CHAPTER 2

Literature review

2.1 Anaerobic digestion

Anaerobic digestion is the biological process used for treating wastewater under anaerobic condition. It has been used after 19th century in rural China and India. Simple reactor constructions have long been used to treat manure and agricultural wastes with the main purpose of recovering energy for cooking and lighting (Gijzen, 2002). Anaerobic digestion in large scale plants using more advanced technology was introduced in the 1860s in France (McCarty, 2001). Until the 1970s, the anaerobic digestion was more attention in term of both research and technology development (Levén, 2006). The main product of this process is biogas which consists of 50-75% methane (CH_4), 25-45% carbon dioxide (CO_2) and small volume of hydrogen (H_2) 0-1%, nitrogen (N_2) 0-2% and hydrogen sulfide (H_2S) 0-1%. Methane is energy-rich component in biogas and can be used as renewable energy respond to growing demand of energy. Biogas can be used directly in electricity and heat production and also in various technological processes e.g. production of methanol. Moreover, biogas can be also purified and upgraded for use as vehicle fuel (Ziemiński and Frąc, 2012). Most biogas is produced during the middle of digestion, after bacterial population has grown (Kangle *et al*., 2012).

Anaerobic digestion has several advantages compared with aerobic processes, including biogas production and high-strength wastewater treatment (Lettinga, 1995). Anaerobic system has a higher loading rate of 5-20 kg COD/m^3/d whereas aerobic system is about 0.5-3.0 kg COD/m^3/d. Due to its high loading rate, anaerobic system operation user smaller reactor volume that lead to lower installation cost (Lema and Omill, 2001). Another advantage of anaerobic digestion is low sludge production, and biogas residue can be used as fertilizer in agricultural land which contributes to recycling of nutrient and reduce the use of synthetic fertilizer. Anaerobic digestion has

also been reported to remove xenobiotics such as phenolic compounds, benzene, toluene and chlorinated hydrocarbon including pathogens in wastewater depending on temperature (Bitton, 2011). However, anaerobic digestion has some disadvantages such as long start-up period, high sensitivity of methanogenic bacteria to change in environment conditions and many chemical compounds which easily result in a system failure. In addition, sulfurous compound can lead to unpleasant odor. Anaerobic technology in middle and low income countries is still relatively new and under development for specific applications (Kangle *et al*., 2012). Nevertheless, anaerobic digestion is still interesting as methane production from this process is an useful source of renewable energy.

2.2 Microbial consortium and microbiological process

The efficiency of anaerobic digestion to biogas depends on combinations of metabolic activity of different microbial groups (Figure 2.1). Complete insight about the microbial community in anaerobic digestion process is essential to make the effective control of the reactor performance. Microbial consortium has complex nutritional requirement and specialized role in a reactor. These different microbial groups work in sequence, the metabolic products of one microbial group are assimilated by another microbial group. The consecutive stages consist of four main steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Figure 2.2) whereas, microorganisms involved in each stage of anaerobic digestion namely hydrolytic, acidogenic, acetogenic and methanogenic bacteria, respectively (Gerardi, 2003).

