THE STUDY FOR INCREASING OF METHANE CONTENT
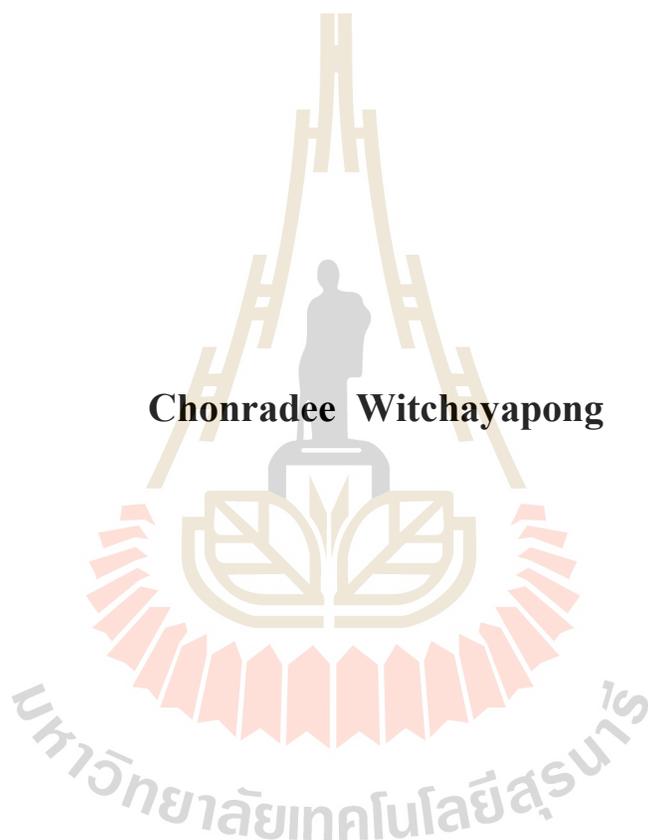
IN BIOGAS PRODUCED FROM CASSAVA PULP

Chonradee Witchayapong

A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Master of Science in Biotechnology

Suranaree University of Technology

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การศึกษาเพื่อเพิ่มปริมาณสัดส่วนมีเทนในไบโอแก๊สที่ผลิตจาก
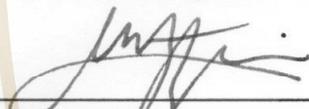# กากมันสำปะหลัง

นางสาวชนรดี วิชญพงศ์

THE STUDY FOR INCREASING OF METHANE CONTENT IN

BIOGAS PRODUCED FROM CASSAVA PULP

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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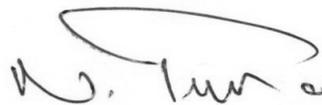(Assoc. Prof. Dr. Apichat Boontawan)

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   กากมันสำปะหลังเป็นของเสียจำนวนมากที่พบในอุตสาหกรรมแป้งในประเทศไทย ซึ่งสามารถ
นำมาเป็นเชื้อเพลิงในการผลิตก๊าซชีวภาพได้ อย่างไรก็ตามกากมันสำปะหลังนั้น มีไนโตรเจนเป็น
องค์ประกอบค่อนข้างน้อย ซึ่งเป็นข้อจำกัดในการย่อยสลายของจุลินทรีย์ นำไปสู่อัตราการย่อยสลาย
ที่ต่ำลงรวมถึงการผลิตก๊าซชีวภาพ และมีเทนอีกด้วยด้วย งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาอัตรา
การผสมของกากมันสำปะหลังกับน้ำเสียจากกระบวนการกลั่นเอทานอลที่ 0:1, 1: 0.5, 1:1, 0.5:1
และ 0:1 ถังหมักไร้อากาศแบบกะถูกแสดงในสภาวะอุณหภูมิเมโซฟีลิกที่ 35 ± 1 องศาเซลเซียส ผล
การทดสอบบ่งชี้ว่า ผลผลิตของก๊าซชีวภาพและมีเทนจากการย่อยแบบร่วม มีค่าสูงกว่าการย่อยแบบ
เดี่ยว ยิ่งไปกว่านั้นยังพบว่า อัตราส่วนที่เหมาะสมของกากมันสำปะหลังกับน้ำเสียจากกระบวนการ
กลั่น เอทานอลคือ 1:1 ที่อัตราส่วนดังกล่าวมีค่าผลผลิตสะสมของก๊าซชีวภาพและมีเทนเท่ากับ
918.73   มิลลิลิตรต่อกรัมของแข็งระเหยง่าย และ 685.10 มิลลิลิตรต่อกรัมของแข็งระเหยง่าย
ตามลำดับ จากอัตราส่วนที่เหมาะสมดังกล่าวสามารถเพิ่มผลผลิตสะสมของก๊าซชีวภาพ 146.54% ใน
กาก มันสำปะหลัง และ 100.64% ในน้ำเสียจากกระบวนการกลั่นเอทานอลเช่นเดียวกับผลการผลิต
สะสมของมีเทนที่เพิ่มขึ้น 222.19% ในกากมันสำปะหลัง และ 105.70%ในน้ำเสียจากกระบวนการกลั่น
เอทานอลตามลำดับ
   นอกจากนี้การศึกษาพบว่า กลุ่มประชากรของแบคทีเรีย และอาร์เคียในระบบการผลิตก๊าซ
ชีวภาพที่เกิดจากส่วนผสมของกากมันสำปะหลัง และน้ำเสียจากกระบวนการกลั่นเอทานอล เมื่อ
วิเคราะห์ด้วย Denaturing Gradient Gel Electrophoresis (DGGE) พบว่า ประชากรเด่นในกลุ่ม
แบคทีเรียคือ *Bacteroidetes, Firmicutes* และ *Chloroflexi* ประชากรเด่นในกลุ่มอาร์เคียที่สร้างก๊าซมีเทน
(Methanogen) คือ*Methanosarcina (Methanosarcina barkeri)* ซึ่งจัดอยู่ในสกุล *Methanosarcinaceae*
ส่วนประชากรของอาร์เคีย เมทาโนเจนกลุ่มย่อยที่พบคือ *Metanosaeta, Methanomicrobiales* และ
Methanobacteriales ผลผลิตก๊าซชีวภาพ และมีเทนที่ต่ำลงของน้ำเสียจากกระบวนการกลั่นเอทานอล
สอดคล้องกับลักษณะของประชากรแบคทีเรียโดยเฉพาะอย่างยิ่งการตรวจพบเชื้อในกลุ่ม sulfate
reducing bacteria อย่างไรก็ตามสำหรับการย่อยแบบเดี่ยวของกากมันที่มีผลผลิตของก๊าซชีวภาพและ
มีเทนที่ต่ำลง มีสาเหตุจากสารตั้งต้นที่มีไนโตรเจนต่ำทำให้ตรวจไม่พบกลุ่มประชากรแบคทีเรีย
ในช่วง 12 วันจากการทดสอบด้วยวิธี DGGE ในการศึกษานี้ค่า H index ของ *Methanosarcina* ถูกพบ
ในการย่อยแบบร่วมในอัตราส่วนกากมันสำปะหลังกับน้ำเสียจากกระบวนการกลั่นเอทานอลที่ 1:1

เป็นการยืนยันอย่างชัดเจนว่าที่อัตราส่วนนี้เป็นอัตราส่วนที่เหมาะสมสำหรับกระบวนการย่อยสลาย
แบบไร้อากาศ

     ความสำเร็จของการผสมกากมันสำปะหลังกับน้ำเสียจากกระบวนการกลั่นเอทานอลด้วย
อัตราส่วนที่เหมาะสมคือ 1:1 ส่งผลให้ผลผลิตก๊าซชีวภาพและมีเทนสูงขึ้นพร้อมกับเสถียรภาพของ
การย่อยแบบไร้อากาศ ซึ่งเป็นผลจากการพัฒนาของการย่อยแบบร่วม
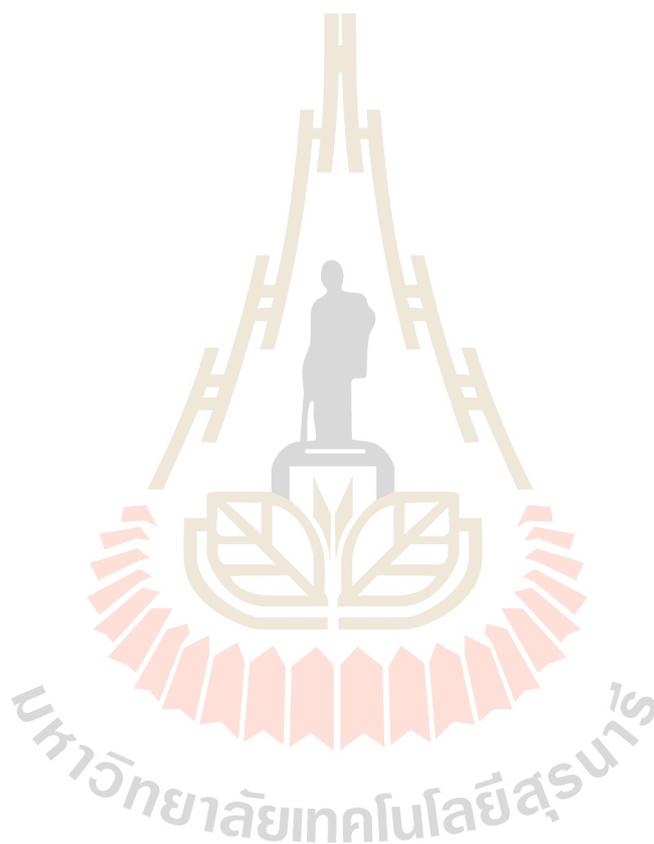
CHONRADEE WITCHAYAPONG : THE STUDY FOR INCREASING OF
METHANE CONTENT IN BIOGAS PRODUCED FROM CASSAVA PULP.
THESIS ADVISOR : ASSOC. PROF. APICHAT BOONTAWAN,
Ph.D., 101 PP.

BIOGAS/ANAEROBIC CO-DIGESTION/CASSAVA PULP/DISTILLERY
STILLAGE/DENATURING GRADIENT GEL ELECTROPHORLYSIS (DGGE)

Cassava pulp is a widely available waste in large amounts in the starch industry in Thailand and can be used for the production of biogas. However, its low nitrogen content is limited microbial degradation leads to a low degradation rate and low biogas and methane production. The potential of cassava pulp: distillery stillage (CP:DS) ratio of 0:1, 1:0.5, 1:1, 0.5:1 and 0:1 was measured in this study. Batch anaerobic digesters were presented in terms of mesophilic condition at $35 \pm 1ºC$. Experimental results showed that the biogas and methane yield from co-digestion were higher than the mono-digestion of CP and DS. Moreover, the optimal of CP: DS ratio was obtained at 1:1. With 1:1 of CP: DS ratio, the cumulative biogas and methane yield, and VS removal rate were 918.73 mL/g VS and 685.10 mL/g VS, respectively. Compared with the mono-digestion of CP only and DS only, the cumulative biogas yield of co-digestion at optimal condition was incresined 146.54 % and 100.64 %, respectively; the same as the cumulative methane yield was 222.19% and 105.70% .

Furthermore, the bacterial and archaeal communities were analyzed by denaturing gradient gel electrophoresis (DGGE). The bacterial community of all CP:DS ratios was dominated by the phyla *Bacteroidetes*, *Firmicutes* and *Chloroflexi*.

Moreover, the methanogenic archaeal community of all CP:DS ratios was dominated by the genus *Methanosarcina (Methanosarcina barkeri)*, which belongs to the family *Methanosarcinaceae.* The genera *Metanosaeta, Methanomicrobiales,* and *Methanobacteriales* were the minor groups represented in the archaeal community. The low biogas and methane production yield of mono-digestion DS was corresponded with the characterization of the microbial community, especially the presence of sulfate reducing bacteria. However, for the mono-digestion of CP, the low biogas and methane production yields were caused by the low initial nitrogen content in substrate (proved by the disappear of bands during 12 days using DGGE method) . In this study, the maximum H´ index value of *Methanosarcina* was obsesered in co-digestion on the CP:DS 1:1 ratio. The high H´ index strongly confirmed that the 1:1 CP:DS ratio was the optimal condition for anearobic digestion process.

Higher biogas and methane production yield at optimal ratio of CP:DS 1:1 was achieved. A high stability of the anaerobic digestion was the result of the adaptation of co-digestion (CP and DS).

School of Biotechnology

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Student's Signature

Advisor's Signature

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Chonradee Witchayapong

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LIST OF ABBREVIATIONS

AD	=	Anaerobic Digestion
APHA	=	American Public Health Association
CP	=	Cassava pulp
S: I	=	Substrate: Inoculum
DS	=	Distillery stillage
BLAST	=	Basic Local Alignment Search Tool
DGGE	=	Denaturing Gradient Gel Electrophoresis
DNA	=	Deoxyribonucleic acid
HRT	=	Hydraulic Retention Time
PCR	=	Polymerase Chain Reaction
TS	=	Total Solids
STR	=	Stirred tank reactor
VFA	=	Volatile Fatty Acids
VS	=	Volatile Solids
SRB	=	Sulfate Reducing Bacteria
H′	=	Shannon diversity index
C: N	=	Carbon: Nitrogen
^{o}C	=	Degree Celsius
wt.%	=	Weight by weigh

CHAPTER I

INTRODUCTION

1.1 Significance of the study

Cassava is the third largest sources of carbohydrates for human consumption in the world with an estimated annual world production of about 210 million metric tons (Ghimire et al., 2015). In Thailand, cassava is mostly used to produce starch and methanol. The starch production process is energy as well as water intensive, however it also generate waste water and solid waste such as cassava pulp (CP). About 0.33 ton of cassava pulp waste is generated from 1 ton of cassava root processed (Chavalparit and Ongwandee, 2009). Most of CP is currently used as a low cost animal feed (60 US$ t^{-1}). However, the low protein of cassava pulp is unsuitable to use as animal feeding for growth promoter. Thus, some of them has become a major problem of solid waste and ground water (Zhang et al., 2015). Consequently, the management of cassava pulp has become an increasingly challenging problem. On the other hand, CP contains 50.77% starch and 39.91% lignocellulose, which are great potential feedstocks for biogas production (Zhang et al., 2015). Therefore, the utilizing cassava pulp effectively could solve the pollution problem convert it into valuable source in future.

Anaerobic digestion (AD) is an attractive waste treatment option for CP due to its high starch contents (50-60% dry matter and 60-70% moisture content) (Sriroth et al., 2000). However, biogas production from cassava pulp might be limited due to its

very high carbon to nitrogen ratio (C/N ratio = 210), resulting in providing insufficient nitrogen to maintain cell biomass and leads to fast nitrogen degradation by microbes (Panichnumsin et al., 2010). Thus, biogas production from CP is very low.

The co-digestion method is more desirable than an addition of synthetic nitrogen source (such as urea), because several wastes can be treated as the same time, resulting in the reduction of operational cost. Recently, several studies have investigated the co-digestion of CP with different co-digestion. Zhang et al. (2015) found that methane production of co-digestion (cassava pulp and sewage sludge) higher than mono-digestion by 33-60%. While, Panichnumsin et al. (2010) reported that the co-digestion of cassava pulp and pig manure enhanced methane production by 41-60%. It is indicated that the co-digestion is advantageous over the mono-digestion for biogas production and methane enhancement.

The distillery stillage (DS) is waste water from bioethanol producing process, contained a large nitrogen content from yeast cell and organic material. Hence, co-digestion of CP with distillery stillage could achieve in the balance of nutrients, at an appropriate C/N ratio and a stable pH needed to increase the methane production. Therefore, this study investigated the effect of the anaerobic co-digestion of CP and DS to biogas and methane production. To further understanding the microbial ecology corresponding to anaerobic digestion, denaturing gradient gel electrophoresis (DGGE) analysis and the Shanmon diversity index (H) analysis were used to investigate the microbial community associated with the process performance of mono-digestion and co-digestion.

In this work, biogas production of CP using distillery stillage as co-digestion was studied. In addition, the effect of CP: DS in different mass ratios of 1:0, 1:0.5, 1:1,

0.5:1, 0:1 to biogas production on anaerobic digestion producing was investigated.

1.2 Research objectives

1.2.1 To evaluate the optimal substrate: inoculum before fermentations.

1.2.2 To study the effect of different CP: DS ratios in term of biogas and methane production yield, pH, volatile fatty acid (VFA) content, ammonia content, volatile solid removal rate (VS removal rate) under mesophilic condition, using anaerobic sludge as inoculum.

1.2.3 To study the effect of *Bacteria* and *Archaea* community structure inside the digesting reactor at different CP: DS ratios using PCR-DGGE (polymerase chain reaction-denaturing gradient gel electrophoresis), phylogenetic analysis

1.2.4 To determine the *Bacteria* and *Archaea* diversity by Shanmon diversity index (H)

1.2.5 To evaluated stability of biogas and methane production process of co-digestion (CP and DS).

1.3 Expected results

1.3.1 High biogas and methane production yield from cassava pulp can be achieved using distillery stillage from ethanol production condition which leads to C/N ratio improvement and provide optimum condition for anaerobic process.

1.3.2 Archaeal and bacterial communities can be responded to the presence in batch anaerobic digestion (AD)

1.3.3 Diversity indexes of methanogens can be obtained higher at co-digestion than mono-digestion

1.3.4   The stability of the anaerobic digestion can be achieved by adaptation of co-digestion (CP and DS) which helps to increase the buffering capacity, and prevents VFAs accumulation
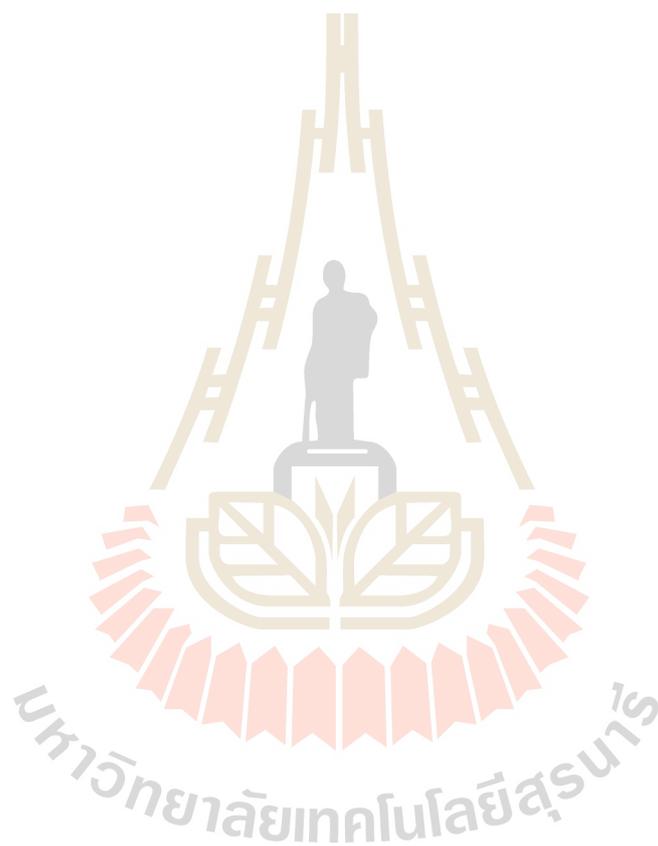
CHAPTER II

LITERATURE OF REVIEW

2.1 History of anaerobic digestion

Anaerobic digestion is historically one of the oldest processing technologies used by mankind. In modern age, after the discovery of methane emissions from natural anaerobic habitats by Volta in 1776, people started to collect the natural biogas and used it as a fuel, basically for lighting. In 1808, Sir Humphry Davy demonstrated the production of methane by the anaerobic digestion of cattle manure. The first anaerobic digestion plant was reported to have been built at a leper colony in Bombay, India in 1859. Anaerobic digestion reached England in 1895, when biogas was recovered from sewage treatment facility to fuel street lamps in Exeter (Lusk, 1997). The development of microbiology as a science led to research by Buswell and others in 1930's to identify anaerobic bacteria and conditions that promote methane production. The primary aim of waste stabilization in due course of time led to the basic municipal sludge digester. However, it took until the end of the 19th century when anaerobic digestion was applied for the treatment of wastewater and solid waste (Gijzen, 2002).

2.2 Anaerobic digestion process and communities involved

Anaerobic digestion is a biological process where the organic matter is decomposed by different communities of microorganisms in the absence of oxygen, finally producing a gas with a high energy content called biogas. Which the anaerobic

process of complex organic polymers is divided into four interrelated steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Fig. 2.1).
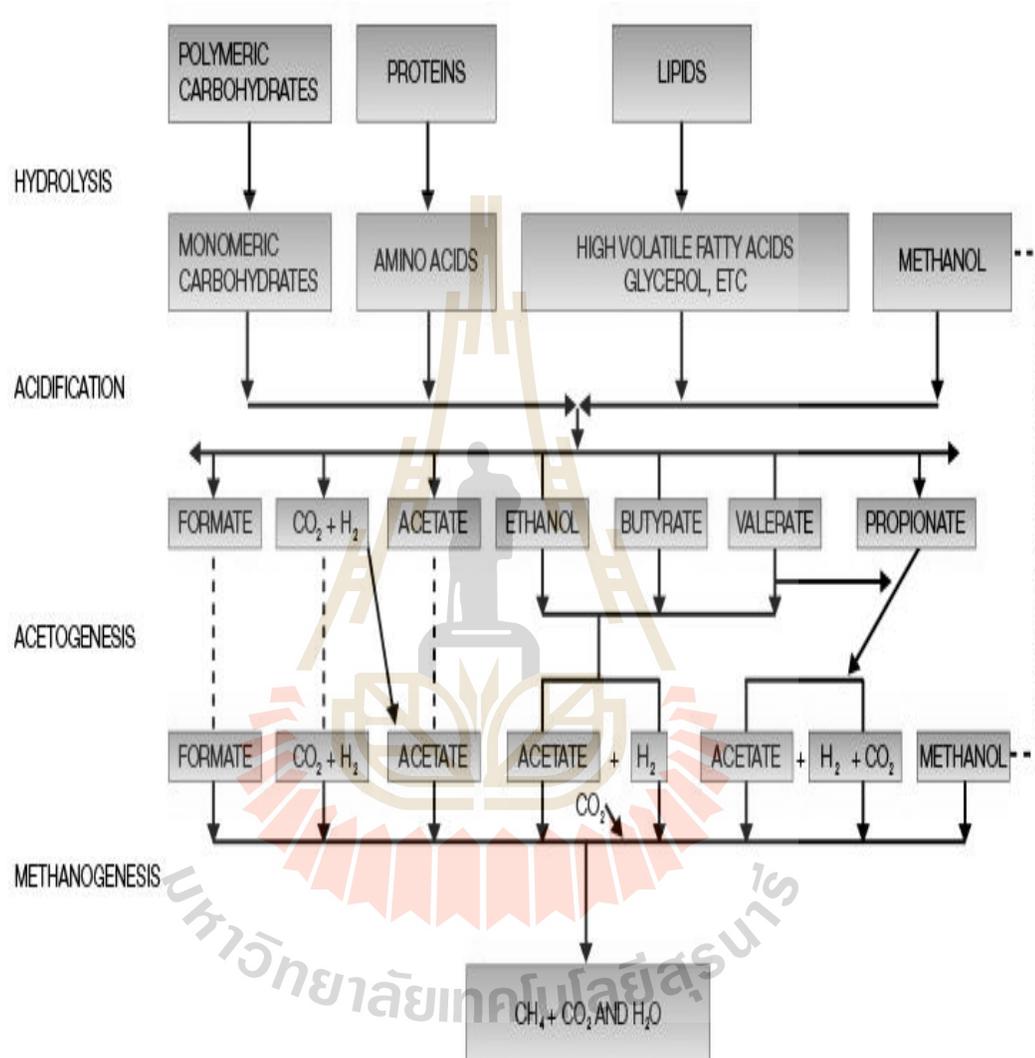
Figure 2.1 Anaerobic digestion scheme with the different stages.

