	TVFA
0	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-	-
21	-	-	-	-	-	-	-	-	-	-
28	-	-	-	-	-	-	-	-	-	-
35	-	-	-	-	-	-	-	-	-	-
42	-	-	-	-	-	-	-	-	-	-
49	-	-	-	-	-	-	-	-	-	-
56	-	-	-	-	-	-	-	-	-	-
63	-	-	-	-	-	-	-	-	-	-
70	-	-	-	-	-	-	-	-	-	-
77	-	-	-	-	-	-	-	-	-	-
84	-	-	-	-	-	-	-	-	-	-
89	-	-	-	-	-	-	-	-	-	-
94	-	-	-	-	-	-	-	-	-	-
99	-	-	-	-	-	-	-	-	-	-
102	-	-	-	-	-	-	-	-	-	-
106	-	-	-	-	-	-	-	-	-	-
109	-	-	-	-	-	-	-	-	-	-
115	-	-	-	-	-	-	-	-	-	-

Table C.5 Individual volatile fatty acid (VFAs) from regular feeding (RF) reactor and periodic feeding (PF) reactor at different solid loading (continued)

Day	Regular feeding (RF)					Periodic feeding (PF)				
	HAc	HPr	iHBu+ Hbu	iHVa+ Hva	TVFA	HAc	HPr	iHBu+ Hbu	iHVa+ Hva	TVFA
123	5.70	-	-	-	5.70	6.26	11.57	-	-	17.83
131	7.20	-	-	-	7.20	6.09	-	-	-	6.09
134	-	-	-	-	-	-	-	-	-	-
137	-	-	-	-	-	-	-	-	-	-
140	-	-	-	-	-	-	-	-	-	-
143	-	-	-	-	-	-	-	-	-	-
153	-	-	-	-	-	-	-	-	-	-
163	-	-	-	-	-	-	-	-	-	-
166	-	-	-	-	-	-	-	-	-	-
173	-	-	-	-	-	-	-	-	-	-
180	-	-	-	-	-	-	-	-	-	-
187	-	-	-	-	-	-	-	-	-	-
194	-	-	-	-	-	-	-	-	-	-
198	-	-	-	-	-	-	-	-	-	-
202	10.26	-	-	-	10.26	7.29	-	-	-	7.29
205	7.38	-	-	-	7.38	-	-	-	-	-
209	9.60	-	-	-	9.60	-	-	-	-	-
213	9.65	11.94	-	-	21.59	-	-	-	-	-
218	9.87	-	-	-	9.87	-	-	-	-	-
225	-	-	-	-	-	-	-	-	-	-

Table C.5 Individual volatile fatty acid (VFAs) from regular feeding (RF) reactor and periodic feeding (PF) reactor at different solid loading (continued)

Day	Continuous feeding (RF)					Periodic feeding (PF)				
	HAc	HPr	iHBu+ Hbu	iHVa+ Hva	TVFA	HAc	HPr	iHBu+ Hbu	iHVa+ Hva	TVFA
232	7.45	-	-	-	7.45	8.26	-	-	-	8.26
236	7.01	-	-	-	7.01	7.57	13.33	-	-	20.90
240	-	-	-	-	-	7.68	-	-	-	7.68
244	7.25	-	-	-	7.25	-	-	-	-	-
248	9.21	13.20	18.85	-	41.27	7.79	12.68	18.50	-	38.97
255	9.09	0.00	18.45	-	27.55	15.97	14.54	-	-	30.51
260	10.30	13.55	21.88	41.27	87.00	8.55	-	-	-	8.55
267	8.54	-	-	-	8.54	-	-	-	-	-
273	9.29	-	-	-	9.29	-	-	-	-	-
276	12.57	-	-	-	12.57	37.78	50.12	43.78	44.81	176.49
280	15.32	12.21	18.39	-	45.92	70.73	131.98	53.78	56.11	312.60
288	10.19	12.68	18.68	-	41.55	12.07	14.04	18.43	40.92	85.45
296	11.10	-	18.68	-	29.78	8.55	-	19.01	-	27.56
299	15.24	-	22.17	-	37.41	8.81	-	18.59	-	27.40
303	12.85	-	20.10	-	32.95	86.01	13.44	42.61	27.54	69.59
308	14.15	-	20.70	-	34.85	192.60	139.88	55.75	55.30	443.52
320	10.72	-	19.77	-	30.49	9.61	-	18.85	-	28.46
325	21.81	-	20.79	-	42.59	8.91	-	18.67	-	27.58
330	14.95	-	21.25	-	36.19	67.11	65.54	34.16	45.56	212.37
334	14.35	-	21.22	-	35.57	54.01	103.20	48.65	54.00	259.86
350	24.72	-	21.46	-	46.17	10.69	-	19.22	-	29.91

Table C.6 Hydrolysis yield at 0, 2, 4, and 8 %PG from regular feeding pattern and periodic feeding pattern