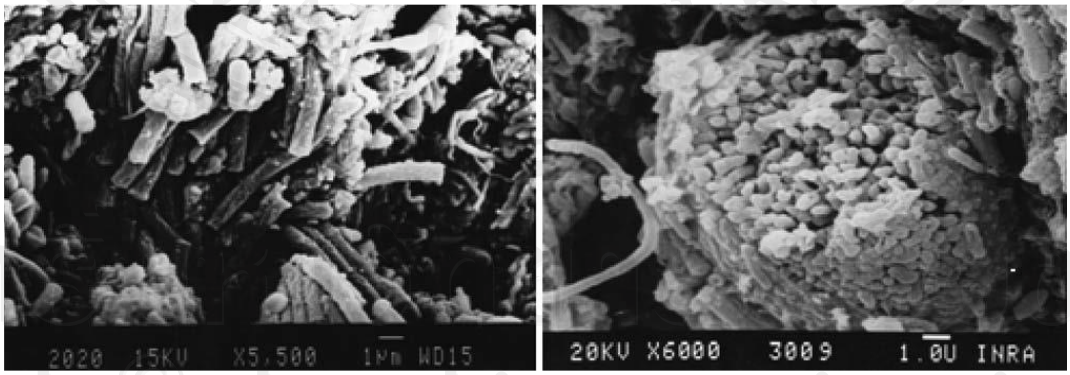

Figure 2.1 Electron micrographs of microbial communities in anaerobic methanogenic granules (Čater *et al*., 2013).

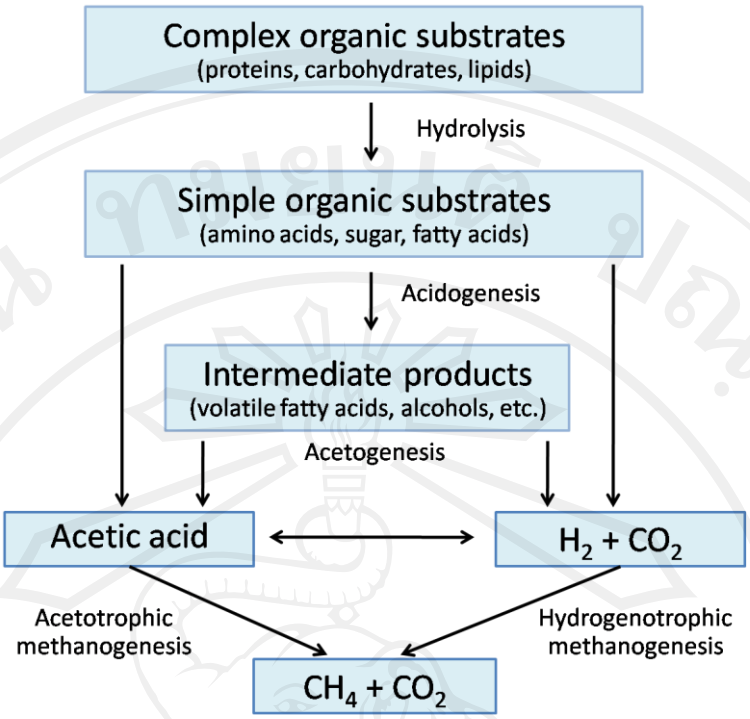

Figure 2.2 Schematic overview of four main steps in the anaerobic digestion process (adapted from Schnürer and Jarvis, 2010).

2.2.1 Hydrolytic bacteria

Hydrolytic bacteria play an important role in hydrolysis which is the first essential step in anaerobic degradation since complex organic compounds cannot be directly utilized by acetogenic and methanogenic bacteria. Hydrolytic bacteria break down complex organic compounds (carbohydrate, protein and lipid) into simple organic compounds such as sugar, amino acid and fatty acid by extracellular enzymes such as protease, lipase, cellulose, pectinase, amylase and chitinase. Major component in reactor depends on the prevalence of substrates in the digester feed. After that, the simple organic compounds are entered into bacterial cells through cell membrane and degraded (Gerardi, 2003). Some hydrolytic bacteria found in anaerobic reactor have ability to break down more than one substrate such as proteolytic bacteria can also brake down carbohydrate (Stronach *et al.*, 1986). In general, lipid shows slower conversion rates than carbohydrate and protein. However, the hydrolysis of polysaccharide, particularly cellulose, occur slowly and is considered to be late limiting step of the anaerobic digestion (Esposito *et al.*, 2012; Hassan and Nelson, 2012). Hydrolytic bacteria in anaerobic reactor are about 10^8-10^9 CFU/ml comprising both facultative and obligate anaerobes.