(http://www. watrec.com).

2.2.1 Hydrolysis

The first stage of anaerobic digestion process and consists in the disintegration and hydrolysis of complex organic polymers such as lipids, proteins and carbohydrates to soluble compounds as simple sugars, long chain fatty acids, amino acids and alcohols, which can pass through the cell membrane (Batstone et al., 2000). These reactions are catalyzed by the action of extracellular enzymes (proteases, lipases and cellulases), excreted by hydrolytic fermentative bacteria.

Several factors can affect the degree and rate whereby the substrate is hydrolyzed (Lettinga, 1996), such as composition of the substrate (lignin content for example), the particle size, pH and temperature. The concentration of hydrolytic biomass is also important (Pavlostathis and Giraldo-Gomez, 1991).

Microorganisms from different genera are responsible for the reactions that occur in the hydrolysis step, as Propionibacterium, Bacteroides, Lactobacillus, Sporobacterium, Megasphaera, Sphingomonas and Bifidobacterium (Deublein and Steinhauser, 2011).

With complex substrates, hydrolysis can be the rate limiting step of the whole process (Miron et al., 2000; Pavlostathis and Giraldo-Gomez, 1991) and in some cases it is necessary to apply an initial pre-treatment to the residue to decrease the particle size, increasing the surface area for adsorption of hydrolytic enzymes. By means of pre-treatments lower hydraulic retention times and smaller reactor volumes could be achieved. The rate of hydrolysis, generally, is also increased with increasing temperature (Pavlostathis and Giraldo-Gomez, 1991; Veeken and Hamelers, 1999).

2.2.2 Acidogenic

Soluble compounds obtained in the previous phase are converted by acidogenic bacteria into different fermentation products as volatile fatty acids (VFA), hydrogen gas, alcohols, some organic nitrogen compounds and organic sulphur compounds. The most important of the organic acids is acetate since it can be used directly as a substrate by methanogenic bacteria. Acidogens have notably high growth rates compared to the methanogens and can survive in extreme conditions such as low pH, high temperature and high OLRs (Ahring et al., 2001).

Many of the bacteria that are able to perform this stage are also involved in the hydrolysis (Deublein and Steinhauser, 2011) and they belong to the taxonomic groups of Clostridia, Bacilli, Bacteroidetes and Actinobacteria (Souidi et al., 2007; Krause et al., 2008). However, for the digestion of crops in the presence of manure, members of the class Chloroflexi and thegenus Clostridium usually associated with hydrogen production, were found as dominant bacterial groups (Krakat et al., 2010)

2.2.3 Acetogenic

The low molecular weight volatile fatty acids generated in the acidogenic phase are oxidized by proton-reducing acetogenic bacteria to an appropriate substrate for microorganisms of the methanogenic stage, such as acetic acid, hydrogen and carbon dioxide. This requirement is achieved by syntrophic association with hydrogenotrophic methanogenesis that maintains the hydrogen partial pressure at low levels, allowing syntrophic acetogenesis to be active.

Syntrophic acetogenesis is obligated hydrogen producers and survived only at very low concentration of hydrogen in the environment. They can only survive if their metabolic waste hydrogen is continuously removed. This is achieved because

of their symbiotic relationship with hydrogen utilizing bacteria or methane forming bacteria. The most common representatives of this group belong to these orders: *Syntrophomonas*, *Syntrophobacter*, *Clostridium* and *Acetobacterium* (Hattori, 2008; Weiland, 2010). If hydrogen is not consumed, acetogenesis is inhibited, causing the accumulation of degradation intermediates (VFA), followed by the decreasing pH and methanogenesis inhibition. As examples of acetogenic bacteria, it can be mentioned *Syntrophobacter wolinii* that decomposes propionic acid and *Syntrophomonas wolfei*, which decomposes butyric acid (Boone and Bryant, 1980).

2.2.4 Methanogenic

The final stage of the anaerobic digestion process, in which the methanogenic bacteria, using a limited number of substrates such as acetic acid, hydrogen, carbon dioxide, formic acid, methanol, methylamines and carbon monoxide to produce methane. According to their affinity for the substrate, methanogenic microorganisms are divided into two main groups: acetoclastics, methane-forming microorganisms from acetic acid or methanol, and hydrogenotrophic, which produce methane from hydrogen and carbon dioxide. The methanogens, the most sensitive group of microorganisms in the anaerobic digestion process, could be classified in one of these orders: Methanobacteriales, Methanomicrobiales, Methanosarcinales, Methanococcales and Methanopyrales (Garrity and Holt, 2001), having the microorganisms of the order Methanosarcinales the widest range of substrate utilization. This taxonomic group is divided into two families, Methanosarcinaceae and Methanosaetaceae. Methanosaeta may have lower yields and be more sensitive to pH compared with Methanosarcina. Methanosarcina has a higher growth rate, while Methanosaeta need higher solids retention times but can operate at lower acetate

concentrations, because it has a higher affinity for the substrate (Deublein and Steinhauser, 2011). The genera most frequently determined of hydrogenotrophic bacteria are Methanobacterium, Methanoculleus, and Methanospirillum (Leclerc et al., 2004).

2.3   Factors affecting in biogas production

To secure a stable biogas process with high efficiency, it is exsential that the process is managed in a way that allows the growth of different groups of microorganisms engaged in the whole process. The process factors described below including various significant parameters in biogas production (pH, carbon to nitrogen ratio (C/N ratio), particle size of substrate, temperature, retention time and inhibition)

2.3.1   pH

The pH of the digestion play as an important indicator of the performance and the stability of an anaerobic digestion. The pH level changes in response to biological conversions during the different processes of anaerobic digestion. A stable pH indicates system equilibrium and digestion stability. The optimal pH range for hydrolytic fermentative bacteria and acidogenic bacteria is between 4.5 and 6.3. In contrast, methanogenic bacteria and acetogenic bacteria need a pH in the range of 6.5-8.5 with an optimal methane production at a pH interval of 7.0-8.0 (Schattauer & Weiland, 2006; Weiland, 2010). However, the hydrolytic fermentative bacteria and acidogenic bacteria can occur at pH 7.0. Therefore, the optimal pH range at anaerobic digestion process is obtained at 6.8 to 7.2 (Schattauer & Weiland, 2006). Zhang et al. (2007) reported that anaerobic digestion of kitchen wastes with controlled pH value at 7.0 resulted in a relatively high rate of hydrolysis

and acetogenesis with about 86% of total organic carbon and 82% of chemical oxygen demand were solubilized. The pH in an anaerobic digestion initially was decreased with the production of volatile acids. However, methanogenic bacteria consumed the volatile acids, lead to alkalinity is produced, the pH of the digestion increasesd and then stabilized. At hydraulic retention time of above five days, the methanogenic bacteria began to rapidly consume the volatile acids. In a properly operating anaerobic digestion, at pH between 6.8 and 7.2 volatile acids were converted to methane (CH_4) and carbon dioxide (CO_2).

Alkalinity is also important for the process monitoring, as it is an indicator of the buffering capacity within the reactor. The alkalinity is the result of the release of amino groups ($-NH_2$) and production of ammonia (NH_3) as degraded of the proteinaceous wastes. Alkalinity is present primarily in the form of bicarbonates that are in equilibrium with carbon dioxide in the biogas at a given pH. A high and constant alkalinity could be able to maintain the pH within the neutral or slightly above neutral range, even in case of acid accumulation (Chandra et al., 2012). For monitoring, alkalinity changes prior to pH and can be used as an early indicator of the risk of acidification (Drosg, 2013).

2.3.2 Carbon to Nitrogen ratio (C/N ratio)

A balanced of C/N ratio availability of nutrients for the growth of the microorganisms in biogas digestion is important for the process performance, including stability and substrate utilization (Takashima and Speece, 1989). A high C/N ratio induces a low protein solubilization rate and leads to it unsuitable for bacterial growth due to the deficiency of nitrogen, resulting in lower biogas production and solid degradation. On the other hand, substrates with an excessively low C/N ratio

increase the risk of ammonia inhibition. Ammonia accumulation is toxic to methanogens and causes insufficient utilization of carbon sources (Hartmann and Ahring, 2006). The optimal C/N ratio for anaerobic digestion has been shown to be between 20 and 30 or between 20 and 35, with the most commonly used ratio of 25 (Punal et al., 2000; Zhang et al., 2015)

2.3.3 The particle size of substrate

The particle size plays as an important role in anaerobic digestion, especially during hydrolysis since a smaller particle size provides a greater area for enzymatic attack (Hartmann and Ahring, 2006). The increasing of the average particle size in anaerobic digestion of substrate was reported to decrease the maximum substrate utilization rate coefficient (Kim et al., 2000).

2.3.4 Temperature

Temperature is one of the major factors affecting to microbial activity in anaerobic digestion, and methane production is strongly temperature dependence. Temperature determines the rate of an anaerobic degradation processes particularly the rates of hydrolysis and methanogenesis. There are two range of temperature anaerobic digestion as mesophilic (30-40ºC) and thermophilic (55-70ºC) (Bowen et al., 2014). Thermophilic digestion range has a rate advantage over mesophilic digestion as a result of its faster reaction rate and higher-load bearing capacity and, consequently, exhibits higher productivity compared with mesophilic anaerobic digestion. However, thermophilic digestion is more sensitive to toxic substance and changes in operational parameters (Mata Alvarez, 2002). Furthermore, the fast solid degradation during thermophilic process could lead to volatile fatty acids accumulation and methanogenesis inhibition. As a result, mesophilic temperatures are the preferred

choice for anaerobic treatment (Yacob et al., 2005; Sulaiman et al., 2009). Mesophilic bacteria are supposed to be more robust and can tolerate greater changes in the environmental parameters, expecially temperature. Although it requires longer retention time, the stability of the mesophilic process makes it more marketable in current anaerobic facilities (Zaher et al., 2007).

2.3.5 Retention time (HRT)

The retention time is the time required to complete the degradation of the organic matter. The average HRT is typically in range 15-30 days under mesophilic condition and 10-20 days under thermophilic condition (Angelidaki et al., 2011). Obtaining an effective HRT depends on the organic loading rate (OLR) and reactor volume. OLR is commonly calculated for the amount of material that is added to process per unit of time.

The decreasing of HRT usually leads to VFA accumulation, whereas, a longer than optimal HRT results in insufficient utilization of digester components. For algal biomass, an HRT below 10 days results in low methane productivity (Kwietniewska et al., 2014). The digestion stability of food waste decreased at 8 days of HRT (Kim et al., 2006). In summary, long HRT and low OLR provide the best strategy for achieving constant and maximal methane yield. Due to its allowing extensive contact time between the microorganisms and the substrate, the degree of digestion and methane yield were improved and enhanced from given substrate. The degree of digestion is defined as the percentage of the organic material degraded and converted to biogas during a specific period of time and varies with the substrate (Angelidaki et al., 2011).

2.3.6 Inhibition

Inhibition is usually indicated by a decrease in the microbial population and methane production. A wide variety of substances have been reported to be inhibitory to the anaerobic digestion processes. These kinds of substances can be found as components of the feeding substrate or as by-products of the metabolic activities of bacterial consortium in the digester. A material may be judged as an inhibitory when it causes an adverse shift in the microbial population or inhibition of bacterial growth. Significant differences in inhibition or toxicity levels have been reported for the various substances because of the natural complexity of the anaerobic digestion process and various biochemical mechanisms that cause inhibition, such as antagonism, synergism and acclimation (Chen et al., 2008). Antagonism is defined as a reduction of toxic effect of one substance by the presence of another, whereas synergism is an increase in the toxic effect of one substance by the presence of another. Acclimation is the ability of microorganisms to rearrange their metabolic resources to overcome the metabolic block produced by the inhibitory or toxic substances when the concentrations of these substances are slowly increased within the environment. The four main inhibition of anaerobic process are described below.

2.3.6.1 Ammonia

Ammonia is produced by the biological degradation of the nitrogenous matter, mostly in the form of proteins and urea (Kayhanian, 1999). Several mechanisms for ammonia inhibition have been proposed, such as a change in the intracellular pH, increase of maintenance energy requirement and inhibition of a specific enzyme reaction (Whittmann et al., 1995). Ammonium ion (NH_4^+) and free ammonia (NH_3) are the two principal forms of inorganic ammonia nitrogen in aqueous

solution. Free ammonia (1700 mg/l) is the most toxic because it can pass through a cell membrane, causing a proton imbalance and potassium deficiency (Sung and Liu, 2003). Ionic ammonia is less toxic; a concentration around 5000 mg/l affects acidogens and decreases the activity of methanogens by 50% (Sung and Liu, 2003). An increase in pH will result in a higher toxicity level due to a higher ratio of free ammonia to its ionized form. It is generally believed that ammonia concentrations below 200 mg/l are beneficial to anaerobic process since nitrogen is an essential nutrient for anaerobic microorganisms (Liu and Sang, 2002). The presence of other ions, such as Na^+, K^+, Ca^+ and Mg_2^+, were found to be antagonistic to ammonia inhibitions (Hendriksen and Ahring, 1991).

2.3.6.2 Sulfide

In anaerobic reactor, sulphate is reduced to sulphide by the sulphate reducing bacteria. Sulphate reduction is performed by two major groups of sulphate reducing bacteria including incomplete oxidizers, which reduce compounds such as lactate to acetate and acetate to carbon dioxide, and complete oxidizers, which completely convert acetate to CO_2 and HCO_3^-. Two stages of inhibition exist as a result of sulphate reduction. Primary inhibition is due to the competition for common organic and inorganic substrates from sulphate reducing bacteria, which suppresses methane production. Secondary inhibition results from the toxicity of sulphide to various bacteria groups (Oude Elferink et al., 1994).

2.3.6.3 Light metal ions

The light metal ions including sodium, potassium, calcium and magnesium are commonly present in the digestion of anaerobic digesters. They may be produced by the degradation of organic matter in the feeding substrate or by chemicals

addition for pH adjustment. Moderate concentrations of these ions are needed to stimulate microbial growth, however excessive amounts will slow down the growth, and even higher concentrations can cause severe inhibition or toxicity. Toxicity due to salt is primarily associated with bacterial cells dehydration due to osmotic pressure (Nayano et al., 2010). Although the cations of salts in solution must always be associated with the anions, the toxic action of salts was found to be predominantly determined by the cation. The role of anions was relatively minor and largely associated with their effect on properties such as the pH of the media.

2.3.6.4 Heavy metals

The presence of heavy metals in trace concentration will stimulate the growth of anaerobic digestion. However, unlike other toxic substances, heavy metals are not biodegradable and can accumulate to potentially toxic concentrations. An extensive study on the performance of anaerobic digestion found that heavy metal toxicity is one of the major causes of anaerobic digestion upset or failure (Chen et al., 2008). The toxic effect of heavy metals is attributed to their ability to inactivate a wide range of enzyme functions and structures by binding of the metals with thiol and other groups on protein molecules or by replacing naturally occurring metals in prosthetic groups of enzymes.

2.4 Batch and continuous feeding systems

Biogas production from cassava pulp requires selection of an appropriate system from various digestion techniques together with proper digester design. Fermentation for biogas production is performed in anaerobic digesters. There are different digester-configuration depending on the process condition selected, as follow

2.4.1 Digestion process

There are generally two digestion process in anaerobic digestion of solid waste: the batch and continuous system. Batch system is a common digestion. The cassava pulp is added to the digester at the beginning while the gas production was produced depend on time and several unit must be operated at the same time to maintain a constant gas supply. However, disadvantage of batch system is large retention time and no constant gas production comparing to the continuous process. Conversely, this process can be run at high solids content (larger than 25% total solid (TS)) and able to digest fibrous and difficult wastes. After digestion, the effluent was removed and new process was started with the new feed in using cassava pulp (El-Halwagi, 2012). Such a simple batch system was used to produce biogas production from cassava pulp (Anunputtikul, 2004). In the continuous process, the cassava pulp is added constantly into the digester and constantly removed at the same time. Ideally, the amount of raw materials is equal to the amount of slurry leaving the digester. Therefore, the digester needs to consider design of the inlet and outlet of the raw cassava pulp and slurry, respectively. Since it effects of the constant amount of gas production. For example, Sirirote et al. (2014) used a continuous stirred tank reactor for cassava biogas production.

2.4.2 Stage digestion process

There are two types of stage digestion process including single and two stage digestion, which depends on the number of used digestion. The single stage digestion process composes of one digester and retention time for single stage digestion process was varied from 30 to 60 days (Metcalf and Eddy, 1991). On the other hand, in the two stage digestion process, the function of two digesters was to

separate the anaerobic food chain into two microbiological processes; hydrolytic/acidogenic phase (first stage) and acetogenic/methanogenic phase (second stage). Retention time for two stage digestion process varied from15 days (Metcalf and Eddy, 1991). The study of Schievano et al. (2012), both two stage anaerobic digestion and single stage anaerobic digestion were fed with identical organic substrate and loading rate. It's resulted that the two stage system could have potentially led to higher energy recoveries than the single stage system. However, the two stage system should be operated in optimal process condition (HRT, OLR, pH, temperature, etc.) to ensure the microbial communities efficiency and stability. Thus, the operating of two stage system is complicated.

2.5 Process enhancement

In recent years, significant efforts has been dedicated in finding ways of improving the performance of digesters treating different wastes, especially solid wastes because of the obvious link between successful pre-treatments and improved yields (Mata Alvarez et al., 2000). Many studies have been conducted regarding to the overall aspect of anaerobic digestion of solid waste, which is useful for process improvement. The aspects of process enhancement include co-digestion with other wastes, pretreatment of substrate and the use of microbial stimulants.

2.5.1 Co-digestion

Co-digestion of food waste with other types of waste is an interesting alternative substrate to improve biogas production and to obtain an efficient process. The use of a co-substrate improves the biogas yield due to the positive synergisms established in the digestion medium and the supply of missing nutrients by co-

substrates. Various types of solid wastes streams such as sewage sludge, pig manure have been used as co-substrate for anaerobic digestion of cassava pulp. Zhang *et al.* (2015) found an increasing of methane production (33-60%) in mixing of cassava pulp and sewage sludge. Whilst, Panichnumsin *et al.* (2010) reported that the co-digestion of cassava pulp and pig manure enhanced the methane production by 41-60 %. In this study, distillery stillage was used in co-digestion to improve methane production.

2.5.2 Pretreatments

Cassava residues are wastes industrial byproduct with the main components of lignocellulosics (cellulose, hemicellulose and lignin), which form a recalcitrant lignocellulose complex that resists assault of the microbial and enzyme in hydrolysis reaction (Himmel et al., 2007). Therefore, it is necessary to pretreat these lignocellulosic materials prior to applying in anaerobic digestion to enhance their bio-digestibility. Various pretreatment methods, such as acid (Liu and Cheng, 2009), alkaline (Lin et al., 2009; Zheng et al., 2009), thermal (Wang et al., 2010) and ultrasound (Hogan et al., 2004) pretreatment, which have different effects on enhancing the digestibility of lignocellulosic substrates have been carried out, in order to obtain higher biogas yield. During recent years, several studies about pretreatment of cassava pulp have been conducted. Zhang et al. (2011) analyzed the thermal pretreatment of cassava with acid addition. Sulphuric acid was used in concentrations of 1.32-4.68% (w/w) and the temperature was 150-170ºC. The reaction time was 10-36 minutes. A 57% higher gas yield was found for pre-treated cassava residues compared with untreated.

2.6 Characteristics of cassava pulp and its biogas production in Thailand

Cassava is a root crop, also known as tapioca and manioc (Cassava, 2014). The cassava plant is a woody plant with erect stems and spirally arranged simple lobed leaves with petioles (leaf stems) up to 30 cm in length (TTDI, 2013) and tubercle 5 to 10 cm in diameter and 15 to 35 cm in length (Fig. 2.2) (Kuiper et al., 2007).

Figure 2.2   Cassava tubers: (A) with stems attached (B) without stems.

(Kuiper et al., 2007).