Day	regular feeding (RF)	periodic feeding (PF)
64	27.90	33.36
71	28.09	32.34
77	24.31	34.75
83	29.77	31.40
89	26.70	28.53
90	24.69	15.62
93	30.34	37.85
96	22.31	27.71
100	22.78	21.05
103	26.34	14.94
107	24.25	6.68
110	23.83	9.23
113	28.19	8.67
115	24.75	8.34
119	17.08	17.73
123	21.05	26.94
127	22.37	19.32
131	11.85	14.45
134	22.37	8.63
137	22.79	5.68
141	24.55	5.36
142	24.94	7.50
145	25.34	18.40
148	25.49	30.05
152	32.08	32.15
159	28.62	11.17
163	30.30	8.55
167	27.35	7.23
171	26.34	23.35
176	26.00	33.36
180	27.48	16.72
184	33.75	9.19
189	30.08	5.51
193	29.34	4.83
196	19.38	13.59
200	24.80	21.12
204	29.90	23.79
208	26.71	12.17

Table C.6 Hydrolysis yield at 0, 2, 4, and 8 %PG from regular feeding pattern and periodic feeding pattern (continued)

Day	regular feeding (RF)	periodic feeding (PF)
212	35.55	9.52
215	35.90	7.25
219	32.19	6.12
221	26.99	10.05
225	27.83	21.01
228	31.08	26.56
232	31.24	19.91
236	33.48	11.86
240	31.46	9.49
245	33.11	7.29
246	24.33	9.40
249	29.58	16.20
253	28.93	23.20
257	30.38	21.82
261	32.47	15.79
264	32.97	12.11
268	30.94	10.38
271	30.44	8.93
274	25.32	12.65
278	23.82	19.29
283	10.32	21.80
288	12.39	13.00
293	14.81	12.12
297	18.60	8.37
301	14.57	11.12
306	14.53	17.78
311	18.15	19.02
317	19.73	11.97
323	16.63	7.28
327	12.98	11.96
332	17.95	19.40
338	15.10	20.11
343	16.55	10.38
349	14.39	6.55

Table C.7 Acidification yield at 0, 2, 4, and 8 %PG from regular feeding pattern and periodic feeding pattern

Day	regular feeding (RF)	periodic feeding (PF)
64	21.27	20.86
71	21.82	22.14
77	18.29	23.13
84	20.88	20.04
89	21.08	18.11
94	20.23	25.86
99	18.10	17.18
102	18.87	11.91
106	19.94	4.39
109	17.38	3.09
115	18.72	1.88
119	16.34	16.22
124	21.98	26.35
130	15.53	14.62
134	20.90	6.46
137	21.42	3.64
141	21.67	2.71
142	22.75	4.87
152	28.70	28.77
162	28.07	5.29
167	23.35	2.75
172	25.06	21.09
179	24.92	19.07
186	23.41	4.36
193	26.39	2.53
197	21.49	14.86
201	23.41	20.02
204	28.35	22.10
208	24.97	10.48
212	33.17	7.67

Table C.7 Acidification yield at 0, 2, 4, and 8 %PG from regular feeding pattern and periodic feeding pattern (continued)

Day	regular feeding (RF)	periodic feeding (PF)
216	25.78	4.79
219	30.03	4.36
223	21.78	14.63
230	28.21	24.64
234	29.63	11.80
238	30.91	7.82
242	28.87	5.35
245	29.62	3.11
246	20.51	4.95
253	25.02	19.18
258	27.13	16.32
265	26.44	6.76
271	25.78	4.44
274	23.32	9.85
278	21.05	15.00
285	5.42	13.75
292	11.11	9.28
297	15.58	4.04
300	10.97	6.63
305	11.81	10.53
310	12.57	12.00
317	16.97	8.73
323	14.09	4.20
327	10.50	9.01
332	15.29	15.82
338	12.45	17.13
344	15.04	5.45
349	11.78	3.00