In Thailand, cassava tubers are processed further into chips or pellets for local animal feeding and for export to other parts of the world. Cassava is also used for producing more than 10 million tons of starch (Chauynarong et al., 2015) using the process including of root preparing and washing, rasping, extraction, drying and packing (centrifugation), and dressing and packing (Fig. 2.3a and b).
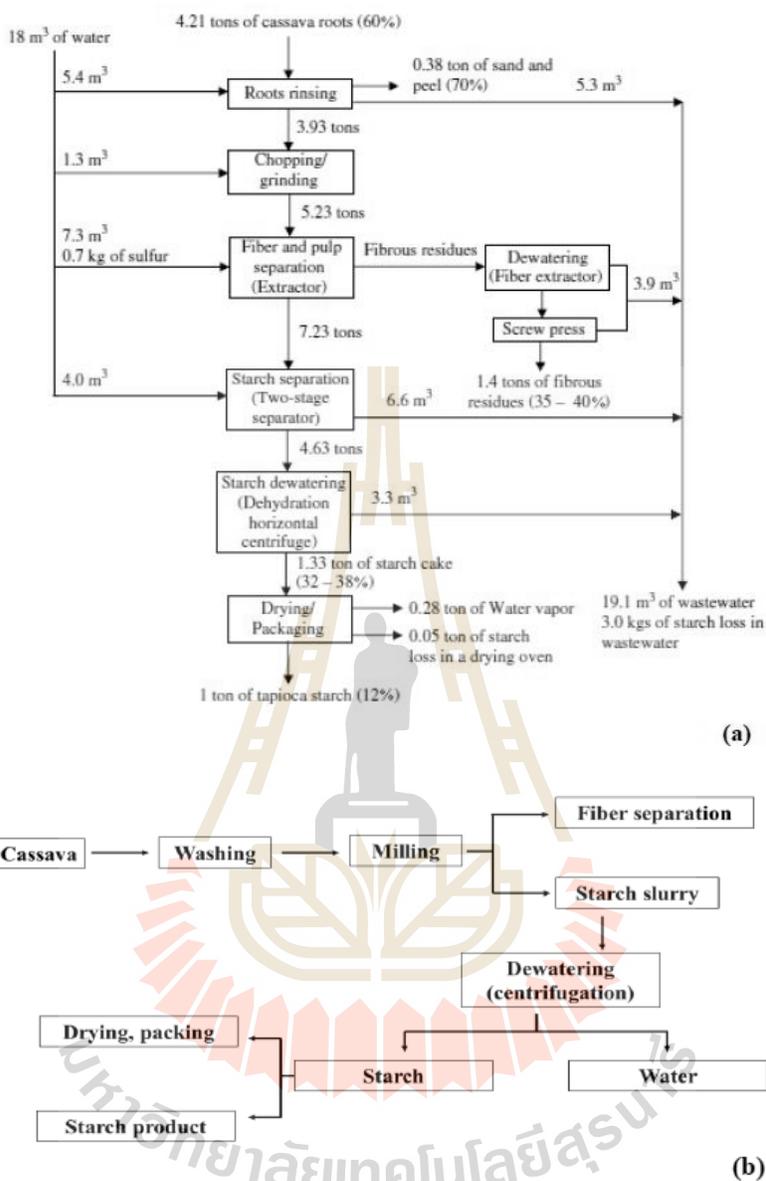
Figure 2.3 Process of tapioca starch production and water mess balance (Chavalparit and Ongwandee, 2009) (a) and simple flow chart of tapioca starch extraction process (b).

As a result, 5.2 million tons of cassava pulp could be produced in cassava starch industry Thailand (Panichnumsin et al., 2010). Generally, the cassava pulp are used as cheap animals feeding. Due to its limitation of low protein content, it is untutored to use as animal feeding for growth promoter (Table 2.1).

Recently, The wastes from cassava industry can be convert into biogas by mixing of cassava wastewater, cassava pulp and nutrients (nitrogen and phosphorus) in a bio-digester. This energy recovery can be applied forward to the cassava factory. In this study biogas production was produced using cassava pulp as a main substrate. Moreover, different from factory, distillery stillage was added into bio-digester as nutrient to make more suitable in renewable energy production.

Table 2.1 Nutrient composition of cassava pulp form four different sources (Chauynarong et al., 2015).

Sources (g kg-1)	Dry matter	GE (MJ kg^{-1})	Fat	CP	CF
Chacho-sun	881.0	16.63	4.8	19.5	104.0
Chacho-oven	868.7	17.18	3.7	13.9	119.1
Chonburi	892.2	16.61	3.6	17.0	144.3
Kalasin	884.6	16.42	1.2	24.5	186.4

DM: Dry matter, GE: Gross energy, CP: Crude protein, CF: Crude fiber

2.7 Methods for assessing microbial diversity

Microbial diversity describes the complex mix of microorganisms at different levels of biological organization. Studies of microbial communities raise questions

about their compositions, structures, and stabilities and about the function and activity of their individual members (Luxmy et al., 2000). Different techniques have already been used to study microbial diversity in bioreactors such as Denaturing Gradient Gel Electrophoresis (DGGE) (Luxmy et al., 2000), Terminal Restriction Fragment Length Polymorphism (T-RFLP) (Liu et al., 1997; Satoh et al., 2012), Fluorescent in situ hybridization (FISH), Real-time PCR (Luxmy et al., 2000; Amann et al., 1990). In this study, DGGE was used to analyze community of total bacteria and methanogens inside reactor.

DGGE is a commonly used method for community characterizations that are based on the melting behaviour of DNA fragments in a special polyacrylamid gel (Spiegelman et al., 2005). The gel contains a linear gradient of denaturant, for example urea or formamide, which causes the denaturation of DNA fragment according to its GC content and nucleotide sequence. It is thereby possible to achieve a separation based on single base pair differences of the fragments, provided that the gel is well calibrated (Zhou et al., 2011). Apart from the high resolution, DGGE is able to provide a good comparison of methanogenic communities as it is possible to compare the different band patterns of the gel. Wang et al. (2010) used this approach of comparing DGGE profiles to analyse the microbial community in a biogas reactor. They analysed the change in the archaeal community while changing the ratio of the added substrates, namely grass silage and cow manure. Thereby it's demonstrated that the archaeal community was only affected to a minimal extend by changes in the substrate composition. Moreover, Wang et al. (2010) stated that they regarded DGGE a potent approach to analyse the connection of bioreactor performance and microbial community structure. Furthermore, the insurance analysis of the whole diversity of the

methanogenic community the primer selection in the DGGE experiment is very important. Hwang et al. (2008) constructed methanogenic profiles by DGGE in anaerobic sludge digestion and found that using universal archaeal primers several methanogens involved in the degradation process were not detected. In order to provide insight into the whole methanogenic community it is therefore necessary to use primers targeting lower taxonomical levels, for example at the order level (Hwang et al., 2008).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Inoculum

The inoculum was sludge, obtained sludge from a modified covered lagoon (MCL) from a cassava starch factory (Fig. 3.1).

Figure 3.1 The modified covered lagoon (MCL) from a cassava starch factory.

The inoculum was kept at mesophilic temperature for one week under anaerobic conditions in order to reduce the residual biogas production. The

characteristics of the two substrates and inoculums are presented in Table 3.1. The main advantage of the inoculum used in this study is its rich in pH, and bicarbonate alkalinity content, thus indicating that is able to maintain pH in an optimal range, and reduce VFAs accumulating VFAs accumulation indicating that it can help to maintain pH in an optimal range, and can reduce VFAs accumulating in the AD process.

Table 3.1 The characteristics of substrate and inoculum.

Parameters	CP	DS	Inoculum
TS (%)	92.45	11.63	2.78
VS (%)	89.48	8.83	2.01
TS/VS (%)	96.78	75.89	2.14
pH	NA	4.95	8.41
Alkalinity(mg/L HCO^{-3})	NA	NA	956.84
NH_4-N	NA	140	326.75
C (% TS)	40.35	41.65	36.62
N (% TS)	0.34	2.47	6.86
C/N	118.68	16.86	5.34

(NA not analysis)

3.1.2 Substrates (CP and DS)

CP was collected from a cassava starch factory whilst DS was obtained from an ethanol production plant in Nakhon Ratchasima, Thailand. Subsequently, collected, the CP was dried at 60°C for 24 h, and crushed into small particles of less

than 1 mm in size (Fig. 3.2). DS was used without further treatment. Both substrates were stored at 4°C in cold- room before using.

Figure 3.2   Cassava pulp (particles < 1 mm).

3.2   Methods

3.2.1   Substrate: inoculum ratio testing using stirred tank reactor (STR)

Experimental was carried out using a 20 liters of STR with an operational volume of 15 liters as shown in Fig. 3.3. The digester was made from glass reactor vessel (SCHOTT DURAN, Germany) composed of feed inlet valve, sampling outlet valve, gas sampling vale, and gas collecting valve connected to a gas collecting tank to measure biogas and methane production.

The reactors were continuously stirred with motors (RW 20 digital, IKA) at 100 rpm and placed in a digital thermostatic water bath at a mesophilic temperature (35°C). Experimental was carried out in triplicate, 15d for each experiment. Each

reactors was flushed with nitrogen gas for 5 min to remove oxygen before tightly inserting the lid. As substrates, different inoculum ratio (S: I ratio) of 1:1, 1:2, 2:1 were maintained for the batch mode (Table 3.2.).
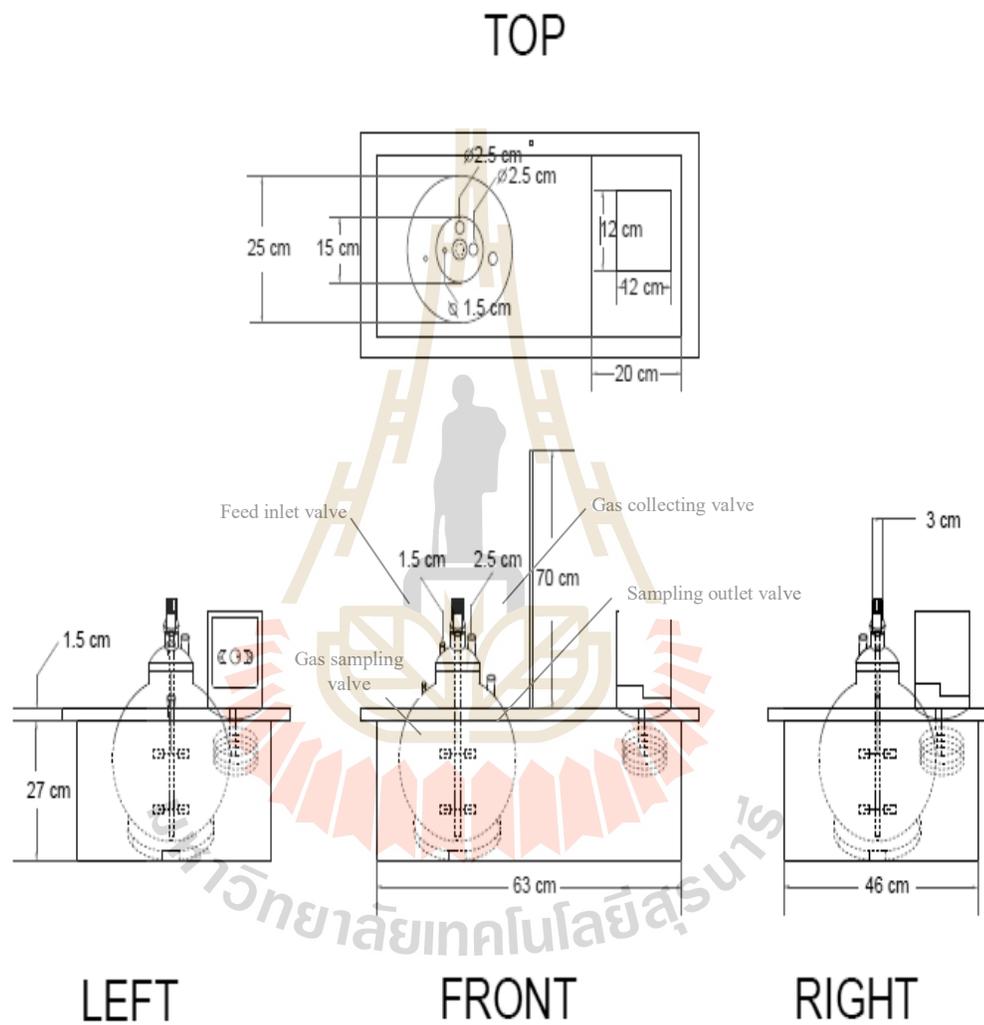
Figure 3.3   STR reactor for testing of substrates: inoculum ratio.

Table 3.2 Experiment design for STR.

S:I	Mass of VS (g)				Mass (g)				
	CP	DS	I	Total	CP	DS	I	Water	Total
1:1	7.5	7.5	15.0	30.0	8.4	85.2	750.0	656.4	1500.0
1:2	5.0	5.0	20.0	30.0	5.6	56.8	1000.0	437.6	1500.0
2:1	10.0	10.0	10.0	30.0	11.2	113.6	500.0	875.2	1500.0

3.2.2 CP: DS ratio analysis using batch reactor

Batch digester was conducted in a 60 L reactor with the working volume of 50 L as shown in Fig. 3.4. The digester tank was made from polyethylene and composed of feed inlet valve, sampling outlet valve, gas sampling vale, and gas collecting valve connected to a gas collecting tank. After that, the reactor was flushed with nitrogen for 10 min to ensure anaerobic condition. All experiments were operated at mesophilic condition (35 ± 1ºC). The produced biogas from the AD reactor was kept in a gas collecting tank using water displacement method (Zhang et al., 2014; Huang et al., 2016).
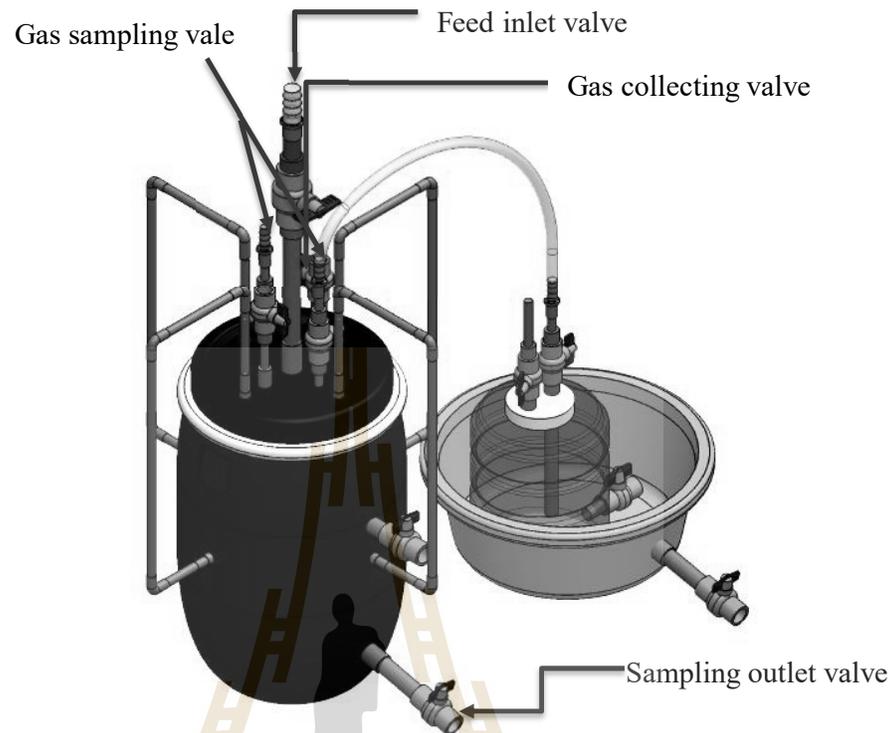
Figure 3.4 Batch reactor for CP: DS ratio analysis.

The same substrate concentration (10g VS/L) was added to each reactors. The substrate/inoculum (S/I) ratio was 1:1 based on the VS. This ratio of S/I was selected according to our previous experiment. The addition of various CP/DS ratios (1:0, 1:0.5, 1:1, 0.5:1, and 0:1, respectively) in each reactor is given in Table 3.3. All batch digesters were performed in triplicate.

Table 3.3 Experimental design for batch digester.

CP/DS	Mass of VS (kg)				Mass (kg)				
ratios	CP	DS	I	Total	CP	DS	I	water	Total
1:0	0.50	0.00	0.50	1.00	0.56	0.00	25.00	24.44	50.00
1:0.5	0.40	0.10	0.50	1.00	0.42	1.42	25.00	23.16	50.00
1:1	0.25	0.25	0.50	1.00	0.28	2.84	25.00	21.88	50.00
0.5:1	0.10	0.40	0.50	1.00	0.14	4.26	25.00	20.60	50.00
0:1	0.00	0.5	0.50	1.00	0.00	5.68	25.00	19.32	50.00

3.2.3 Analytical methods

A daily monitoring of reactors and system performance were conducted by undertaking various laboratory analyses: pH, VFAs, volume of gas, methane content (%). Total solids (TS), total volatile solids (VS), VS removal (%) and NH_4-N of CP and DS were analyzed before and after degradation period. Carbon and nitrogen contents were measured prior to fermentation to calculate the C/N ratio in each experiments. All of methods were described below.

3.2.3.1 pH determination

The pH meter was calibrated, using two buffer solutions, of one is neutral (pH 7.0) and the other is pH 4.0. The pH was measured with a pH700 Oakton. 50 ml each sample in the sample container was well shaken to allow a homogenous mixture and poured into 100 ml beakers. The probe was then inserted and the pH value was measured digitally and recorded.

3.2.3.2 VFAs

.The VFAs contents (acetic acid, propionic acid and butyric acid) of each sample was analyzed from the fraction of total peak area attributed by the mass spectrum libraries. The analyses were conducted on a 6820 GC system gas chromatography, Aligent Techndogy. A FFAP capillary column was used, 30m x 250 µm x 0.25 µm (Quaclrex Corporation). Carrier gas-nitrogen flow 1.0 ml/ per min, detector-FID, temperature program used: 60-200ºC (20ºC/min, 10 min), injector-250 ºC, detector-350ºC. The injector was equipped with a glass liner to glass wool to separate particles of dirt from the sample. The sample were closed by HT 300A automatic closing device at an injection size of 1 µl using the split method and a 30:1 splitting ratio. The analysis time is approximately 15 min.

3.2.3.3 Volume of gas production

The gas production of each sample was analyzed daily at 9.00 am using water displacement method (Zhang et al., 2014; Huang et al., 2016).

3.2.3.4 Methane content (%)

The methane content (%) of each sample was analyzed once a day 9 using a gas analyzer (Geotech, Biogas 5000, USA) (Fig. 3.5).

Figure 3.5 Gas analyzer in this study.

3.2.3.5 Total solids (TS)

The percentage of total solids (TS) presented in the feed suspensions as well as in the reactor effluents were measured by evaporating to dryness 100 mL aliquots of the 200 ml refrigerated and acidified samples to constant weight in previously tared beakers at 105ºC for at least 48 hrs. Then, the beakers were cooled and stored in a desiccator until weighing. The amount of total solids was measured by subtracting the tare weights of the beakers. Total solids were expressed as grams of TS per 100 mL of sample (APHA, 1999) as formula below:

$$\text{Total Solids (\%)} = \frac{(A - B) \times 1000}{\text{Sample volume, ml}} \times 100$$

Which,   A = Weight of dried residue + dish (mg)

B = Weight of dish, mg.

3.2.3.6 Total volatile solids (VS)

To determine the volatile solids (VS), approximately 200-300 mg total dried solids were placed in tared ceramic crucibles, previously baked at 550 ºC prior to tarring. This method is a modification of the standard method for water and wastewater analysis (APHA, 1999). The crucible plus total solids were weighed to 3 decimal place precision then burned in a muffle oven at 550ºC for at least 48 hours. Once constant weight was achieved, the sample crucibles were cooled in a desiccator and stored until final weighing. Once the tare weight was subtracted, the weight loss of each sample was used to calculate the percent of volatile solids presented in the initial total solids. The remaining non-volatile solids were scored as ash. The VS percentage was calculated via following equation:

$$\text{Total Volatile Solids (\%)} = \frac{(A - B) \times 1000}{\text{Sample volume, ml}} \times 100$$

Which,   A = Weight of residue and dish before ignition (mg)

B = Weight of residue and dish or filter after ignition (mg)

C = Weight and dish (mg)

3.2.3.7 VS removal rate (%)

VS removal rate (%) is the efficiency calculation of digest substrate rate during anaerobic digestion. The calculation of VS removal rate (%) was shown below (APHA, 1999):

$$\text{VS removal rate (\%)} = \frac{(VSi - VSe)}{VSi} \times 100$$

Which, VS_i = Influent of VS (mg l^{-1})

VS_e = Effluent of VS (mg l^{-1})

3.2.3.8 NH$_4$-N

NH$_4$-N was analyzed by spectrophotometer according to Baethgen and Alley. (1989).

3.2.3.9 Carbon and nitrogen content

Total carbon and total nitrogen levels were determined using the CHN628 Series Elemental Determinator (LECO, CHN628, USA) (Fig 3.6) in order to calculate the C/N ratio.

Figure 3.6   The CHN628 Series Elemental Determinator.

3.2.4   Molecular analytical methods for genomic DNA extraction

3.2.4.1   Sampling

Samples were taken from different anaerobic digesters work a day. Collected in sterile 50 ml centrifuge tube were used for microbiological analysis. However, the PCR-DGGE analyzing was only carried out at day 3, 5, 6, 12, 15 and 21.

3.2.4.2   Extraction of DNA

In the soil samples, DNA was extracted from approximately 0.25 g of soil using ZR Soil Microbe DNA Miniprep Kit. DNA was extracted according to the manufacturer's instructions using Bead. According to the protocol 0.25 mg of soil sample and 750 µl lysis solution were added to a ZR BashingBead Lysis Tube. ZR BashingBead Lysis Tube was mixed by vortex for 10 min and spun down at 10,000 x g for 1 min. 400 µl supernatant was added to Zymo-spin IV Spin Filter in collection tube and centrifuge at 7,000 x g for 1 min. 1200 µl Soil DNA

Binding Buffer was filled to the Zymo-spin IV Spin Filter. Its mixture was transferred to a Zymo-spin IIC Column in collection tube, centrifuge at 10,000 x g for 1 min and discarded the flow through. 200 µl Soil DNA Pre-Wash Buffer was added to Zymo-spin IIC Column in new collection tube and spun down at 10,000 x g for 1 min. After that, the addition of 500 µl Soil DNA Wash Buffer was done in Zymo-spin IIC Column and centrifuge at 10,000 x g for 1 min. Zymo-spin IIC Column was moved to 1.5 ml microcentrifuge tube and added 100 µl DNA Elution Buffer directly to the column matrix. Centrifugation of 1.5 ml microcentrifuge tube was performed at 10,000 x g for 30 s to elute the DNA. Last step, the eluted DNA was transferred to Zymo-Spin IV-HRC Spin Fillter in new 1.5 ml microcentrifuge tube and centrifuged at 8,000 x g for 1 min. Gel electrophoresis was used to verify the presence of DNA. 5 µL of extracted DNA was mixed with 1 µL 6x DNA Loading dye (Biolab) and loaded onto a 1% agarose gel stained with ViSafe Red gel stain (Vivantis). Extracted DNA was stored at -20°C for further experiment.

3.2.4.3 Denaturing Gradient Gel Electrophoresis

Bacterial and Archaeal 16S rRNA genes were amplified by PCR with the domain-level universal primers (Table 3.4) (Lee et al., 2008; Shin et al., 2008).

Table 3.4 Universal primers for PCR-DGGE.