APPENDIX D

Microbial analysis

D1. Bacterial analysis

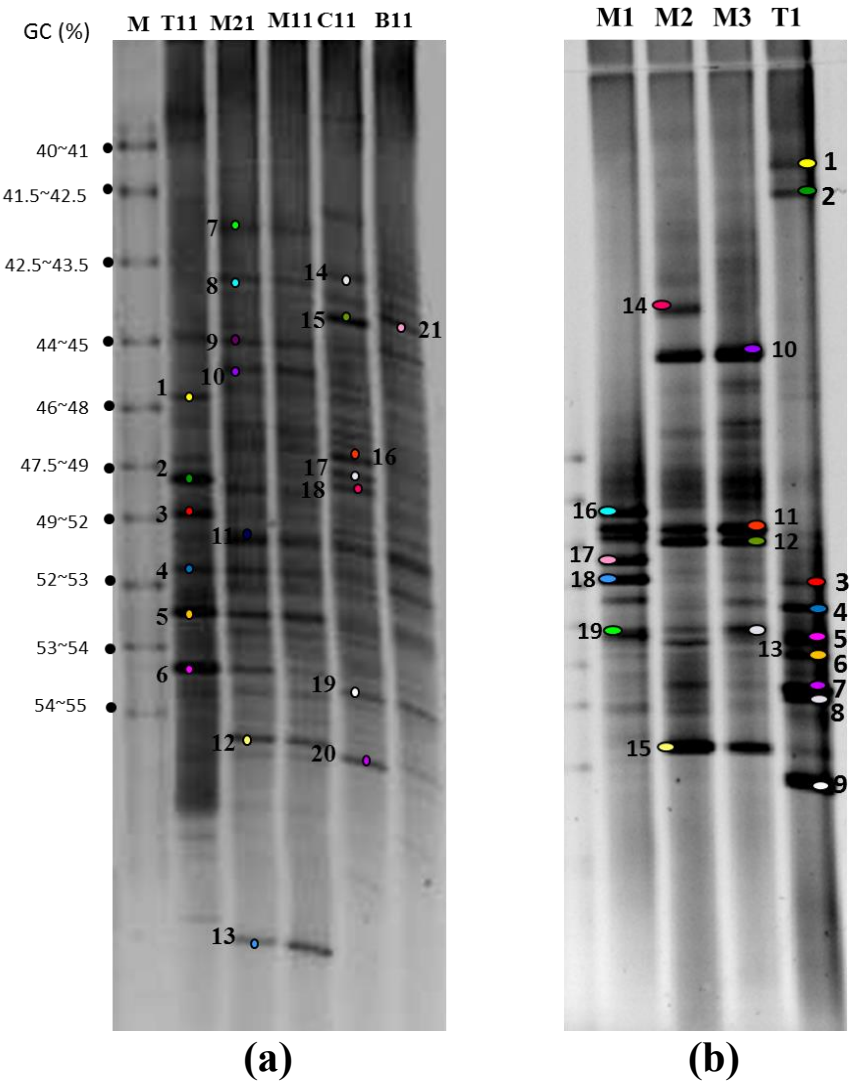


Figure D.1 Bacterial community profile determined with PCR-DGGE of partial 16s rRNA genes fragments; (a) 0.101 gVS/L/d (0%PG) and (b) 1.931 gVS/L/d (4%PG)

Table D.1 Sequencing of bacterial from reactor that feed with pig manure only (0%PG) or 0.101 gVS/L/d

Band	Size	Matching	Sequences
1	120	Uncultured *Deltaproteobacteria*	CCCTTCGGAGGGCGCAGTGGGGGATAATTGGCAAATGGGAAAACCCGAACCAGGCACCC CCCCGAAAAAAAAAAAGTCCTTGGAGTTAAAATCCGTTGGCAGGAGAAGGAAGTGACGG ACC
2	116	*Saccharofermentans* sp.	CCTACGGGAGGCAGCCGTGGGGAATATTGGGCAATGGGCGAAAGCCTGACCCAGCGACG CCGCGGAAGAAAAAAGATCCTCGGATTTAACTTCAGTGCAGGGCAAAAAGATGACGG
3	125	*Thermoanaerobacterium sp.*	CCTACGGGAGGCAGCAGTGGGGAATCTTGCGCAATGGGGGGAACCCTGACGCAGCGACG CCGCGTGGACGAAGAAGGCCTTCGGGTTGTAAAGTCCTGTAGATGGGGAAGAAGTAGAG ACGGACC
4	132	*Halanaerobium* sp.	CCTACGGGAGGCAGCAGTGGGGATCCTTCCACAATCCACCATTCGTTTCTTACCCAAACC AATCCCCAAACACCCCCACCCCGTAAAAGCCAAAACCCCTCAAAGTAATCTTTCATCTAA AAAAGTGCCTTC
5	123	*Clostridium* sp.	TCCTACGGGAGGCAGCAGTGGGGAATCTTGCGCAATGGAGAAACCCCTGACGCACCGAC GCCGCGTGAAGAAAGAAGGCCTTCGGGTTGTAAACTCCCTTGGCCAGCAGAGAAAAAGA CGGTC
6	162	*Tepidiphilus* sp.	TTCCTACGGGAGGCAGCAGTGGGGAATCTTGGACAATGGGGGCAAGCCTGATCCAGCAA TGCCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCATTCGGCGGGGAAGAAATCGG TCAGGCGAATCGTAGGGAGATGACGTACGGCAGAGAGACACCAC
7	140	*Sulfobacillus* sp.	TCCTACGGGAGGCAGCAGTGGGGAATTCTTCGCGAGGGCAGAAGGGGGGAACAGCAAC GCCGGGGGAAAGCGGAAGGCGTAGGGCCGAAAGCTGATACGCCTAGAAAACAAGAGGG TAAAAGGCCAAAAGGACGGGCCGC

Table D.1 Sequencing of bacterial from reactor that feed with pig manure only (0%PG) or 0.101 gVS/L/d (continued)