Primer name	Sequencing
F: BAC338F	ACTCCTACGGGAGGCAG
R: BAC805R	GACTACCAGGGTATCTAATCC
F: ARC787F	ATTAGATACCCSBGTAGTCC
R: ARC1059R	GCCATGCACCWCCTCT

The 5′-ends of BAC338F and ARC787F were added with 40-bp GC-clamps,5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3′ and 5′-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG-3′, respectively. All primers were synthesized by Pacific Science (Thailand). Deoxyribonucleotide triphosphates (dNTPs) were used from Vivantis (Malaysia). DreamTaq DNA Polymerase were used from Thermo scientific (Fermentas, Glen Burnie, MD, USA). The PCR reactions (total volume of 25 μL) were prepared as described in Table 3.5.

Table 3.5 PCR reaction components for DGGE analysis.

Components	Volume/Concentration
Template	Approx. 100 ng of genomic DNA
10X DreamTaq Buffer*	2 μl
dNTP Mix, 10 mM	0.25 μl
Forward primer	1 μM
Reverse primer	1 μM
DreamTaq DNA Polymerase	0.625 U
Water, nuclease-free	To 25 μl

*10X DreamTaq Buffer contains 20 mm $MgCl_2$, which is optimal for most applications.

A touch-down PCR was performed to amplify genes from genomic DNA using a T100 Thermalcycler (Bio-RAD). The amplification protocol was as follows: initial denaturation at 94ºC for 10 min; 20 cycles of denaturation at 94 ºC for 30 s, annealing at 65 to 55ºC (reducing the temperature by 0.5ºC per cycle) for

30 s, and extension at 72ºC for 1 min; 15 additional cycles of 94ºC for 30 s, 55ºC for 30 s, and 72ºC for 1 min; final extension at 72ºC for 7 min. The expected PCR product size was 540 bp of *Bacteria* and 340 bp of *Archaea*.

Table 3.6 A denaturing gradient gel ranging from 40-60% solution.

Components	40% DGGE solution	60% DGGE solution
40% Acrylamide Bis	20 ml	20 ml
50x TAE buffer	2 ml	2 ml
Formamide	16	24
Urea	16.8	25.2
ddH$_2$O	to 100 ml	to 100 ml

*All components were added to a 100-mL volumetric flask. dH$_2$O was added to obtain 100 mL. The solutions were filtered through a 0.2 μm filter (PALL) and stored in amber glass bottles at 4°C.

Denaturing Gradient Gel Electrophoresis (DGGE) was used to screen the microbial communities in the different zones in the lava tube caves. A denaturant gradient gel is a continuous gradient gel that separates DNA fragments based on their size and melting point by running them through a gradient of the denaturants urea and formamide. A denaturing gradient gel ranging from 40-60% denaturant was used in this study. A 100% denaturant solution corresponds to 7 M urea and 40% (v/v) formamide. The solutions required to make a denaturing gel were prepared as described in table 3.6. 300 μL of a 10% ammonium persulfate solution, APS (Sigma), and 30 μL of tetramethylethylenediamine, TEMED (Sigma), were added to 30 mL of each solution

immediately prior to casting the gel. The 8% polyacrylamide gel was poured on to the DGGE system (DCode system, Bio-Rad, Hercules, CA) using a dispensing needle. This pouring was stopped, when the denaturing solution was approximately 1 cm under the comb level. After gel polymerization (1 hour), the comb was removed from the gel and cleaned.
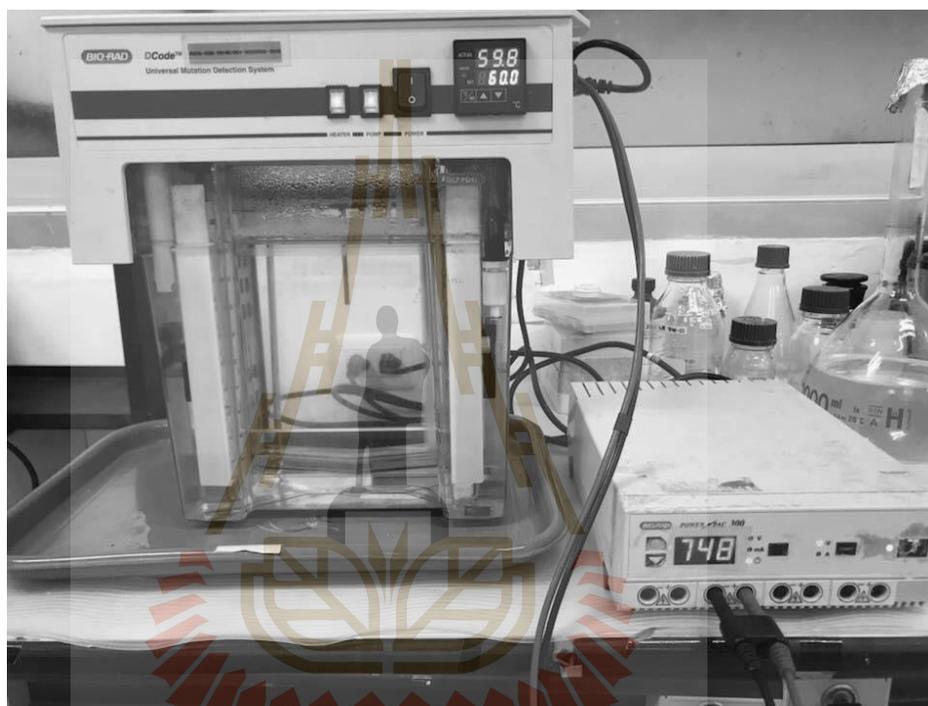
Figure 3.7 The DGGE, DCode system.

The amount of amplified DNA, loaded for each sample was determined visually from the gel electrophoresis of amplified PCR products. Volumes ranged from 20 µL of sample. Loading buffer was added to each sample in order to load 25 µL in each well. The loading buffer was contained 5 mL TAE buffer, 5 mL glycerol (100%) and 200 µL of 0.5% bromophenol blue with xylene. The polyacrylamide gel was run for 16 h in DGGE system (DCode system, Bio-Rad, Hercules, CA) (Fig. 3.7) filled

with 7 L 1X TAE buffer heated to 60°C at 100 V. The gel was stained in 30ml ddH$_2$O to which 6 μL SYBR Gold (Invitrogen, Carlsbad, CA) for at least 1 hour. The gel was visualized using the BioRad Gel DOC XRS. The visible bands were excised and eluted with distilled water for 1 day.

3.2.4.4 PCR for cloning

The PCR was used to insert fragment of cloning (Chapter 3.2.4.5). The bands eluded (Chapter 3.2.4.3) were used as template. Bacteria was amplified by BAC338F and BAC805R. Archaeal was amplified by ARC787F and ARC1059R without GC-clamp (Table 3.4). PCR reaction components were used at the same concentration in Chapter 3.2.4.3. The amplification protocol of *Bacteria* was as follows: initial denaturation at 94ºC for 3 min; 34 cycles of denaturation at 94ºC for 30 s, annealing at 59.5ºC for 30 s, and extension at 72ºC for 1 min; final extension at 72 ºC for 5 min. Whilst, the amplification protocol of *Archaea* was as follows: initial denaturation at 94ºC for 3 min; 34 cycles of denaturation at 94ºC for 30 s, annealing at 55ºC for 30 s, and extension at 72ºC for 1 min; final extension at 72ºC for 5 min. The brands were observed at 500 bp for *Bacteria* and 300 bp for *Archaea*.

3.2.4.5   Cloning of *Bacteria* and *Archaea*

PCR products of amplified 16S rrna genes (Chapter 3.2.4.4) were cloned using the pGEM-T easy vectors kit (Promega, USA). The kit provided by Promega contains linearized plasmid DNA that carries 3´-terminal thymidine at both ends. PCR products amplified with Dream Taq DNA polymerase that generates 3´-dA overhangs, so that PCR fragments can be directly ligated with the vector. The pGEM-T vectors allows ampicillin (100 μg ml^{-1}) selection and furthermore blue/white colony screening (Chapter 3.2.4.8).

3.2.4.6   Competent *E. coli* DH5α cells

Cold competent cells of *E. coli* DH5α were prepared according to the protocol of Inoue et al. (1990) for subsequent heat shock transformation (Chapter 3.2.4.7). *E. coli* DH5α cells were grown overnight in LB medium (5 ml) and thereafter transferred into 250 ml of SOB medium (2 l Erlenmeyer flask). The culture was grown at 18 °C and 60 rpm to an optical density of 0.6 at a wavelength of 600 nm (OD600). The flask was placed on ice for 10 min, and cells were concentrated by centrifugation (4,000 × g, 10 min, 4°C). The cell pellet was suspended in 80 ml ice-cold TB buffer and sun down as above. At last, the cell pellet was gently suspended in 20 ml TB buffer, DMSO was added to a final concentration of 7 % and the cell solution was incubated on ice for 10 min. Aliquots of the cell suspension were frozen in liquid nitrogen and stored at -80°C. SOB medium (Sambrook and Russell, 2001) and TB buffer (Inoue et al., 1990) were shown below.

SOB medium (Sambrook and Russell, 2001)

Tryptone 5 g 2.0 % [w/v]

Yeast extract 1.5 g 0.5 % [w/v]

NaCl 140 mg 10 mM

KCl 480 mg 2.5 mM

ddH$_2$O ad 125 ml

pH 7.0 with NaOH

TB buffer (Inoue et al., 1990)

PIPES 3.02 g 10 mM

CaCl$_2$× 2 H$_2$O 2.2 g 15 mM

KCl 18.6 g 250 mM

MnCl$_2$× 4 H$_2$O 6.9 g 55 mM

ddH$_2$O ad 1000 ml

pH 6.7 with KOH

filtrated, stored at 4°C

3.2.4.7 Heat shock transformation

Cold competent *E. coli* DH5α cells (Chapter 3.2.4.6) were used for heat shock transformation of plasmid DNA. 5 μl of plasmid DNA was added to 50 μl competent *E. coli* DH5α cells. Mix of plasmid DNA and competent *E. coli* DH5α cells were defrosted on ice for 30 min. After heat shocking (42°C for 30 s) cells were directly chilled on ice. LB medium (400 μl) was added for cell regeneration, and cells were incubated for 1 h at 37°C and 225 rpm.

3.2.4.8 Blue white screening

The pGEM-T easy vectors (Promega, USA) contains the lacZ gene within the multiple cloning site. In the presence of IPTG (Isopropyl β-D-1-thiogalactopyranoside) the respective enzyme, the β-galactosidase, catalyzed the alteration of X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) into blue 5-bromo-4 chloroindole. Accordingly, successful ligation of DNA fragments indicated by white *E. coli* colonies after transformation, since the ligation of a DNA fragment into the lacZ gene inhibits the generation of operational β-galactosidase. On the other hand, unsuccessful ligation is indicated by *E. coli* blue colonies. LB agar plates (100 μg ml^{-1} ampicillin), covered with 80 μl X-Gal (98 μM) and 40 μl IPTG (200 μM), were used for screening.

3.2.4.9 Verification of transforment colonies

All of transformant colonies were verified by colony PCR amplification. Single colony was collected to dissolve in 50 μl of sterile water, boiled for 10 min, cooled on ice, and centrifuged (13,000 × g, 1 min). The cell-free supernatant contained the DNA was used as template in PCR reaction. The respective DNA fragment cloned into the The pGEM-T easy vectors (Promega, USA) was amplified with

13M primer (Table 3.7). PCR reaction components were used at the same concentration in Chapter 3.2.4.3. The amplification protocol was as follows: initial denaturation at 94ºC for 3 min; 34 cycles of denaturation at 94ºC for 30 s, annealing at 55ºC for 30 s, and extension at 72ºC for 1 min; final extension at 72ºC for 5 min. The expected size of PCR products were 500 bp of *Bacteria* and 300 bp of *Archaea*.

Table 3.7 Universal primers for Colonies PCR analyzing.

Primer name	Sequencing
F: M13F	TGTAAAACGACGGCCAGT
R: M13R	CAGGAAACAGCTATGACC

3.2.4.10 Plasmid extraction

Plasmid was extracted using Presto Mini Plasmid Kit (Geneaid). Plasmid was extracted according to the manufacturer's instructions as follow protocol: 1.5 ml of cultured bacterial cells was transferred to a 1.5 ml microcentrifuge tube and centrifuged at 14,000 x g for 1 min. Then, the supernatant was discarded and 200 µl of PD1 buffer was added to 1.5 ml microcentrifuge tube containing the cell pellet. The cell pellet was vortexed until all traces dissolving. 200 µl of PD1 buffer was added to resuspended sample and then mixed gently by inverting the tube 10 times. After that, 300 µl of PD3 buffer was mixed immediately by inverting the tube 10 times and centrifuge at 14,000 x g for 3 minutes at room temperature. All of the supernatant was transferred to the PDH column and then added 400 µl W1 into the PDH Column. The water inside the PDH Column was discarded and placed back to the 2 ml collection. Wash Buffer (600 µl) was added into the PDH Column and centrifuge at 14,000 x g for

6 seconds. The water inside the PDH column was discarded and placed into new 1.5 ml microcentrifuge tube. Finally, 50 µl of elution buffer was added into the center of the column matrix and centrifuge at 14,000 x g for 2 minutes at room temperature to elute the purified DNA.

Gel electrophoresis was used to verify presence of plasmid. 5 µL of extracted plasmid was mixed with 1 µL 6x DNA loading dye (Biolab) and loaded onto a 1% agarose gel stained with ViSafe Red gel stain (Vivantis). Extracted DNA was stored at -20°C for further processing.

3.2.4.11 Plasmid verification using restriction enzyme digestion.

Restriction enzyme was used to confirm recombinant plasmid (Chapter 3.2.4.8) with cut plasmid by EcoRI specific site on the cloning vector (pGEM-T easy vectors). The expected size of enzyme digestion was 500 bp of bacterial inserted fragment and 3015 bp of plasmid. While, the expected size of enzyme digestion was 300 bp of archaeal inserted fragment and 3015 bp of plasmid

3.2.4.12 DNA Sequencing

Sequencing of plasmid DNA was analyzed by the Macrogen (Korea).

3.2.4.13 Phylogenetic trees

The phylogenetic trees of bacteria and archaea were constructed using the MEGA-X program with neighbor-joining method.

3.2.4.14 Analysis of DGGE Gel Images

The Shannon diversity index (H´) was calculated using the Microsoft excel, based on the quantity and relative intensity of each band, which were obtained by the software Quantity One (ver. 4.6.2; Discovery Series, Bio-Rad) (Lou et al., 2004)

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Biogas and methane production from different S: I ratio

4.1.1 Daily biogas and cumulative biogas production yield

The daily biogas production as a function of time with different S: I ratio was shown on Fig. 4.1. The daily biogas production was increased within 1-2 days in both different S:I ratios. Then by, it was reduced gradually. This could be explained by the fast consumption of organic material by bacteria during anaerobic digestion (Espinoza-Escalante et al., 2009). The maximum yields of biogas production in S:I ratio of 1:1, 1:2 and 2:1 were achieved at 98, 110 and 130 ml/g VS, respectively.
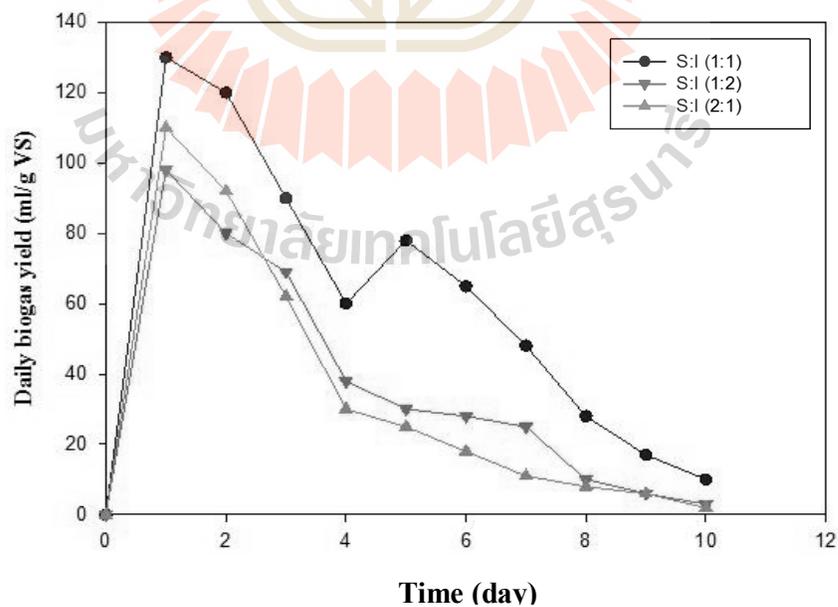
Figure 4.1 Daily biogas production yield at different S: I ratio of 1:1, 1:2, 2:1.

However, in the S:I ratio of 1:1, the biogas yield was increased again on day 5 of digestion. It was discussed in the following part.

As shown in Fig. 4.2, the highest cumulative biogas production was observed at 1:1 S:I ratio with 646 mL/g VS. Whist, the cumulative biogas production at S: I ratio of 1:2 and 2:1 were 387 and 384 mL/g VS, respectively. This implied that the S: I ratio of 1:1 had synergistic effect on biogas production, compared to S: I ratios of 1:2 and 2:1.
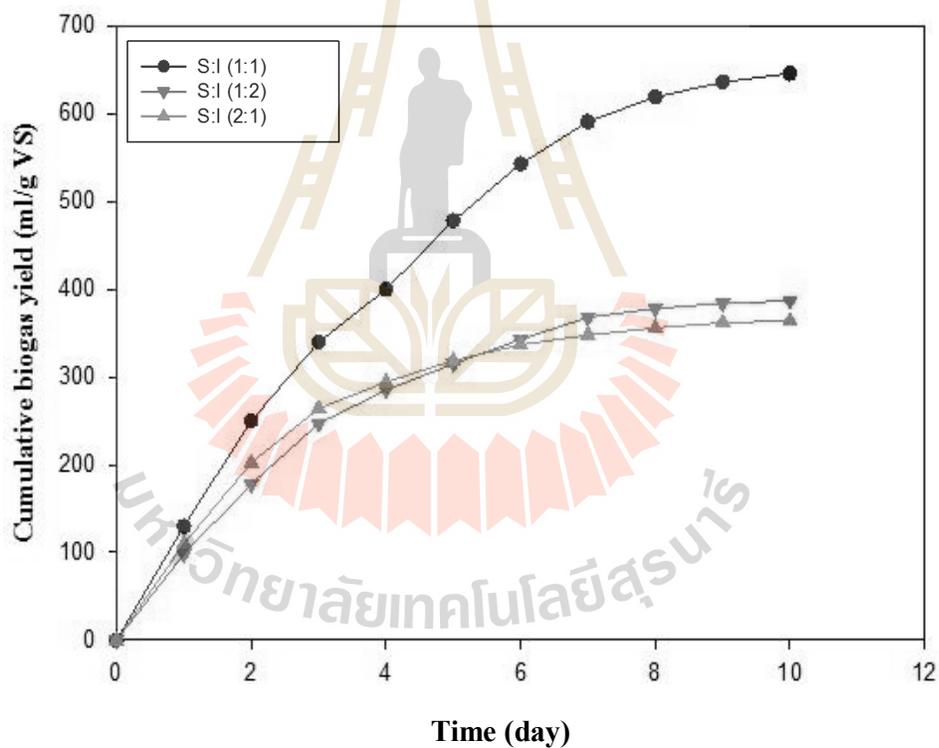
Figure 4.2 The cumulative biogas production at different S: I ratios of 1:1, 1:2, 2:1.

4.1.2 Daily and cumulative methane production yield

Figure 4.3 shows the daily methane production yield, as a function of time with different S: I ratios. The methane production of all experiments accreted immediately on the first day of digestion. This could be described by the fact that the substrate was instantaneously consumed by the acetogenic bacteria and the organic acids were used by methanogenic bacteria as food sources in the short term. The highest methane production of all experiments was 81.6, 52, 61.6 ml/g VS at S:I ratios of 1:1, 2:1, 1:2, respectively, and leveled off thereafter at all S:I ratios since the substrate was almost completely consumed by the bacteria consortium. Same as biogas production yield, methane yield was increased in addition in the S:I ratio of 1:1 at 5 days digestion.
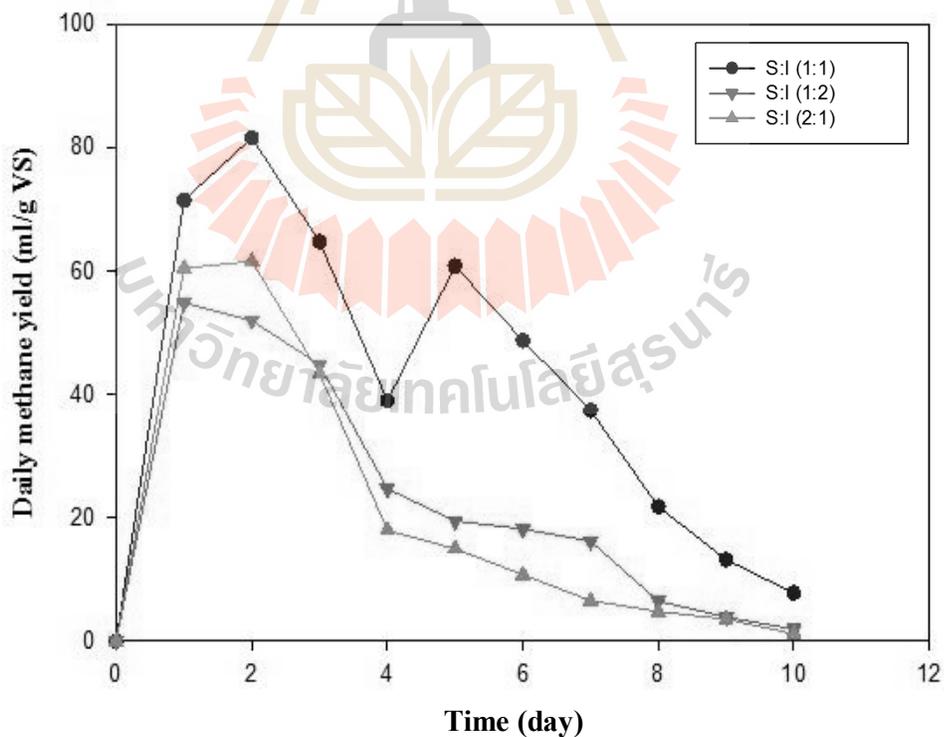
Figure 4.3   Daily methane production yield at different S: I ratio of 1:1, 1:2, 2:1.