Band	Size	Matching	Sequences
8	134	*Ruminococcaceae* sp.	CCTACGCGAGGCAGCAGCAGGGAATCCTTACGAGGGCAATGGGGGGGAATAGCAA CAACGGGGGAAAGCAGGAAGAGTACCGCTGAAGAAGATACGCCTTGGAAAGCAAA AGTTATCACGCCGAAATGACGTAC
9	137	*Lachnospiraceae* sp.	CCTACGGGAGGCAGCAGCGGGGACTCCTACGGGGGGCCACAGGAGGGGATTTTGCA ACAGGGGGGAAAACCAGAAACAGACACCCCGCAGAAAAAAAAGCCTTCAGTATCC CCTGGGTATCATACCAACATGACGGT
10	141	*Rhodobacteraceae* sp.	CCTCCTGCGGGCGGCAGCAGGGGCTCCCCAGGAACAACAGCAACGGCATGAACCCA CAACGCGGAAAGCCAGAAAACAGTAGCAAGATAAAAAAAAAGACTCCTAATCTAA CCACTCCTCCAAAAACAGTTAATGACGTTC
11	145	*Acinetobacter* sp.	CCTACGGGAGGCAGCAGTGAGGAATCCTTACCAAAGGCGGCAGGGGGGAATATGC AACGACGGGAGAAAGCGGAAAGTATTAAGGCCGTAAAGGTGAAGACGGCCTTGGG TAGACAAGCTCTTCCTCACGACGATAAGACGTACT
12	145	Uncultured *Syntrophaceae bacterium*	TCCTACGGGAGGCAGCAGTGAGGAATCCTGCGCAATGGGGGAAACCCTGACGCAGC AACGCCGGGTGAGTGAGGAAGGTCTTCGGGTCGTAAAGCTCATCAGGTGGGAAGAA ATGCAAGAGGTGTACAGCCGCCATGAAGACGGA
13	145	*Geobacter* sp.	TCCTACGGGAGGCAGCAGTGGGGAATTTTGCGCAATGGGGGCAACCCTGACGCAGC AACGCCGCGTGAGTGACGACGCCCTTCGGGGTGTAAAGCTCTGTTGCCCGGGACGA AGCCTGGGAGGTAACAGCCTTCTAAATGACGGT
14	133	*Staphylothermus* sp.	CTACGGGAGGCGGCACAGGGGACTCCCTCCCCAGGCCCCCAGCCCCTATTTTGCCCC GCCCCAACCTTTACGCCCCCGCCCCCCGTAACCCCTTCCCCCTCCTACATCCTCCCCC ACGACCCCTTCAGGCCAG

Table D.1 Sequencing of bacterial from reactor that feed with pig manure only (0%PG) or 0.101 gVS/L/d (continued)

Band	Size	Matching	Sequences
15	120	*Clostridium* sp.	GGACTCCTACGGGAGGCCGCAGTGGGGAATATTGCGCAATCACGAAAGCTT ACGCCCAACGCCGCGTGCGACGAAGCTCTTCGATCGTAACCCTCTCTAAGAA AGGAAGGACATCCGCTG
16	136	Uncultured *Clostridium* sp.	TCCTACGGGAGGCAGCAGTGGGGAACCTTGCCCAATGCCACTAACCCTTACG CACAACGCCGCAAGATGATACACCCTTCCCGTGTGATCCTTTCTTTTAGGGG CTTCTAGTGCTATACCCTCTTGCTGGACGTTC
17	135	*Gemmatimonas* sp.	CCTACGGGAGGCAGCAGTGGGGACTCCTGGGCGGGGCCAGAACCGATATGC AGCAACGCGGCAAACCGGGGACAGCGTTGCGGTGTAAAGATGAGGACGTTG GTATAGCCTATGGCTATTCCCGAACATGACGGT
18	161	*Pelotomaculum* sp.	CTTTCCTGGGGGGGGGCGGCGGGGGTCCTCCGGGGGGCGGCAGCGGGGAAT ATTGACAAAGGCGAAAGCCGCGCCGCCACCCCGCGTGAAGAAAAAAGGCCT TCGGAAATAAAACCTGTCCCAGGGCAGGGATGACACGTACTGAAGAGTTTT GGTTTACC
19	135	*Geobacter* sp.	CCCCTACGGGGGGCCACAGGGGGGTATTTTGCACAAGGGGAAAACGTGTGA CACCGCCACGCCGCGAGGGAAAGAACGCCTTGGGGATAAATCTCTGTCGAG AGGGCGAGAAAGACGTACTCAGAGAGCTATCCC
20	120	*Desulfotomaculum* sp.	CTCCTACGGGAGGCAGCAGGGGGGGATTTTGGGCAATGGGCGAAAGCCTGA CCGAGCGACGCCGCGTGAGCGAAGAAGGCCTTGGGGTGTAAAGCTCTGTTA GGGGACGAGCAATGACGT
21	131	*Flavobacterium* sp.	CCTATCGTACGGGAGGCAGCGGTGTCGTATATTGGGCACGGGTGGTATGTGC ACCAGCCATACACCCTACACGACGACAACCCTATTATATGGATTCCTTAGTT AGGCTTGGTTCTTCAAACACGGGTGCA

Table D.2 Sequencing of bacterial from reactor that feed with pig manure and 4% of PG as co-substrate (1.931 gVS/L/d)

Band	Size	Matching	Sequences
1	149	*Clostridium stercorarium* subsp. stercorarium	GGGGACTGGACGTCTCATGGGTACTGTCATCTTCTTCTCCCTATCAAAGAGACTTTAAACCCA AAGGCCTTCTTCCTCCACACGGCGTCGCTGCTTCAGG
2	150	Uncultured bacterium	GGGAATGAGCTTCTCATGCGATACCGTCTTCCTTCTTCCCTGTCAAAGGAGTTTCAAACCCAA AGCATTCTTCCTCCACGCGGCGTCGCTGCGTCTCAGGATTCCCCCATTGCGCAAAATTCCCCA CTGCTGCCTCCCGTAGGGGACCCA
3	152	*Clostridium* sp. MF18_Ns	GGGGACTGGACTTCTGATGCGGTACCGTCATCTTTCTTCCCCGCAACATAAAGGATTATATA CCGCAATAGATCTTCTCTCCCACGGCGTCGCTGGATCTCGGGTTCCCCCCTTGTGCAAAATAC CCCCCTGCTGCCTCCCGCAGGAGACCC
4	171	Uncultured *Caloribacterium* sp.	GGGACTGGGCTTCTGATAGGTACCGTCATCGTCTTCTCCCCCAACTACTGGACTTTACCACTC CCAAAGACTTCTTCTTCACCATGCGAACACCTTCTTCGGTTTCCCCCATTGCTCCATATTCCCT TCTGCTGCTGCCCAAAAGTCCCTACTGCTGCCTCCCGTAGGAA
5	147	*Thermophilic anaerobic bacterium* K1L1	GGGACTGGGGCTCTCATGGGTACGTCATCTACTTCTTCCCATCAAAGAGACTTTAAACCCGA AGGCCTTCTTCCTCCACGCGGCGTCGCTGCGTCAGGGTTCCCCCCATTGCGCAAAATTCCCCA CTGCTGCCTCCCGTAGGAAA
6	173	*Thermoanaerobacterium* sp.	GGGAACTGGGGCTTCTGATGTGGTACCGTCTCTTCTTCTTCCCCATCTAACGGACTTTGAACT CCAAGGACTTTTTCCTCCACGAGGCGACGATGTTTCAGGGTTCCCCCCCATTGCGTAACGATTC CCCTTTGGTGCCTCCCGGAGGATTCCCTACTGCTGCCTCCCGTAGGA
7	153	Uncultured bacterium partial 16S rRNA gene, clone	GAGGGACTGCGCTCTCTTGCGGTACCGTCACTTCCTTCGTCCCGACTGACAGAGGTTTACAAT CCAAAGACCTTCTTCCCTCACGCGGCGTCGCTGCATCAGGAGTTTCCTCCATTGTGCAATATC CCCCACTGCTGCCTCCCGTAGGTAAGA
8	148	*Thermoanaerobacterium sp. enrichment culture clone* D5	GGGGATTGGGGCTGTCGATGGGTACGTCATCTACTTCTTCCCCATCTACAGGACTTTACAACC CGAAGGCCTTCTTCGTCCACGCGGCGTCGCTGCGTCAGGGTTCCCCCCATTGCGCAAGATTC CCCACTGCTGCCTCCCGTAGGAA
9	148	Uncultured bacterium	GGACCGTTGCCTTGTTTTCAGGGTACCGTCCTTCCTTCGTCCCCTGCCAAGGAAGTTTAAACC CGAAGGCCTTCTTCCTCACGCGGCGTCGCTGCGTCAGGGTTTCCCCCATTGCGCAAGATTCCC CACAGCTGCCTCCCGTAGAGAA
10	168	*Flavobacterium* sp. 01WB03.1-18	GGACCGGGCCTTTCTGTCGGTACGTCATACACTCACGTATTAGGTAAATGCCCTTCCTCCCAA CTTAAAGTGCTTTACAATCCGAAGACCTTCTTCACACACGCGGCATGGCTGGATCAGGCTTT CGCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGA

Table D.2 Sequencing of bacterial from reactor that feed with pig manure and 4% of PG as co-substrate (1.931 gVS/L/d) (continued)