The cumulative methane yield was evaluated for 10 days, and the cumulative methane yield at different S:I ratio was shown in Fig. 4.4. After digestion, the cumulative methane yield of all digestions was rapidly increased. The cumulative methane yield at S:I ratio of 1:1 was significantly increased until the end of digestion. While, the cumulative methane yield at S:I ratio of 2:1 and 1:2 was remained constantly after 5 days until the end of digestion. As a result, the highest cumulative methane yield was presented on S:I ratio of 1:1. Consistently with study of Li et al. (2015), it was found that the S:I ratio of 1:1 (cassava pulp: pig manure) was the optimal ratio to increase cumulative methane yield.
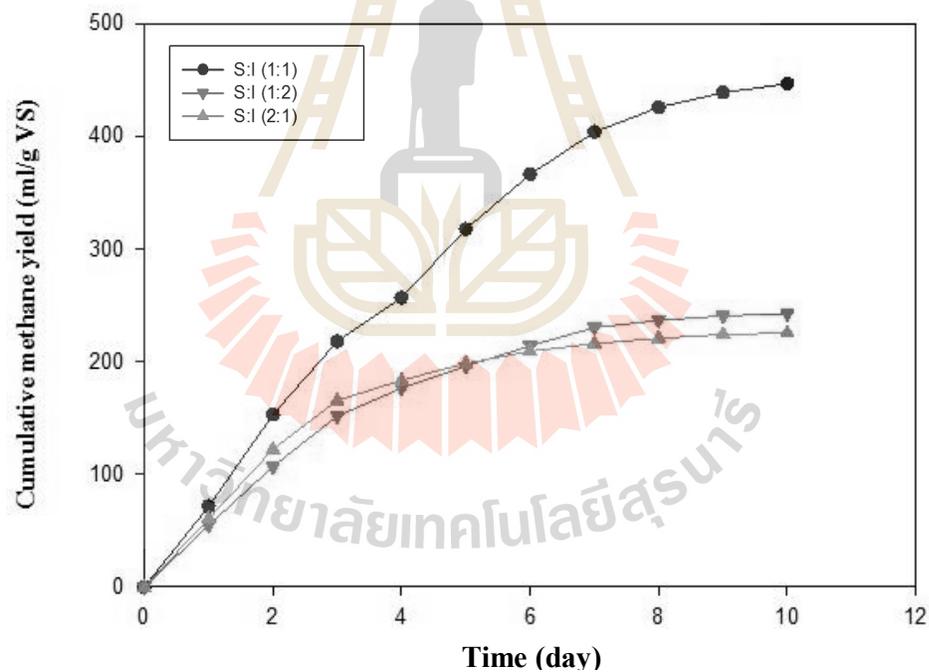
Figure 4.4 Cumulative methane production yield at S: I ratios of 1:1, 1:2, 2:1.

4.1.3 pH value of S: I ratios

The activities of methanogenic and acidogenic microorganisms are depend on the optimal nutritional requirements and optimal pH. The optimal pH for methanogenesis was around 7.0, while it was between 5.5 and 6.5 for hydrolysis and acidogenesis, as reported in numerous studies (Lin et al., 2011; Park and Li, 2012; Wang et al., 2012). Thus, pH of a batch anaerobic system should be maintained within the correct range for methanogenic bacteria (6.8-7.2) to reduce the inhibitory effects of the increased VFA concentrations (Pöschl *et al.*, 2010). The variation of pH values at different S:I ratio was shown in Fig 4.5. All digesters started at the neutral pH value without any adjustments, varied from 7.01 to 7.20. In this experiment, pH values at S: I ratio of 1:1 and 1:2 were dropped during the first 4 days, with values of 6.9 and 6.7, respectively. However, at day 5, the pH value at S: I ratio of 1:1 was increased and maintain stable value while the pH value at S: I ratio of 2:1 was decreased.
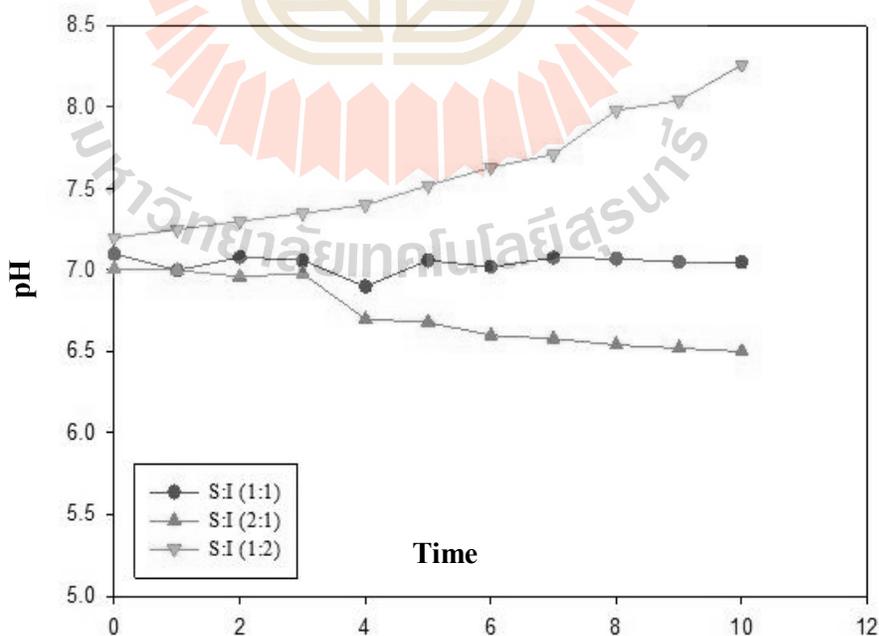
Figure 4.5 pH at different S: I ratios of 1:1, 1:2, 2:1.

The likely reasons of pH dropping at S: I ratio of 2:1 were due to the VFAs accumulation case by the lack of buffer capacity. The pH value at S: I ratio of 1:2 was gradually increased and reached the maximum pH value around 8.26. Alkan-Ozkaynak and Karthikeyan (2011) reported that at pH above 8, unionized form of ammonia tended to be the dominant type, resulting in toxicity issues to methanogenic bacteria. It is highly confirmed from the low biogas and methane production yield data. The final pH values were 7.05, 8.26 and 6.5 at S: I ratio of 1:1, 1:2 and 2:1, respectively.

In addition, the optimal S:I ratio of CP and DS as substrate was obtained at S:Iratio of 1:1 due to the high biogas and methane production yield and stability of process (pH). Therefore, the S:I ratio of 1:1 was used for further experiment.

4.2 Biogas and methane production by co-digestion (CP and DS)

4.2.1 Characteristics of substrates and inoculum

The characteristics of substrates and inoculum including TS (%), VS (%), TS/VS (%), pH, Alkalinity (mg/L HCO^{-3}), NH_4-N (mg/L), C (%TS), N (%TS) and C/N ratio were presented in table 4.1.

The C/N ratios in this experimental work were ranged from 16.86 to 118.86. When the C/N ratio was about 20 to 25, it can be considered that the anaerobic process was appropriate in term of nutrient balance without the risk of acidification (Yen and Brune, 2007).

Table 4.1 Characteristics of substrates and inoculum.

Parameters	CP	DS	Inoculum
TS (%)	92.45	11.63	2.78
VS (%)	89.48	8.83	2.01
TS/VS (%)	96.78	75.89	2.14
pH	NA	4.95	8.41
Alkalinity (mg/L HCO^{-3})	NA	NA	956.84
NH_4-N (mg/L)	NA	140	3267.53
C (% TS)	40.35	41.65	36.62
N (% TS)	0.34	2.47	6.86
C/N	118.68	16.86	5.34

4.2.2 The daily biogas and cumulative biogas yield

The time courses for daily biogas yield and cumulative biogas yield for co-digestion of CP with DS at the CP:DS ratios of 1:0, 0.5:1, 1:1, 0.5:1.5 and 0:1 were shown in Fig. 4.6 (a-e). Most of the batch digestion process were finished within 21 days; however, some digesters took shorter time to finish. The shortest duration was mono-digestion including CP:DS ratios of 1:0 (Fig. 4.6a) and 0:1 (Fig. 4.6e) which took 12 and 16 days, respectively. The reason for a shorter duration of the CP was a high content of lignocellulose portion, which was hard to digest under AD. Whereas, DS contained high amount of short chain molecules that was easy to be degraded by bacteria, lead to a shorter time of AD (Syaichurrozi and Sumardiono, 2013). The highest biogas production was observed since the first day for all experiments, and the value gradually declined for the subsequent days (Fig. 4.6 a-e). The possible cause

includes a fast biodegradability of organic materials under anaerobic condition (Espinoza-Escalante et al., 2009).
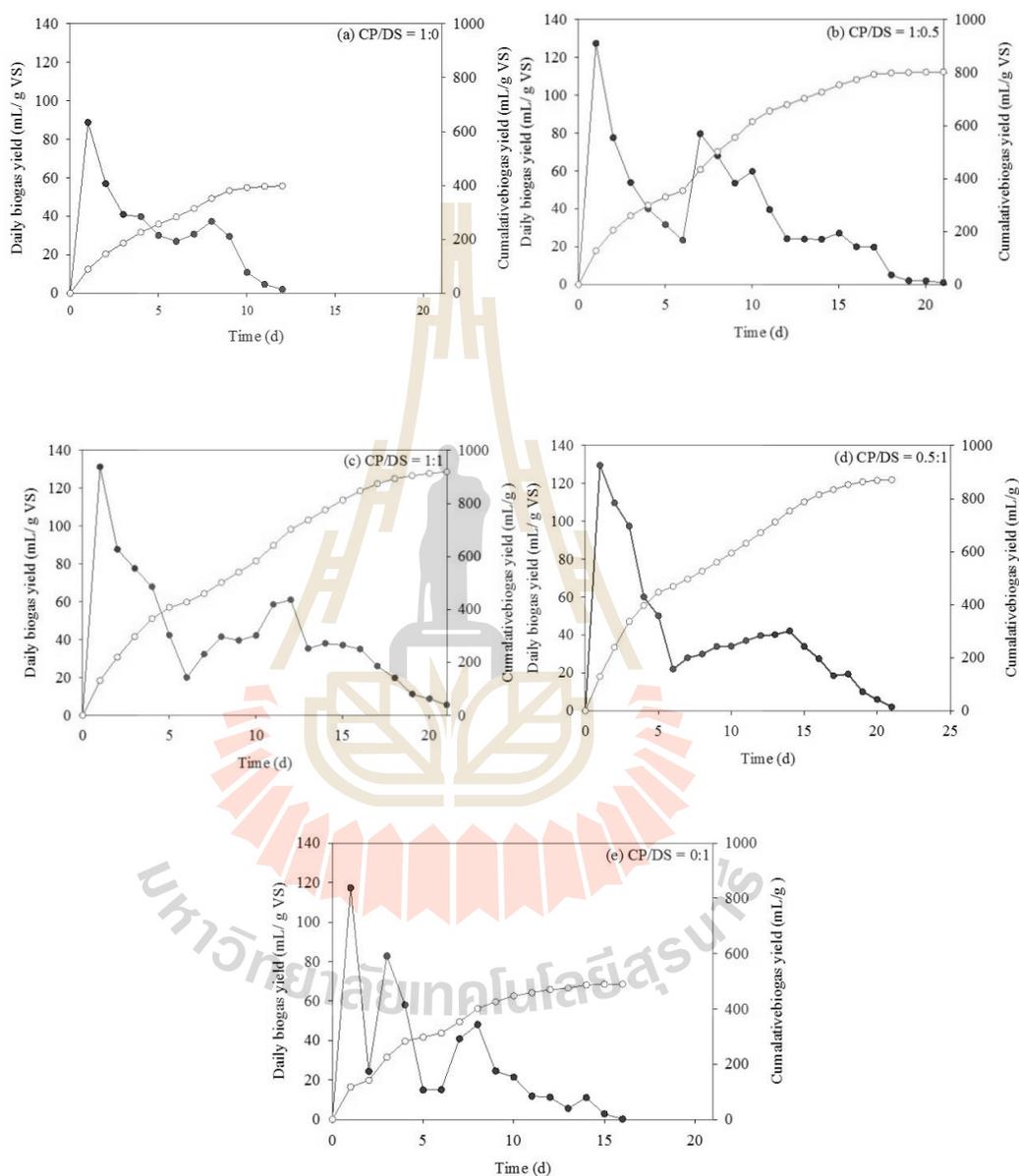
Figure 4.6 Daily biogas yield and cumulative biogas yield of CP with DS at mixing ratios of 1:0, 1:0.5, 1:1, 0.5:1, 0:1 based on.

The cumulative biogas yield of mono-digestion at CP:DS ratios of 1:0, 0:1 were 398.20 mL/g VS (Fig. 4.6a), and 489.30 mL/g VS (Fig. 4.6e), respectively. For the co-digestion experiments, the highest cumulative biogas yield at CP:DS ratios of 1:1, 0.5:1, 1:0.5 were 981.73 mL/g VS, 870.93 mL/g VS and 803.41 mL/g VS as shown in Fig. 4.6c, Fig. 4.6d, and Fig. 4.6b, respectively. All of the co-digestion ratios showed higher biogas yield than mono-digestion, indicating that the balanced nutrients in the mixed substances was enhanced the biodiversity in the reactor and promoted microbial activity. In this work, the optimal CP:DS ratio was obtained at 1:1 where the cumulative biogas yield increased 146.54%, and 100.64% in comparison to CP and DS only.

4.2.3 The daily methane yield and cumulative methane yield

Daily methane production yield of co-digestion of CP with DS at the CP:DS ratios of 1:0, 1:0.5, 1:1, 0.5:1 and 0:1 were shown in Fig 4.7. Two peaks were found in the digestion of CP with DS at the CP:DS ratios of 1:0, 1:0.5, 1:1, 0.5:1 and 0:1 on the 1 and 12 day, respectively, and three peaks were found in the digestion of DS only on the 1, 3 and 8 day, respectively. Kafle and Kim (2013) suggested that two peaks come from the degradation of carbohydrates and crude protein in anaerobic digestion, respectively. Therefore, the lower methane yield of two peak in CP only is attributed to the shortage of crude protein and other macromolecular substances. The co-digestion of CP with DS at ratio 1:1, resulted in the highest maximal daily methane production yield of 76.42 mL/g VS. The maximum daily methane yield (76.42 %) was achieved at CP:DS ratio of 1:1 within one day digestion.

The result of cumulative methane yield was also presented in Fig 4.7. The cumulative methane yield during the co-digestions of CP and DS was increased more rapidly than that of CP and DS alone. At 1:1 ratio of co-digestion of CP and DS, the

highest cumulative methane yield was obtained (685.10 mL/g VS). Compared with mono-digestion of CP (212.64 mL/g VS) and DS (333.06 mL/g VS), the cumulative methane yield was increased 222.19% and 105.70%, respectively. In addition, the optimal CP/DS ratio (1:1) of this study was achieved higher cumulative methane yield superior than previous studies. Panichnumsin et al. (2010) found that the highest cumulative methane yield of 391 mL/g VS, was obtained at 2:3 ratio of CP and pig manure (PM) while other literatures reported that the highest cumulative methane yield of 370 mL/g VS was obtained at 60:40 ratio of CP/PM (Panichnumsin et al., 2012). Moreover, Zhang et al. (2015) found that the cumulative methane yield of 242 and 333 mL/g VS during the co-digestion of CP and sewage sludge inoculum. Therefore, it could be concluded that the co-digestion of CP and DS attained an optimal C/N ratio (29.2) leading to achieving the highest cumulative methane yield.
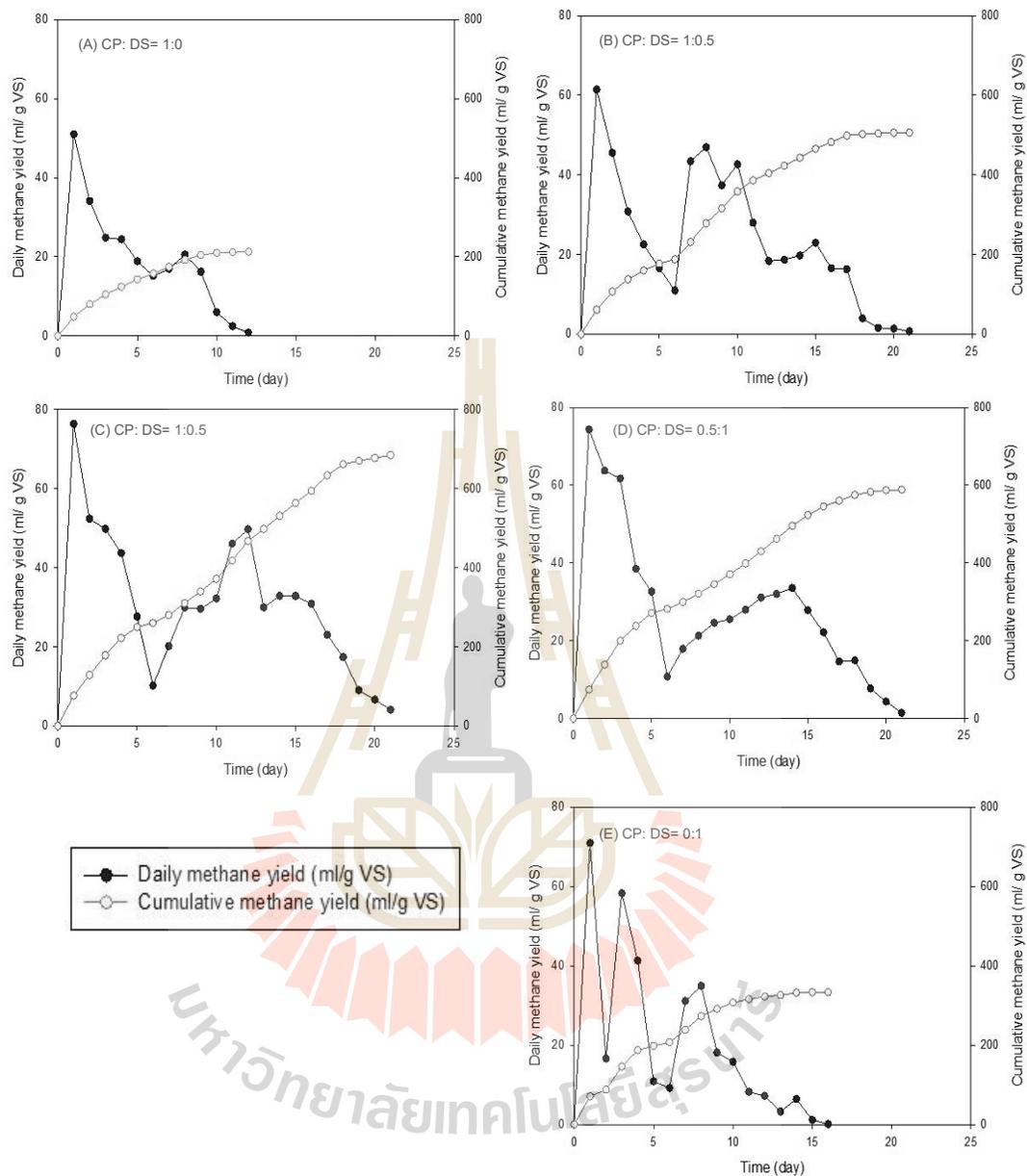
Figure 4.7   Daily methane yield and cumulative methane yield of CP with DS at mixing ratio of 1:0, 1:0.5, 1:1, 0.5:1, 0:1 based on VS.

4.2.4 The methane content

The methane content of biogas produced in all CP: DS ratios was present in Fig 4.8.
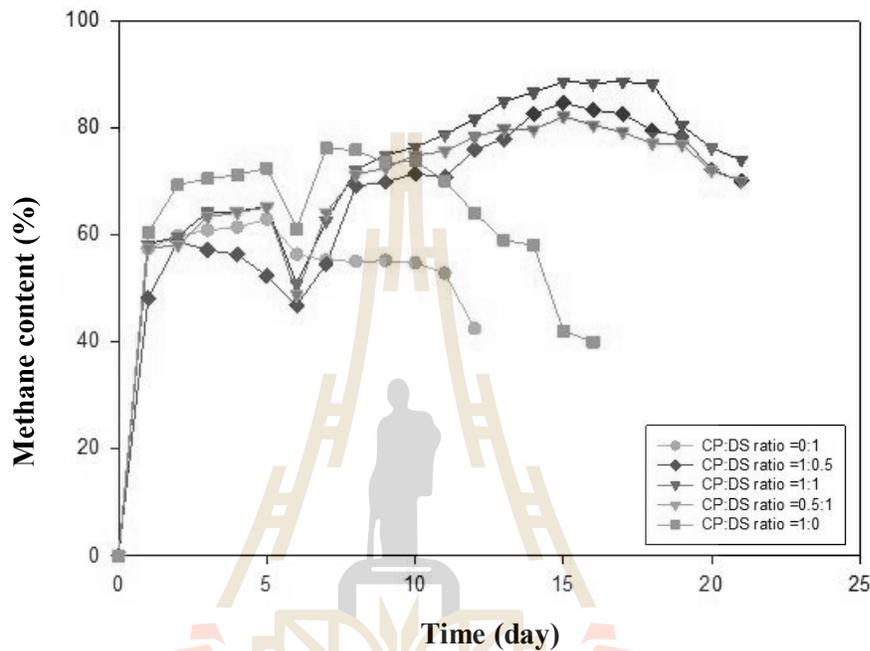
Figure 4.8 Methane content of CP with DS at different ratio of 1:0, 1:0.5, 1:1, 0.5:1, 0:1.

All of co-digestion showed the similar trends that methane content started to increase immediately after inoculating and then decrease during 6 days. However, the methane content after 6 days was kept increasing until the 12 days and then gradually decreased after 18 days. The methane content of mono-digestion was similar with the trends of co-digestion during 1 to 6 days. The methane content of all mono-digestion was declined till the end of experiment. 56.78% and 66.39% of average methane content were obtained in mono-digestion of 1:0, 0:1 CP/DS ratios,

respectively. Moreover, the average methane content of co-digestion of 1:0.5, 1:1 and 0.5:1 CP/DS ratios were 68.64%, 74.57%, 71.96%, respectively. The higher methane content was obtained in co-digestion of CP and DS in comparison to CP or DS only. This phenomena occurred probably because of the balanced nutrient supply and an optimal living environment for the microorganisms (Huang et al., 2016)

4.2.5   pH and VFAs

The time course for pH values at different CP:DS ratios was presented in Fig. 4.9.
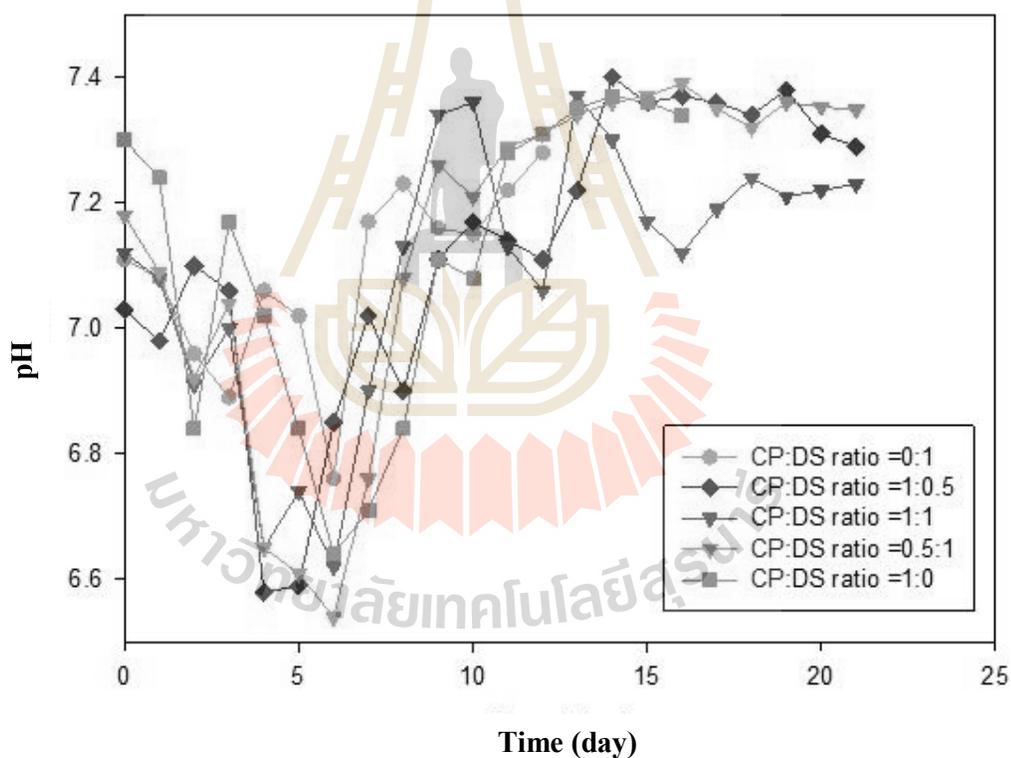
Figure 4.9   pH of CP with DS at different ratios of 1:0, 1:0.5, 1:1, 0.5:1, 0:1.

All pH values of the five reactors were dropped during the 6 days, with the values from 6.5 to 6.8 (Fig 4.9). The initial pH of all reactors was range from 7.0 to

7.3 due to the rich buffer capacity from inoculum. At the end of AD, final pH values were from 7.2 to 7.3. The pH value is one of the main operational factors which greatly affect the anaerobic digestion process (Appels et al., 2008). In biogas production process, generally, there are several groups of microorganisms that require different optimal pH values. The optimal pH of hydrolytic and acidogenic microorganism ranged between 5.5 and 6.5, while methanogenic microorganism required pH around 7.0. However, the suitable pH range for methanogenic bacteria to reduce accumulate VFAs in single-stage anaerobic digestion was 6.8-7.2 (Mei et al., 2016; Dai et al., 2015). The main reason for pH decreasing at the beginning of AD process was due to the conversion of micro-molecular organic matters to organic acids. Consequently, the value of VFAs increased.

The VFAs concentration of CP with DS at different ratios was present in Fig. 4.10. The VFAs concentration of all reactor was high during first 6 days, resulting in the biogas and methane production yield dropped during these days. Moreover, the reactor contained the high concentration of DS (CP:DS ratio 0:1) presented higher VFAs. Due to the high content of short chain molecular, substances were degraded easily into VFAs. However, the VFAs accumulation reached plateau after 6 days, and gradually declined until the end of experiment. The pH values of co-digestion were not extremely different from the mono-digestion, indicates that high buffer capacity of the inoculum could reduce VFAs accumulation, and maintain a stable fermentation environment. According to various studies, there were reported that a large amount of inoculum could help AD to shorten the start-up period, and prevent the acidification caused by accumulation of VFAs (Dang et al., 2013; Yang et al., 2015).

Firstly, the concentration of VFAs concentration including acetic acid, propionic acid and butyric acid was measured and its results was presented in Fig. 4.10 (a-e). At co-digestion, the acetic acid concentration gradually was increased on 1st day fermentation reach the maximum value on 6 day (around 2800 mg/l at CP: DS ratio 1:1). After 6 days, its concentration was declined vigorously and increased over again during 10 to 13 days. Then by, VFAs concentration was gradually decreased til the end of experiment. The butyric acid and propionic acid were remained at low concentration for the entire period.

The VFAs concentration of mono-digestion was displayed on Figure 4.10 (a and e). The dominant VFAs concentration of CP only (Fig 4.10a) was acetic acid followed by butyric acid and propionic acid. The VFAs concentration was increased during first 7 days fermentation afterward decreaseding. In contrast, the dominant VFAs concentration of DS only was acetic acid and propionic acid followed by butyric acid. In mono-digestion fermentation, the highest acetic concentration and propionic concentration (Fig 4.10e) was observed at DS only on 7 days (2,100 mg/ ml) and 6 days (1,440 mg/ ml), respectively and then sharply declined till the end experiment.

4.2.6 NH_4-N and VS removal rate in different CP: DS ratios

One of inhibition factors in AD is ammonia (NH_4-N). The ammonia (NH_4-N) concentration of CP: DS ratios was showed in Table 4.2. The average of ammonia (NH_4-N) concentration was varied from 334.43-503.15 mg/L. At high concentration, NH_4-N was a strong inhibitor for methanogenic bacteria (Lin et al., 2011). When the concentration of the ammonia was in excess of 1500 mg/L NH_4-N, it leads to the process instability, and eventually system failure (Van Velsen, 1979). In this experiment, the initial NH_4-N concentration of all reactors were not above the limit.
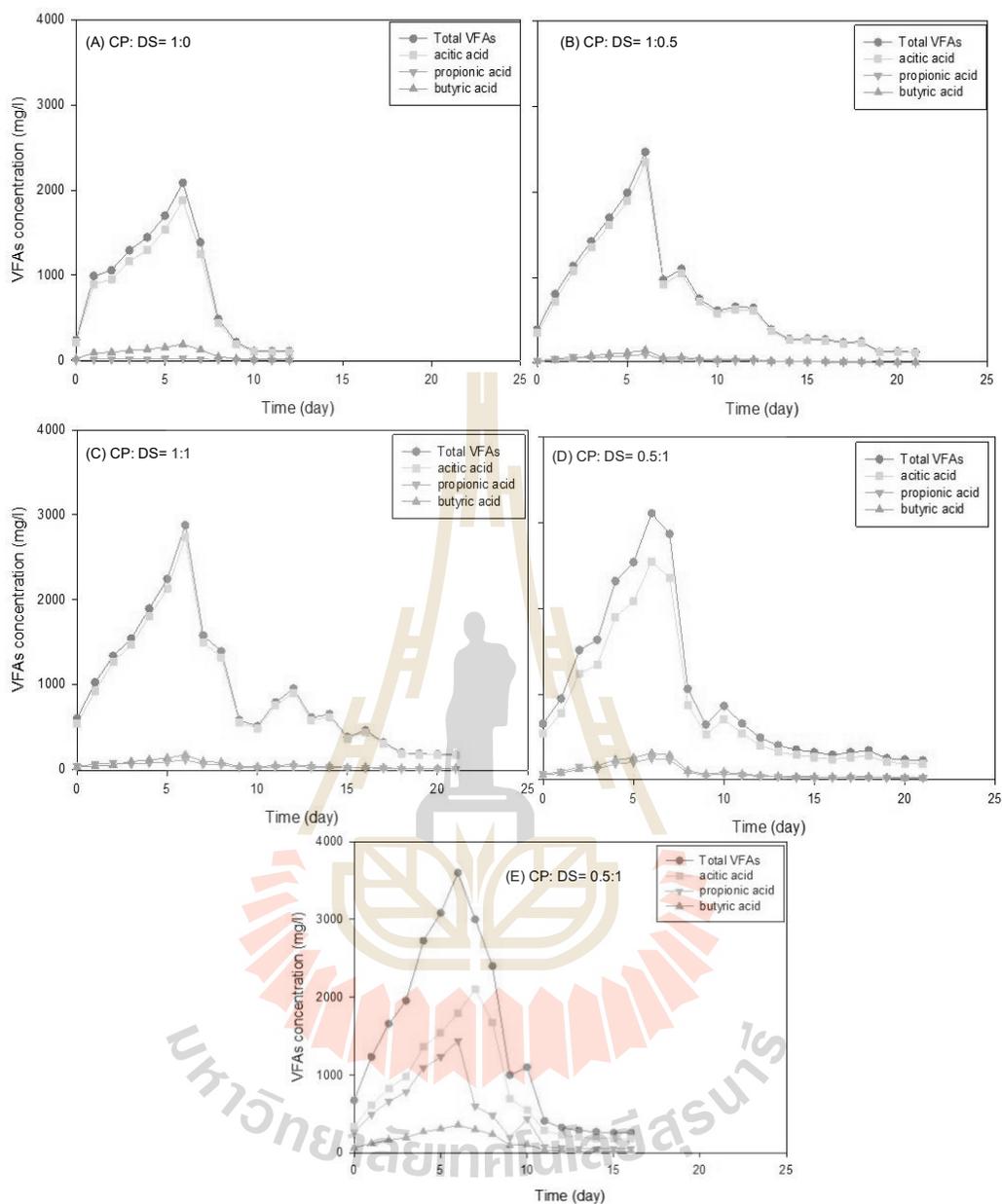
Figure 4.10   VFAs of CP with DS at different ratio of 1:0 (A), 1:0.5 (B), 1:1 (C), 0.5:1 (D), 0:1 (E).

The percentage of VS removal was showed in Table 4.2. The VS removal rate was superior with the co-digestion. The highest VS removal rate of co-digestion (80.2%) at was the 1:1 ratio of CP/DS, followed by the 0.5:1 (77.4%), and 1:0.5

(70.6%), whilst the VS removal rate of mono-digestion was 62.8% and 53.4% at ratio 0:1 and 1:0, respectively. Moreover, the change in methane content is significantly correlated with the VS removal rate. As the VS removal rate increased, the methane content also increased. This consequence was due to the effective utilization rate of organic matter contributed to the methane yield.

Table 4.2 Ammonia concentration and VS removal rate of different CP: DS ratios.

Parameters	CP/DS ratio (base on VS)				
	1:0	1:0.5	1:1	0.5:1	0:1
NH_4-N_{in} (mg/L)	334.43	349.72	360.86	357.33	345.01
NH_4-N_{out} (mg/L)	503.15	495.34	457.26	476.53	416.72
VS removal (%)	53.40	70.60	80.20	77.40	69.80
C/N ratio	118.68	38.84	29.18	23.42	16.86

4.2.7 Microbial analysis

4.2.7.1 Bacterial and archaeal community shifts

DGGE fingerprinting and sequence analysis were conducted to examine the variations in bacterial and archaeal community structures in different trials (Kim and Lee, 2016). The bacterial and archaeal DGGE profiles of the community DNA samples were presented in Fig. 4.11-4.12 Twenty-two bacterial sequences (B1-B22) and nineteen archaeal sequences (A1-A12, A15-A21) were retrieved from the gel, and their thenby sequences and the affiliations were determined by comparison with the GenBank database (Table 4.3).

The dominant populations were represented by B21 (*Lachnospiraceae*), B7 *(Nitrospiraceae)* and B19 (*Prevotellaceae paludivivens*).

B21 was one dominant band which closely related to *Lachnospiraceae* with 99% sequence similarity (Table 4.3). *Lachnospiraceae* is a spore-forming anaerobe which ferments polysaccharides to short-chain fatty acids such as acetate and propionate as fermentation products (Biddle et al., 2013).

Bands B7 and B16 were related to *Nitrospiraceae*. *Nitrospiraceae* (Westerholm et al., 2016) is syntrophic acetate-oxidising bacteria in cooperation with hydrogentrophic methanogen.

B20, and B22 were related to uncultured bacterium with 89% and 94% sequence similarity, respectively (Table 4.3).

The bands intensity of B2, B11, B12, B17, B20, and B22 were increased during the early batch period (3-12 days). The high intensity of bands were observed on 12 days but then gradually decreased after 6 days. This bands belonged to the *Firmicutes* with 99%, 100%, 97%, 88%, 89% and 94% sequence similarity, respectively (Table 4.3). Bands B1 and B14 were both related to *Bacteroideles* with 96% and 99% sequence similarity, respectively (Table 4.3). Similarly with *Firmicutes* band, the high intensity of *Bacteroideles* was also increased during first 12 day, and then gradually reduced. *Bacteroidetes* and *Firmicutes* were widely observed as acid-forming bacteria that degrade cellulose and protein (Whitehead et al., 2005; Ntaikou et al., 2009).

Bands B4, B5, B15 were assigned to *Dehalococoides* sp. with 90%, 91% and 88%, sequence similarity (Table 4.3). The important role of *Dehalococoides* sp. was also reported for degradation of both polysaccharides and monosaccharides as well as the production of acetic acid (Yamada et al., 2005). In this

study, the high detection of these bands were observed during first 6 days. However, therirs bands detection were declined next 6 days.

The sequence similarity of bands B8 and B9 were related to *Atopobium sp.* with 100% and 90% respectively (Table 4.3).

For bands B13, it sequence similarity was related to *Actinomyces* sp. with 99% (Table 4.3).

B18 was related to *Allochomatium viosum* with 91% sequence similarity (Table 4.3). However, its roles in anerobic digestion was relatively unclear.

B3 was costly related to *Alphaphaproteo bacterium* with 96% sequence similarity (Table 4.3).

The band intensity of B19 was observed during the early batch period of co-digestion and CP only. This band intensity was increased during 12 days then gradually decreased. B19 was *Prevotella paludivivens* with 99% sequence similarity (Table 4.3). The important role of *Prevotella paludivivens* have been reported that it could be utilized various saccharides including xylan, xylose, pectin and carboxymethylcellulose and produced acetate and succinate with small amounts of formate and malate (Ueki et al., 2007).

B6 was observed only at first 3 day of co-digestion and all period of digestion of mono-digestion (DS only). B6 was closely related to *Desulfobulbaceae* with 90% sequence similarity (Table 4.3). The role of *Desulfobulbaceae* is a sulfate- reducing bacteria which could oxidize propionate during reduction to produce acetate in the presence of sulfate (Widdel, 1998).

B10 was detected only in the mono-digestion of DS only. B10 sequence was closely (99%) matched with *Desulfotomaclum sp. Desulfotomaclum sp.*

is a sulfate- reducing bacteria which could degrade acetate to produce CO_2 and H_2O in the presence of sulfate (Zhao et al., 2008)

The significantly detection of mono-digestion was dispersed of bands. During 12 days of CP only, the bands (B1-B2, B8, B11-12, B14, B18, and B20-B22) were disappeared. However, during 12 days of DS only, the bands (B1-2, B4-5, B7-8, B11-12, and B16-17) were disappeared. Moreover, during 15 day of DS only, most of bands was disappeared except the bands of B18, B6, B9, and B10.

The archael DGGE profiles showed continuous shifts in archaeal community structure during the batch period (Fig. 4.12). Overall, the archaeal band patterns were less complicated than the bacterial results due to the relatively low diversity of the domain Archaea in most microbial complexes (Curtis and Sloan, 2004). A3, A8 and A9 were the dominant bands of methane production (Fig. 4.12). All of dominant bands were closely related to *Methanosarcina mazei* with 100% sequence similarity. The intensity of *Methanosarcina mazei* bands (A3, A8 and A9) were observed during the first 3 days and then were dropped on 6 days. However, the intensity of these co-digestion bands were increased during 12 days then gradually decreased til the end of digestion. Whilst, the intensity of mono-digestion were dropped on 12 days and 12-15 days for CP and DS only, respectively.

Six bands including A1, A6, A7, A10, A11 and A18 were closely related to *Methanosaeta concilii* (Table 4.3). A1, A6, A7, A10, A11 and A18 bands were appeared during the 15-21 days of co-digestion and DS only. Whilst, CP only were not appeared all of *Methanosaeta concilii* bands.

Four bands A2, A12, A19, and A21 were closely related to hydrogenotrophic methanogens (*Methanomicobiales*) (Table 4.3). These co-digestion

bands were observed at beginning fermentation and still remain until the end of experiments. Whilst, some bands of *Methanomicobiales* (A2, A21) were low intensity on 12 days and disappeared during 15 days of DS only

Two bands (A17 and A4) were also closely related to hydrogenotrophic methanogens (*Methanobacteriales*) (Table 4.3). In co-digestion, these bands were found since the first day till end of experiments. Whilst, the bands (A17 and A4) of DS only were low intensity during 12 day and then disappeared on 15 day.

Among these bands, A5, A15, A16, and A20 were related to *Euryarchaeote*. These bands were observed at beginning fermentation (Fig. 4.12).

Moreover, neighbor-joining trees were constructed to characterize the affiliation of these bacterial and archaeal band sequences to the database sequences (Fig. 4.13-4.14). The bacterial sequences were branched within seven phyla, *Alphaproteobacteria*, *Zetaproteobacteria*, *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Choloflexi*, *Nitrospirae* (Fig. 4.13). In the archaeal neighbor-joining tree, 15 out of 18 bands were assigned to methanogenic orders; three bands within *Methanosarcinales*, six bands within *Methanosaeta*, two bands within *Methanobacteriales* and four bands within *Methanomicobiales* (Fig. 4.14).

Table 4.3 Identification of the 16S rRNA gene sequences from DGGE bands.

Bands	Nearest sequence	Accesion No	% Similarity
B7, B16	Uncultured *Nitrospiraceae bacterium*	KJ127962.1	100%, 99%
B1, B14	Uncultured *Bacteroidales bacterium* HQ2	EU573859.1	96%, 99%
B19	*Prevotellaceae bacterium* WR041	AB298732.2	99%

Table 4.3 Identification of the 16S rRNA gene sequences from DGGE bands (Continued).

Bands	Nearest sequence	Accesion No	% Similarity
B13,	*Actinomyces* sp.	HM099646.1	99%
B8, B9	*Atopobium* sp.	LT625115.1	100%, 90%
B4-B5, B15	Uncultured *Dehalococcoides* sp.	LC000772.1	90%, 91%, 88%
B6	Uncultured *Desulfobulbaceae*	MH252190.1	90%
B18	*Allochromatium vinosum*	NR_074584.1	91%
B3	Uncultured *Alphaproteobacteria bacterium*	CU926303.1	96%
B11	Uncultured *Fimicutes bacterium*	AB780889.1	100%
B21	Uncultured *Lachnospiraceae bacterium*	LT625851.1	99%
B20	Uncultured *bacterium*	MF246420.1	89%
B22	Uncultured *bacterium*	MF268800.1	94%
B10	Uncultured *Desulfotomaculum* sp.	MG803340.1	99%
B17, B12	Uncultured *Fimicutes bacterium*	CU921543.1	88%, 97%
B2	Uncultured *Fimicutes bacterium*	CU925649.1	99%

Table 4.3 Identification of the 16S rRNA gene sequences from DGGE bands (Continued).