Band	Size	Matching	Sequences
11	167	*Pseudomonas* sp. R-45822	GGGACTGGGCGTTTCTGTCGGTACGTCAGACACTAACGTATTAGGTTAATGCCCTTC CTCCCAACTTAAAGTGCTTTACAATCCGAAGACCTTCTTCACACACGCGGCATGGCT GGATCAGGCTTTCGCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGA
12	171	SRB bacterium enrichment culture clone SRtB-otu1-52	GGGAACTGGCGCTTCTCTGTAGGTACTGTCATTATCTTCCCTGATCAAGAGCTTTACT ACCCGAAAGACTTCATCACTCACCCGGCGTAGATGCATCATCGCTTTCGCCCATTGT GCAATATTCCCCACTGCTGCCTCCCGTAGGATCCCCACTGCTGCCTCCCGTAGGAG
13	168	Uncultured bacterium clone KFeR2-13	GGGGACTGGGCTGCTCTTAGGTACTGTCATTATCATCCCTGATCAAGAGCTTTACGA CCCGAAGGCCTTCATCACTCACGCGGCGTTGCTGCATCAGGCTTTCGCCCATTGTGCA ATATTCCCCACTGCTGCCTCCCGTAGGATTCGTAGCTGCTGCCTCCCGTAGGA
14	164	Bacterium C16-Siri110	GGGGGCTGGGGCTTCTTCTTCAGGTACGTCATTCTCTCTCTCTTACTTGACAGCTGAA GTACGAAACCAGAAAGGAATGTCTCCCCTCTCGCAGGCTGTTGTGGCTGGATCTCGC TTCTGCTGAATAATACTCACCCGCGACGGCTAGGATACGGTAGGAGTGC
15	99	*Microbacterium aerolatum* strain KUDC1073	GGCCGCTTCCGCAGGTACCGTCACTCTCGCTTCCTTCCCTGCTAAAGAGGTTTCAACC CGAAGGCCGTCATCCCTCACGCGGCGTTGCTGCATCAGGCTT
16	167	Lachnospiraceae bacterium BSY4	GGGGGACAGGCTTCTTGTCGGTACGTCATTTCCTTCTCCGTTGAGACAGGTTACTTCC CCAAAGACTACTTCGTTCACCCTTAAAGGATGCTTCTGACTCACGCCCATTGTGCAAT ATTCCCCTCTGCTGCCTCGCGCTAGGATCCCCACTGCTGCCTCCCGTAGGA
17	156	*Roseburia* sp. 499	GGGGACCCTGTCTTCTTTGTCGGTACTGTCATTTTCTTCACTGCTGATAGAGCTTTACT TCCCGAAAGACATCTTCGCTCCCCTTAAAAGGCTGCATCACGCTTTCGCCCATTGTGC AATATTCCCCACTGCTGCCTCCCGTAGGATCCCCACTGC
18	165	Bacterium IARFR2495	GGGGACCGGGGCTCTTGTCGGTACGTCATTATCTTCCTGTTGAGAAACGTTTCTTCCT CAAAGACTTCATGCTTCCACTGCGAAGATGCTTCACACTTTCGCCCATTGTGTGATAT TACGCTCTGCTGCCTTGCGCATGTTCCCCACTGCTGCCTCCCGTAGGAA
19	205	Uncultured bacterium clone ambient_alkaline-53	GGGACCTGGGCCTGTCCTTCGGTACTGTCATTATCATCCCTGATCCAGAGCTTTACGA CCCGAAAGCCTTCATCACTCACCCTTTGTAGCTGCATCATGCTTTCGCCCATTGTGCA ATATTCCACACTGCTGCCTCGCGTAAGATTCTTTACTGCTGCCTCCCGTAGGAGAGTT TGTCTTTTCCTTCCTAAGGAAAACTCTGACT

D2. Archaeal analysis

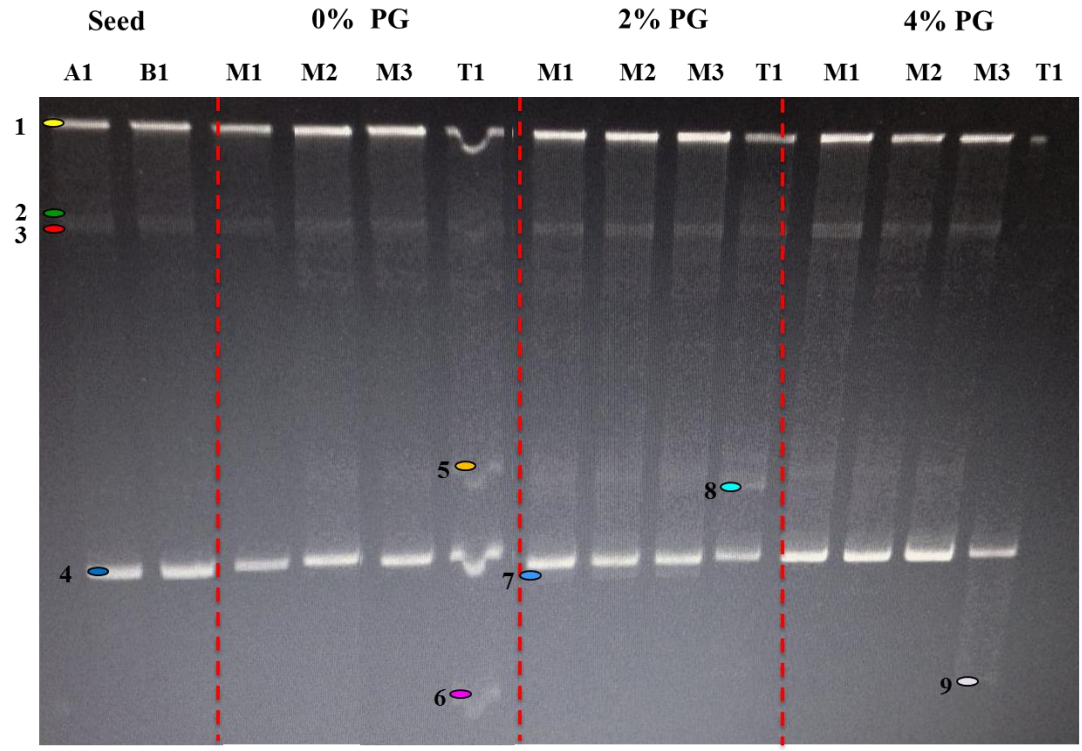


Figure D.2 Archaeal community profile determined with PCR-DGGE of partial 16s rRNA genes fragments