Bands	Nearest sequence	Accesion No	% Similarity
A1, A6, A7,A10, A11, A18	*Methanosaeta concilii* X16932	KM408635.1	100%, 100%, 100%, 100%, 97%, 100%
A3, A9, A8	*Methanosarcina mazei*	AY196685.1	100%, 100%, 100%
A12	Uncultured *Methanomicrobiales archaeon* QEDF1A031	KF198685.1	100%
A2, A19, A21	Uncultured *Methanolinea* sp. SMS-sludge-8	AB479399.1	100%, 99%, 99%
A17	*Methanobacterium beijingense* M4	EU544027.1	99%
A4	*Methanobacterium lacus* 17A1	NR_117917.1	99%
A16, A 15, A20	Uncultured *euryarchaeote* SMS-sludge-6	AB479397.1	100%
A5	Uncultured *archaeon*	GU881711.1	99%
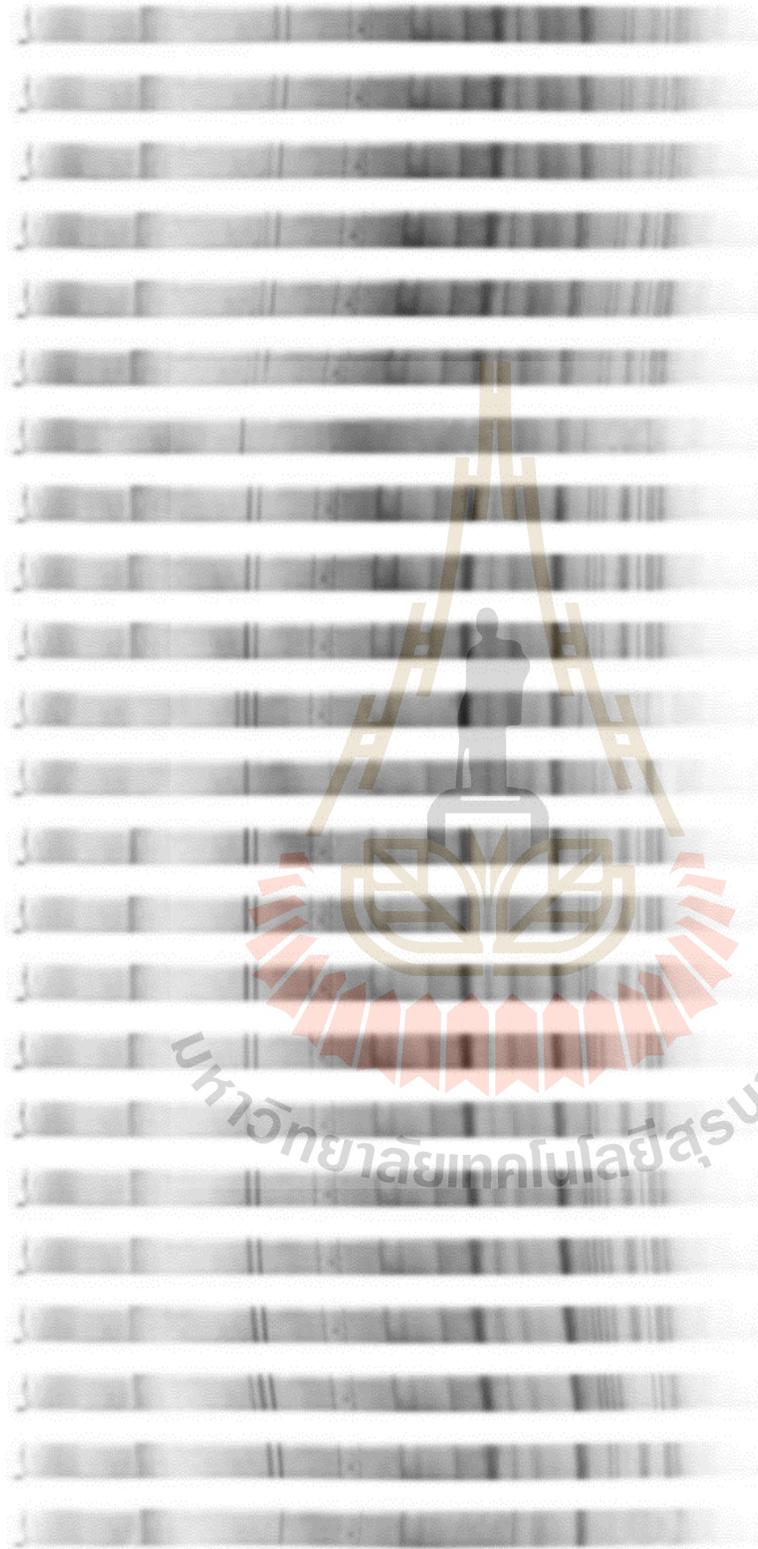
Figure 4.11   DGGE profiles of bacterial 16S rRNA gene fragments of different CP: DS ratios.
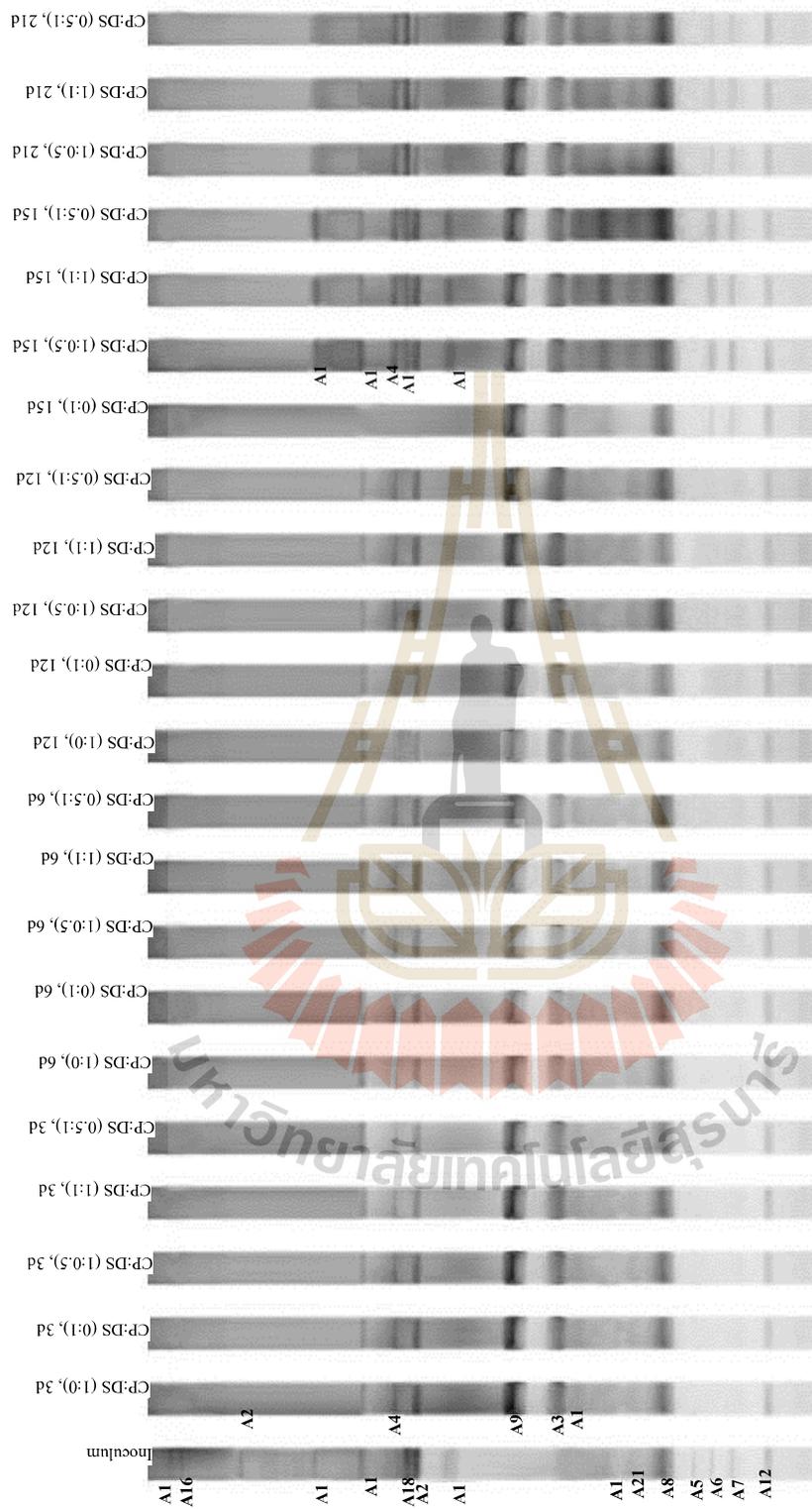
Figure 4.12 DGGE profiles of archaeal 16S rRNA gene fragments of different CP:DS ratios.
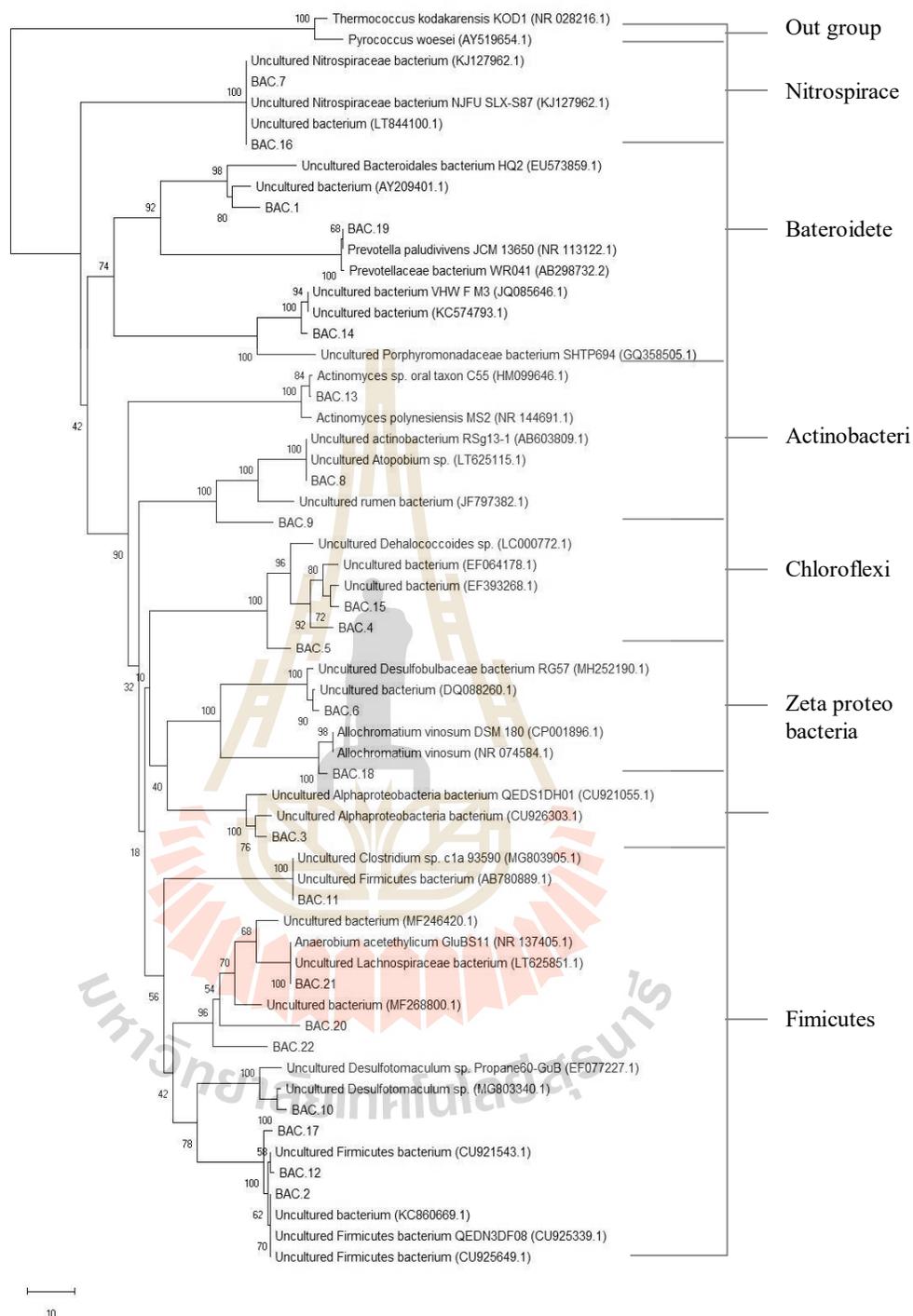
Figure 4.13 Neighbor-joining tree illustrating the phylogenetic identities of the 16S rRNA gene sequences from bacterial DGGE bands.

Figure 4.14   Neighbor-joining tree illustrating the phylogenetic identities of the archaeal 16S rRNA gene sequences from DGGE bands.

4.2.7.2 Shannon index (H´) calculated from DGGE banding patterns of bacterial and archaeal

The major bacterial 16s rRNA gene sequences were similar to several ribotypes in the Chloroflexi, Bacteroidetes, *and* Firmicutes phylum. This finding corresponds to that observed in earlier studies, in which a high abundance of this microbial phylum was found in a stable anaerobic digestion used to ferment various crop and materials (Ren et al., 2014; Zheng et al., 2015; Zhao et al., 2014). This phylum was widely observed as an acid-forming bacteria. Therefore, in this study, pH during the first 6 days of all different CP: DS ratios was dropped. The dropping of pH was significantly with the increased of bands intensity B4, B5, and B15 during 3- 6 days (Fig 4.8) leads to increasing of increased of Chloroflexi phylum as present in Fig 4.15c. The highest Shannon index (H´) value of Chloroflexi phylum was observed during 6 days with 2.91, 3.43, 3.54, 3.46 and 2.63 at CP:DS ratios of 1:0, 1:0.5, 1:1, 0.5:1, and 0:1, respectively. As kown that Chloroflexi phylum (*Dehalococoides* sp.) played an important role in digestion carbohydrate to produce acetate and other short chain fatty acids (Yamada et al., 2005). This result confirmed that one peak of methane production yield during first 6 days shown one peak of carbohydrate digestion. Two peak of digestion was shown significantly with Bacteroidetes and Firmicutes phylum for cellulose and protein digestion (Zhao et al.;2014; Wang Y et al.; 2012). The both Bacteroidetes and Firmicutes phylum was observed during first day of digestion. However, the highest detection of both were 12 days of co-digestion with 1.83 and 2.16, 3.26 and 3.16, 3.38 and 3.32, 3.40 and 3.42, 3.39 and1.88 and 1.58 (Fig 4.15a and b). Compared with the mono-digestion, Bacteroidetes and Firmicutes phylum was decreased due to lack of nitrogen to

maintain bacteria cell of the digestion in CP only and the accumulation of the toxic of metabolic product in DS only.

One of such final product of sulfate-reducing bacteria metabolism is hydrogen sulfide produced in the process of dissimilatory sulfate reduction (Kushkevych, 2016; Kushkevych, 2013) It is known that hydrogen sulfide is toxic for living organisms and can inhibit the enzymes of different groups of microorganisms (Kushkevych, 2016; Kushkevych, 2013). In this study, the sulfate-reducing bacteria (*Desulfobulbaceae* and *Desulfotomaculum*) was found only in 3 days of co-digestion and all of experiment of DS only (Fig.4.11). The reasons of the short period of these bacteria for co-digestion was since the feed sulfate levels were low enough to discourage the proliferation of the sulfate-reducing bacteria (Griffin et al., 1998). The low value of detection of the sulfate-reducing bacteria during 6-8 days was observed the positive result of the increasing of three pack of methane production yield. Since, the sulfate-reducing bacteria could degreased propionate to produce acetate as a substrate of *Methanosarcina mazei*. The high detection of the sulfate-reducing bacteria during 12-15 days were failed in digestion. Due to sulfate-reducing bacteria produce hydrogen sulfide which can toxic for methanogen and fermentative bacteria. Resulting in, the dispersed of most of bacteria (Fig 4.11and 4.15) and methanogenic (Fig 4.12 and 4.16) in case of low methane production yield in DS only.

Acetate is often regarded as the major (70%) methanogenic precursor (Speece, 1996) and can be utilized directly by methanogens. In this study, *Methanosarcinales* was the most abundant methanogenic group. Accordingly, the acetate-utilizing *Methanosarcinales* has been reported to be the dominant methanogenic group in previous studies (Kobayashi et al., 2008; Yu et al., 2005a). At

3 days in this system (Fig 4.12 and 4.16), *Methanosarcuna mazei* (*Methanosarcinales*) A3, A9, and A8 was the only aceticlastic methanogen visualized in the DGGE profile. *Methanosarcuna mazei* was able to only utilize acetate for growth and is widely distributed in nature due to its high affinity to acetate (Smith and Ingram-Smith, 2007). The 16S rRNA gene concentration of *Methanosarcinales* of all different CP:DS ratios was increased on first 3 days when the acetate concentration was increased (Fig 4.16a). The highest H′ index values of co-digestion of *Methanosarcinales* were 2.46, 2.52, and 2.49.of 0.5:1, 1:1, and 0:1 during 12 days. While, the highest H′ index value of mono-digestion of *Methanosarcinales* were declined during 12 days (Fig 4.16a). The reasons of the low of *Methanosarcinales* of mono-digestion were the lack of nitrogen in CP only and some toxic in DS only. However, *Methanosarcinales* was decreased when the acetate concentration was decreased after 12 day, resulting in the *Metanosaeta* remained presently during 15 days due to it likely enviromaent that low acetate concentration (Demirel and Scherer, 2008)

Moreover, Hydrogenotrophic methanogenesis is one of the major methanogenic pathways in anaerobic digesters (Angelidaki and Sanders, 2004). Due to the thermodynamic limitations of the hydrogen-mediated metabolism, low hydrogen partial pressure must be maintained for syntrophic consortia to utilize various intermediates (Batstone et al.,2002). Therefore, efficient removal of hydrogen by hydrogen-utilizing microorganisms is required for acidogenesis and/or acetogenesis to occur. In this study, hydrogen-utilizing *Methanobacteriales* was stable increased during the early reaction period and still remained utill the end of co-digestion (Fig. 4.16d). The *Methanobacteriales* of DS only was deceased and dispersed during 12-15 days with the reason of previous. *Methanomicobiales* of co-digestion was observed

sine the first day till the end of experiments. Whilst, some bands of *Methanomicobiales* (such as A2, A21) were low intensity on 12 days and disappeared during 15 days of DS only (Fig4.16c).with also the reason of previous.
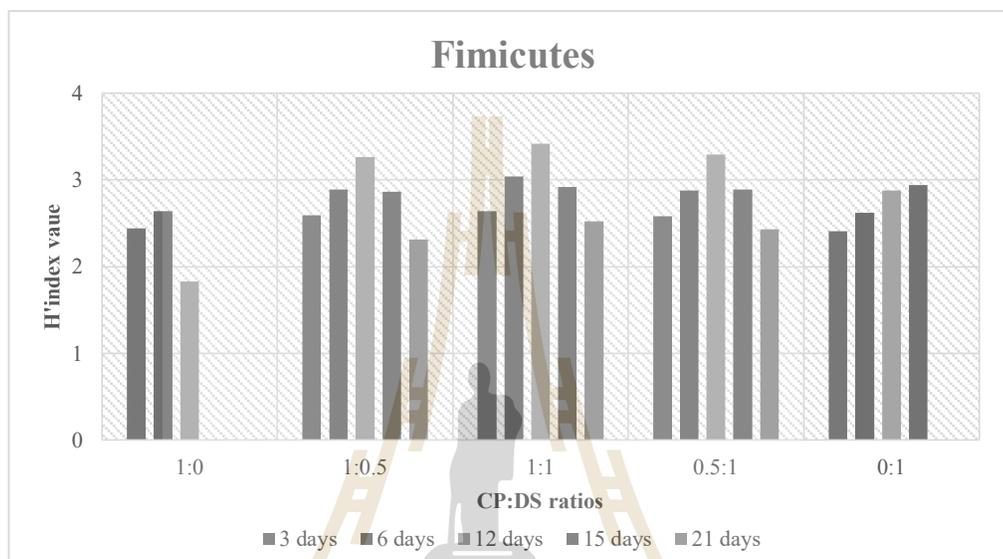
Figure 4.15a   H´ index value of bacteria (*Fimicutes*) from DGGE bands.
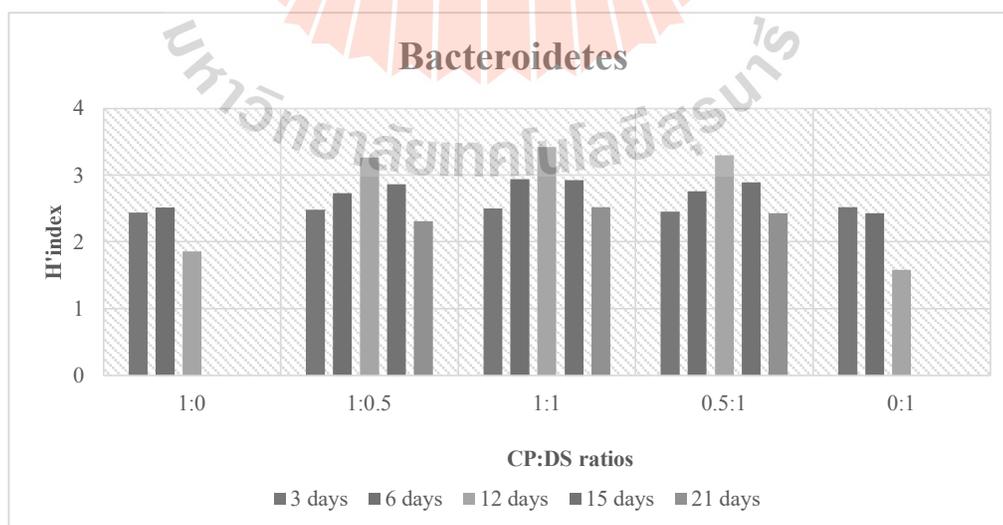
Figure 4.15b   H´ index value of bacteria (*Bacteroidetes*) from DGGE bands.
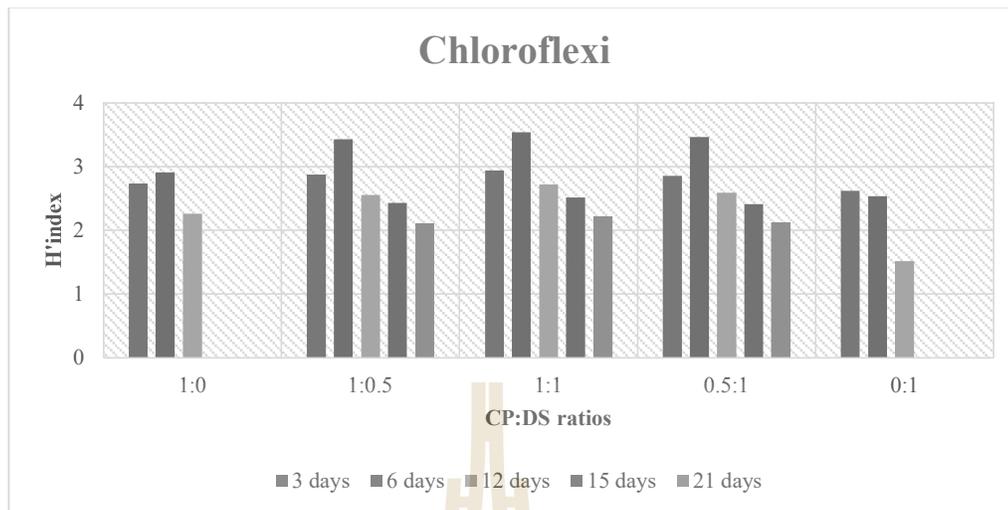
Figure 4.15c H′ index value of bacteria (*Chloroflexi*) from DGGE bands.
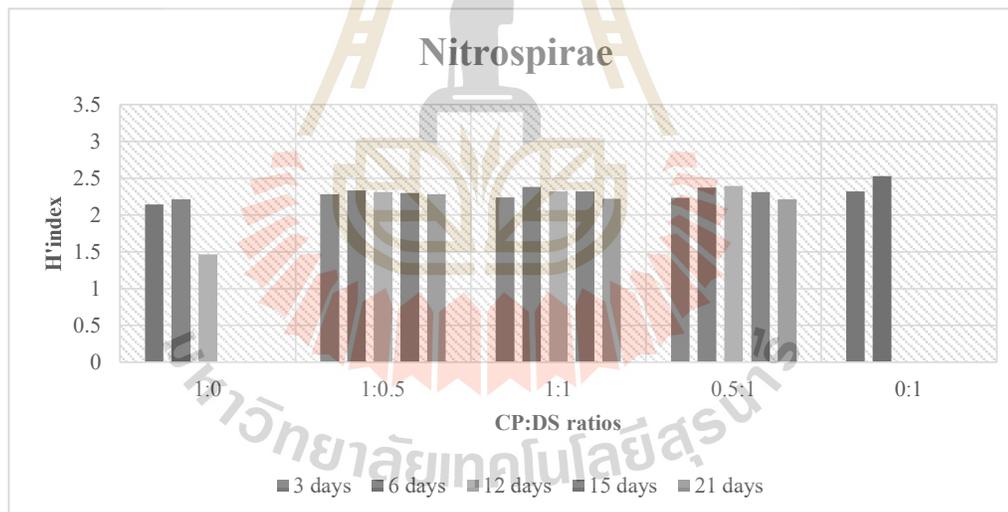
Figure 4.15d H′ index value of bacteria (*Nitrospirae*) from DGGE bands.
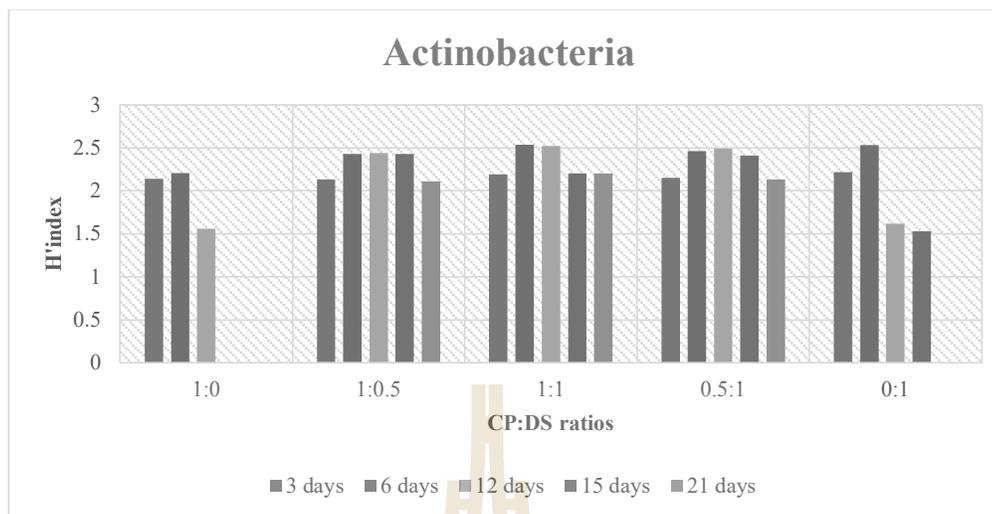
Figure 4.15e   H′ index value of bacteria (*Actinobacteria*) from DGGE bands.
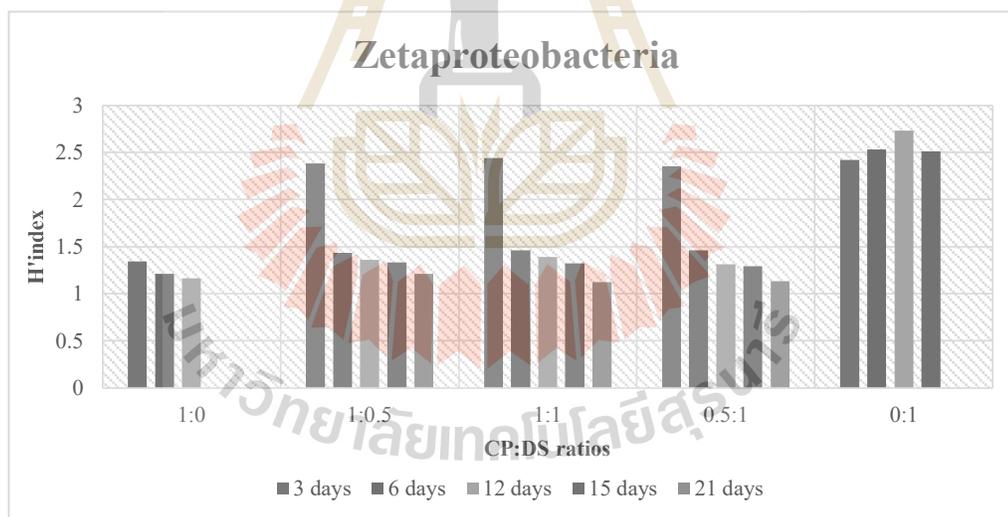
Figure 4.15f   H′ index value of bacteria (*Zetaproteobacteria*) from DGGE bands.
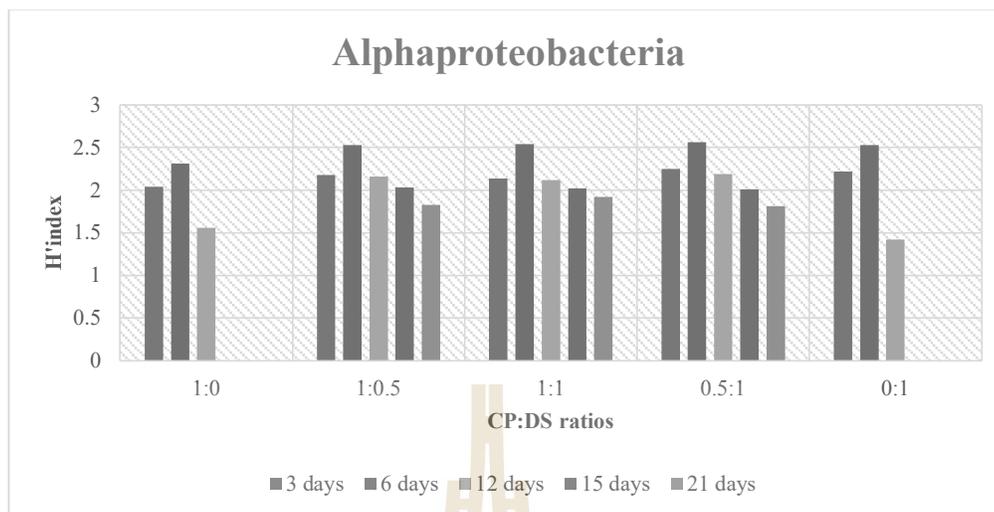
Figure 4.15g H′ index value of bacteria (*Alphaproteobacteria*) from DGGE bands.
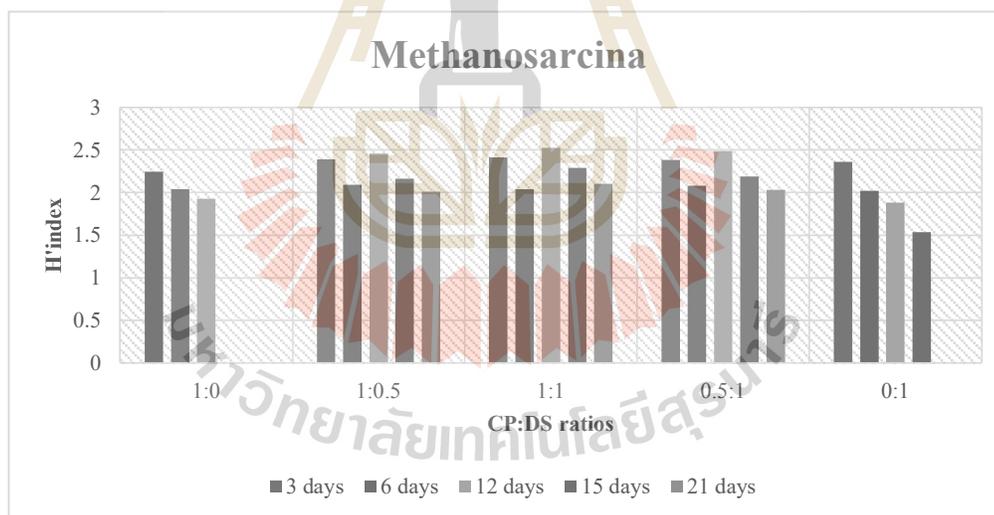
Figure 4.16a H′ index value of archaea (*Methanosarcina*) from DGGE bands.
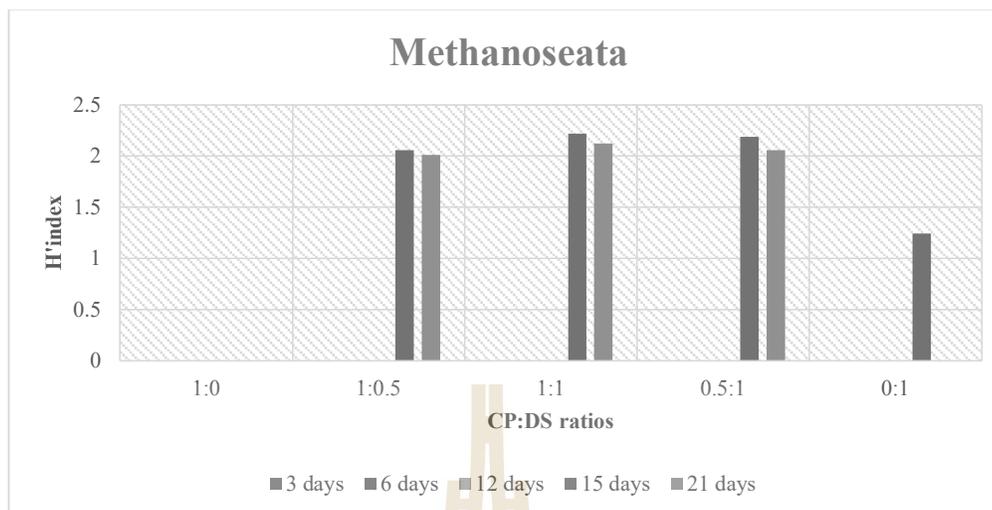
Figure 4.16b   H′ index value of archaea (*Methanoseata*) from DGGE bands.
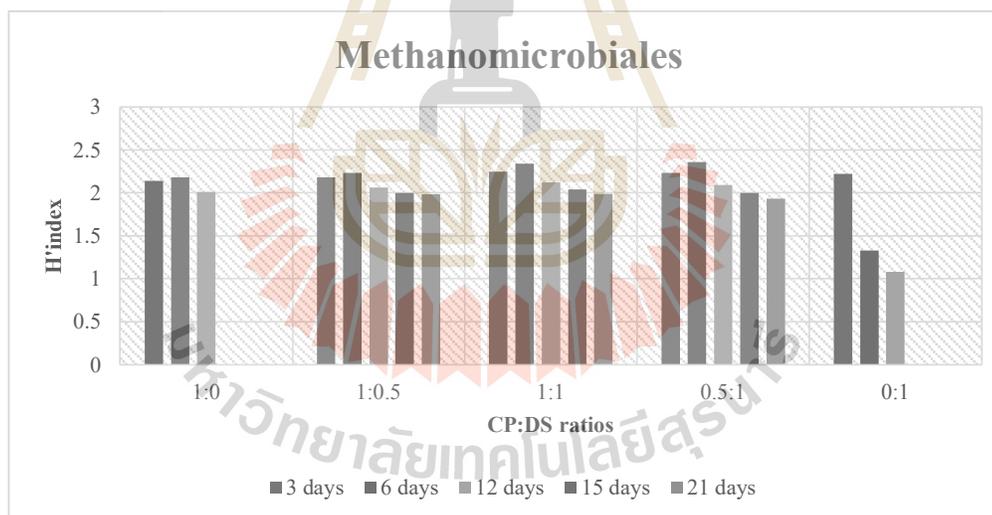
Figure 4.16c   H′ index value of archaea (*Methanomicrobiales*) from DGGE bands.
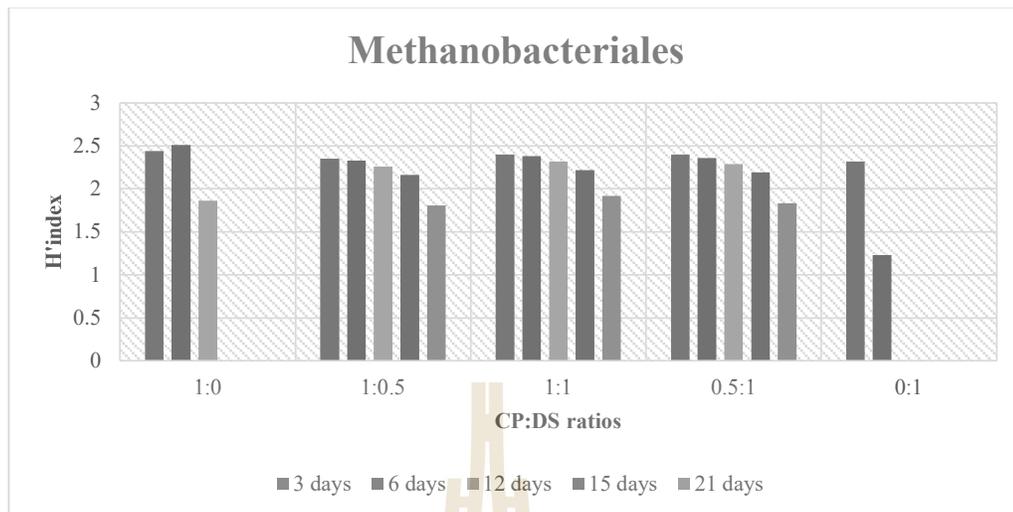
Figure 4.16d H′ index value of archaea (*Methanobacterales*) from DGGE bands**.**

CHAPTER V

CONCLUSION

In Thailand cassava is mostly used to produce starch and methanol. About 0.33 ton of cassava pulp/1 ton cassava root was generated by this process. Cassava pulp has become a major problem of solid waste and ground water. Anaerobic digestion (AD) is an attractive waste treatment option for CP. However, biogas production from cassava pulp is very low nitrogen content resulting in low biogas and methane production yields. Therefore, the fermentation between distillery stillage as co-digestion with cassava pulp at five different CP:DS ratios of 1:0, 1:0.5, 1:1, 0.5:1 and 0:1 was evaluated the examining operation stability, biogas and methane production potentials in term of S:I ratio, biogas and methane production yield, pH, volatile fatty acid (VFA) content, ammonia content, volatile solid removal rate (VS removal rate) and the effect of *Bacteria* and *Archaea* community structure under mesophilic condition.

The substrate: inoculum ratios is important for biogas and methane operation. The effect of several S:I ratios such as 1:1, 1:2 and 2:1 was measured in this study. The optimal S:I ratio was obtained at 1:1 with high biogas and methane production yields and stability of process (pH). Therefore, the S:I ratio of 1:1 was recommended for the study of CP:DS ratios.

All pH and VFAs of all different CP: DS ratios weren't in a rage of operation in inhibition due to high buffer capacity of inoculum leads to reducing VFAs

accumulation. Resulting in, all experiments were maintained as the optimal pH. The average of ammonia (NH_4-N) concentration of different 334.43-503.15 mg/L. These concentration was acceptable within the limitation (1500 mg/L). CP: DS ratios was varied from The co-digestion of CP and DS is a promising approach for increasing the cumulative biogas and methane yield by 64.20%-146.54% and 65.57%-222.19% respectively compared to the mono-digestion of CP. The highest VS removal rate (80.20%), cumulative biogas yield (981.73 mL/g VS) and methane yield (685.10 mL/g VS) were obtained at 1:1 mixing ratio of CP:DS.

The effect of *Bacteria* and *Archaea* community structure was corresponding with result of methane production. The bacterial and archaeal communities were analized by denaturing gradient gel electrophoresis (DGGE). The bacterial community of CP:DS ratio was dominated by Bacteroidetes, Firmicutes and Chloroflexi phylum. The methanogenic archaeal community of CP: DS ratio was dominated by the genus *Methanosarcina (Methanosarcina barkeri)* belonging to the family *Methanosarcinaceae.* The genus *Metanosaeta, Methanomicrobiales, Methanobacteriales* were minor represented in the archaeal community. The *Methanosarcina* was sharply presented in co-digestion especially in CP:DS ratio of 1:1. The low biogas and methane yield of mono-digestion (CP only) are low nitrogen to maintain *Bacteria* and *Archaea* cell resulting in dropping H′ index value during 12 days. Moreover, the sulfate-reducing bacteria was observed in DS only. It produced hydrogen sulfide which is toxic for methanogen and fermentative bacteria. As a result, the band of most of bacteria and methanogenic was dispersed during 12-15 days cause of low methane production yield.

Therefore, it was concluded that a high stability of the anaerobic digestion was the result of adaptation of co-digestion (CP and DS).

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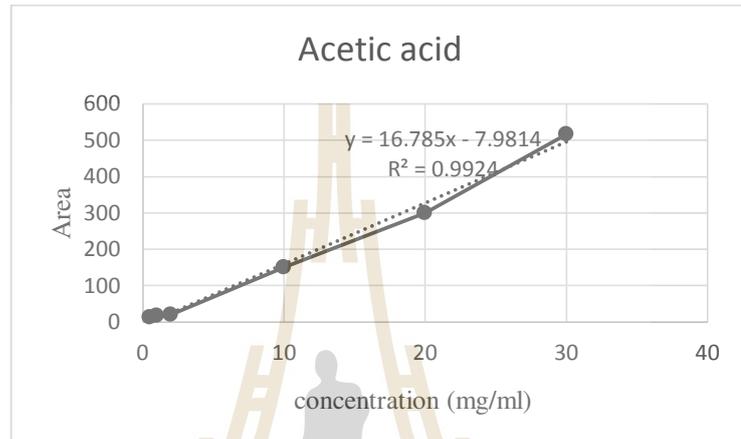
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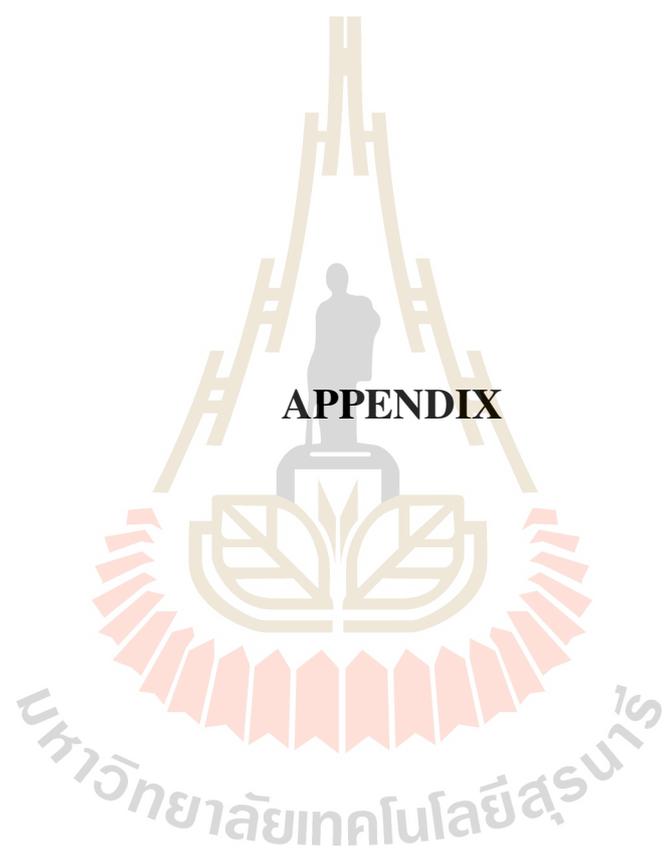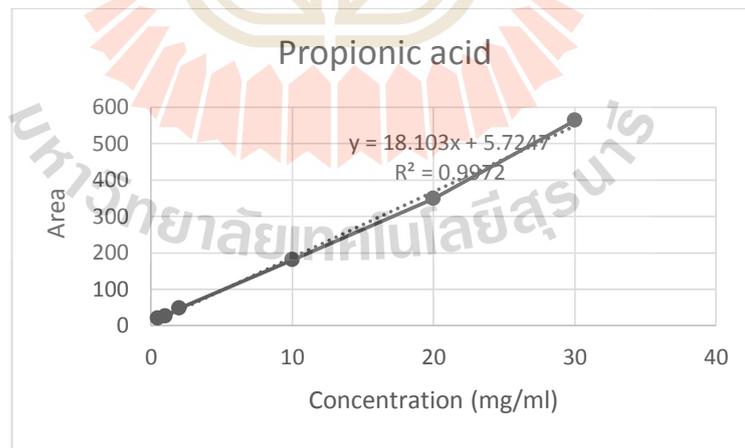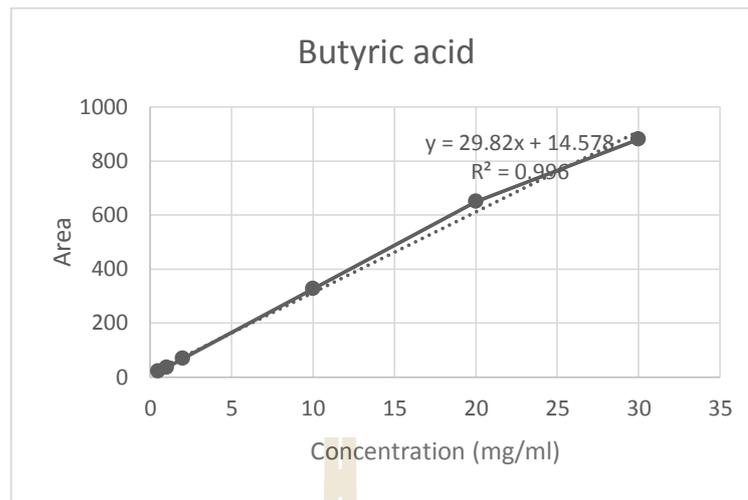
APPENDIX

APPENDIX

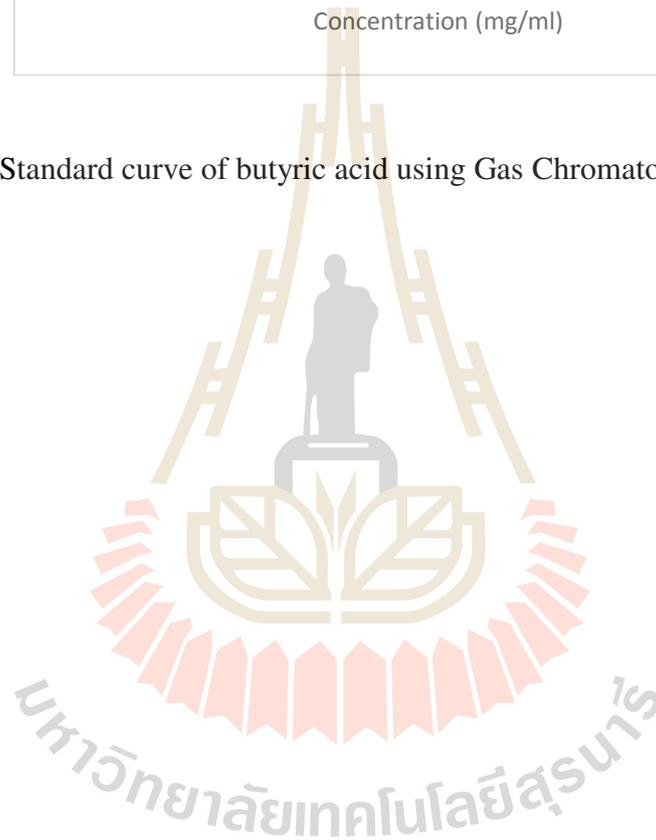## STANDARD VFAs ANALYSIS CURVE

Figure 1A   Standard curve of Acetic acid using Gas Chromatography (GC).

Figure 2A   Standard curve of propionic acid using Gas Chromatography (GC).

Figure 3A   Standard curve of butyric acid using Gas Chromatography (GC).

BIOGRAPHY

Ms. Chonradee witchayapong was born in Chiang Mai, Thailand, in 1993. She received the bachelor's degree in environmental engineering from the University of Ubon Ratchathani, Ubon Ratchathani, Thailand. After graduation, she decided to further study for master degree in the field of Bioenergy at school of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakorn Ratchasima, Thailand. She researches topic was the study for increasing of methane content in biogas produced from cassava pulp. The results from some part of thesis have been presented in International Conference on Bioenergy and Clean Energy (ICBCE 2018), Hong Kong.