Table D.3 Archaeal strains of rubber latex concentrated digester (RLD) inoculum and pig farm digester (PFD) inoculum

Band	Bacterial strains	Accession number	% similarity	Sequences
1	*Methanobacterium formicicum*	JX042445	99	ATTTTACCGCGGCGGCTGGCACCGGTCTTGCCCAGCTCTTATTCCAA AAGCTTTTTACACTTAAGAAAAGCCACCCCGTTAAGAGTGGCACTT GGGTTTCCCCCGTCGCACTTTCGTGCATTGCGGAGGTTTCGCGCCTG CTGCACCCCGTAGGG
2	Uncultured *Methanosaeta* sp.	JX301662	100	ACCGCGGCGGCTGGCACCGGTCTTGCCCGGCCCTTGCTATGCAATG CTTTTTAGGCATCACGACAGCCAGATTTGTAACCTGGCACTCGAGG TCCCCTTATCGCCGTTGCCGGCATTGTAAAGTTTTCGCGCCTGCTGC ACCCCGTAGGG
3	*Methanomethylovorans* sp.	JN836398	99	TTTACCGCGGCGGCTGGCACCGGTCTTGCCCGGCCCTTGCTAACAC ATGTGATTTAGACATATGGACAGCCAACATAGGATGCTGGCACTCG GTGTCCCCTTATCGCGGTTCCCCGCATTGTAAAGTTTTCGCGCCTGG TGCACCCCGTAGGGA
4	Uncultured *Methanolinea* sp.	JN394651	99	CCTACGGGGTGCAGCAGGCGCGAAAACTTTACAATGCGAGAAATC GTGATAAGGGAACCCCGAGTGCCCGTAAATTCGGGCTGTCCGCCAG CATAAATAACTGGTGAAGAAAGGGCCGGGCAAGACCGGTGCCAGC CGCCGCGGTAAA

Table D.4 Archaeal strains from the reactor that feed with 0% of PG (0.101 gVS/L/d)

Band	Bacterial strains	Accession number	% similarity	Sequences
1	*Methanobacterium formicicum*	JX042445	99	ATTTTACCGCGGCGGCTGGCACCGGTCTTGCCCAGCTCTTATTCCAAAAGCT TTTTACACTTAAGAAAAGCCACCCCGTTAAGAGTGGCACTTGGGTTTCCCCC GTCGCACTTTCGTGCATTGCGGAGGTTTCGCGCCTGCTGCACCCCGTAGGG
2	Uncultured *Methanosaeta* sp.	JX301662	100	ACCGCGGCGGCTGGCACCGGTCTTGCCCGGCCCTTGCTATGCAATGCTTTTT AGGCATCACGACAGCCAGATTTGTAACCTGGCACTCGAGGTCCCCTTATCGC CGTTGCCGGCATTGTAAAGTTTTCGCGCCTGCTGCACCCCGTAGGG
3	*Methanomethylovorans* sp.	JN836398	99	TTTACCGCGGCGGCTGGCACCGGTCTTGCCCGGCCCTTGCTAACACATGTGA TTTAGACATATGGACAGCCAACATAGGATGCTGGCACTCGGTGTCCCCTTAT CGCGGTTCCCCGCATTGTAAAGTTTTCGCGCCTGGTGCACCCCGTAGGGA
4	Uncultured *Methanolinea* sp.	JN394651	99	CCTACGGGGTGCAGCAGGCGCGAAAACTTTACAATGCGAGAAATCGTGATA AGGGAACCCCGAGTGCCCGTAAATTCGGGCTGTCCGCCAGCATAAATAACT GGTGAAGAAAGGGCCGGGCAAGACCGGTGCCAGCCGCCGCGGTAAA
5	*Methanocella conradii*	JN048683	99	CCCTACGGGGTGCACCAGGCGCGAAAACTCTACAATGCAGGCAATCTGCGA TAGGGGGACATCGAGTGGCATCTTCTTAAGGTGCCTGTCCAACCGTCTAAAA AACGGTTGTTAGCAAGGGCCGGGTAAGACCGGTGCCAGC
6	Uncultured *Methanosarcinales archaeon*	KF186097	99	ACCGCGGCGGCTGGCACCGGTCTTGCCCGGCCCTTGCTATGCAATGCTTTTT AGGCATCACGACAGCCAGATTTGTAACCTGGCACTCGAGGTCCCCTTATCGC CGTTGCCGGCATTGTAAAGTTTTCGCGCCTGGTGCACCCCGTAGGGAAT

1, 4, 5, 6 : found in Thermophilic reactor

Table D.5 Archaeal strains from the reactor that feed with 2% of PG (1.016 gVS/L/d)

Band	Bacterial strains	Accession number	% similarity	Sequences
1	*Methanobacterium formicicum*	JX042445	99	ATTTTACCGCGGCGGCTGGCACCGGTCTTGCCCAGCTCTTATTCCAAAA GCTTTTTACACTTAAGAAAAGCCACCCCGTTAAGAGTGGCACTTGGGTT TCCCCCGTCGCACTTTCGTGCATTGCGGAGGTTTCGCGCCTGCTGCACCC CGTAGGG
2	Uncultured *Methanosaeta* sp.	JX301662	100	ACCGCGGCGGCTGGCACCGGTCTTGCCCGGCCCTTGCTATGCAATGCTT TTTAGGCATCACGACAGCCAGATTTGTAACCTGGCACTCGAGGTCCCCT TATCGCCGTTGCCGGCATTGTAAAGTTTTCGCGCCTGCTGCACCCCGTA GGG
3	*Methanomethylovorans* sp.	JN836398	99	TTTACCGCGGCGGCTGGCACCGGTCTTGCCCGGCCCTTGCTAACACATG TGATTTAGACATATGGACAGCCAACATAGGATGCTGGCACTCGGTGTCC CCTTATCGCGGTTCCCCGCATTGTAAAGTTTTCGCGCCTGGTGCACCCCG TAGGGA
4	Uncultured *Methanolinea* sp.	JN394651	99	CCTACGGGGTGCAGCAGGCGCGAAAACTTTACAATGCGAGAAATCGTG ATAAGGGAACCCCGAGTGCCCGTAAATTCGGGCTGTCCGCCAGCATAA ATAACTGGTGAAGAAAGGGCCGGGCAAGACCGGTGCCAGCCGCCGCGG TAAA
7	Uncultured *Methanobacterium* sp.	EU812212	99	CCTACGGGGTGCACCAGGCGCGAAACCTCCGCAATGCACGAAAGTGCG ACGGGGGAAACCCAAGTGCCACTCTTAACGGGGTGGCTTTTCTTAAGTG TAAAAAGCTTTTGGAATAGGAGCTGGGCAAGACCGGTGCCAGCCGCC
8	*Methanobacterium palustre*	NR_041713	98	TCCCTACGGGGTGCACCAGGCGCGAAACCTCCGCAATGCACGAAAGTG CGACGGGGGAAACCCAAGTGCCACTCTTAACGGGGTGGCTTTTCTTAAG TGTAAAAGGCTTTTGGAATAAGAGCTGGGCAAGACCGGTGCCAGCCGC CGCGGTAA

1, 4, 8: found in Thermophilic reactor

Table D.6 Archaeal strains from the reactor that feed with 4% of PG (1.931 gVS/L/d)

Band	Bacterial strains	Accession number	% similarity	Sequences
1	*Methanobacterium formicicum*	JX042445	99	ATTTTACCGCGGCGGCTGGCACCGGTCTTGCCCAGCTCTTATTCCAAAAGCTTT TTACACTTAAGAAAAGCCACCCCGTTAAGAGTGGCACTTGGGTTTCCCCCGTC GCACTTTCGTGCATTGCGGAGGTTTCGCGCCTGCTGCACCCCGTAGGG
2	Uncultured *Methanosaeta* sp.	JX301662	100	ACCGCGGCGGCTGGCACCGGTCTTGCCCGGCCCTTGCTATGCAATGCTTTTTAG GCATCACGACAGCCAGATTTGTAACCTGGCACTCGAGGTCCCCTTATCGCCGTT GCCGGCATTGTAAAGTTTTCGCGCCTGCTGCACCCCGTAGGG
3	*Methanomethylovorans* sp.	JN836398	99	TTTACCGCGGCGGCTGGCACCGGTCTTGCCCGGCCCTTGCTAACACATGTGATT TAGACATATGGACAGCCAACATAGGATGCTGGCACTCGGTGTCCCCTTATCGC GGTTCCCCGCATTGTAAAGTTTTCGCGCCTGGTGCACCCCGTAGGGA
4	Uncultured *Methanolinea* sp.	JN394651	99	CCTACGGGGTGCAGCAGGCGCGAAAACTTTACAATGCGAGAAATCGTGATAA GGGAACCCCGAGTGCCCGTAAATTCGGGCTGTCCGCCAGCATAAATAACTGGT GAAGAAAGGGCCGGGCAAGACCGGTGCCAGCCGCCGCGGTAAA
9	Uncultured *Methanobacterium* sp.	HQ231791	98	TTACCGCGGCTGCTGGCACCGGTCTTGCCCAGCCCTTATTCCAAAAGCTTTTTA CACTTAAGAAAAGCCACCCCGTTAAGAGTGGCACTTGGGTTTCCCCCGTCGCA CTTTCGTGCATTGCGGAGGTTTCGCGCCTGGTGCACCCCGTAGGG

1, 4, 9: found in Thermophilic reactor

BIOGRAPHY

Mr. Sumeth Dechrugsa was born on July 05, 1974 in Nakorn Si Thammarat, Thailand. I graduated for the bachelor degree of Science (Animal Science) from Kasetsart University; I graduated for the master degree of Economics from Sukhothai Thammatirat Open University. Then I pursued the Ph. D study in International Postgraduate Program in Environmental Management, Graduate School, Chulalongkorn University, Bangkok, Thailand.

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