Typical hydrolytic bacteria consists of members of phylum *Bacteroidetes*, *Fermicutes* and *Proteobacteria* (Wang *et al*., 2010). Member of the phylum *Choroflexus*, *Spirochaetes* and *Actinobacteria* are also found (Klocke *et al*., 2007, Li *et al*., 2010; Bengelsdorf *et al*., 2013). Table 2.1 lists several hydrolytic bacteria that have been detected in anaerobic reactors.

Table 2.1 Examples of hydrolytic bacteria in anaerobic reactors

Substrates	Microorganisms	References
Protein	*Clostridium, Bacteroides, Bacillus, Proteus, Peptococcus, Vibrio, Butyrivibrio, Fusobacterium, Selenomonas, Campylobacter, Streptococcus, Peptostreptococcus, Staphylococcus, Eubacterium, Sarcina, Propionibacterium, Desulfovibrio*	Stronach *et al*. (1986); Liu *et al*. (2009); Arsova *et al*. (2010); Hassan and Nelson (2012)
Cellulose	*Clostridium, Bacteroides, Pseudomonas, Butyrivibrio, Micromonospora, Eubacterium, Spirochaeta, Thermotoga, Fibrobacter, Ruminococcus, Micromonospora*	Cirne *et al*. (2007); Arsova *et al*. (2010); Insam *et al*. (2010)
Hemicellulose	*Clostridium, Bacteroides, Ruminococcus, Streptococcus*	Arsova *et al*. (2010)
Pectin	*Clostridium, Bacteroides, Streptococcus, Bacillus*	Stronach *et al*. (1986); Arsova *et al*. (2010)
Starch	*Clostridium, Bacteroides, Lactobacillus, Bacillus, Streptococcus, Selenomonas, Succinomonas*	Stronach *et al*. (1986); Arsova *et al*. (2010)
Lipid	*Clostridium, Micrococcus, Staphylococcus, Anaerovibrio*	Stronach *et al*. (1986); Arsova *et al*. (2010)

2.2.2 Acidogenenic bacteria

Acidogenic bacteria play a role in acidogenesis (fermentation). In an equilibrated system, most hydrolysis products are converted to acetate and some of organic substrates are converted to volatile fatty acids (VFAs) (e.g. formic, propionic, butyric or succinic acids), alcohols (e.g. ethanol, methanol), H_2 and CO_2, and new bacterial cells (Gerardi, 2003). Among the products of acidogenesis, ammonia (NH_3) and H_2S are derived from degradation of amino acids causing an intense unpleasant smell. Also, these can be inhibitor for anaerobic digestion (Kangle *et al*., 2012; Ziemiński ans Frąc, 2012). Products of acidogenesis vary with substrate type, microorganism and hydrogen partial pressure (Anderson *et al*., 2003; Schnürer and Jarvis, 2010). Acetate, CO_2 and H_2 are produced in low hydrogen partial pressure condition meanwhile propionic acid, butyric acid, lactic acid and ethanol were produced in high hydrogen partial pressure condition. Excessive activity of acidogenic bacteria can result in reactor failure due to accumulation of organic acids. Most bacteria in this group are strictly anaerobe. The facultative members also help to protect methanogenic bacteria by consuming traces of oxygen. The number of acidogens in anaerobic reactors is in the range of 10^6-10^8 CFU/ml (Anderson *et al*., 2003).

There are various acidogenic bacteria in an anaerobic reactor, including members of the genera: *Clostridium, Bacteroides, Ruminococcus, Butyribacterium, Propionibacterium, Eubacterium, Lactobacillus, Streptococcus, Pseudomonas, Flavobacterium, Fusobacterium, Leuconostoc, Klebsiella, Desulfobacter, Micrococcus, Bacillus* and *Escherichia* (Anderson *et al*., 2003; Hassan and Nelson, 2012).

2.2.3 Acetogenic bacteria

The main function of acetogenic bacteria in anaerobic digestion is the production of acetate, H_2 and CO_2 which are further used by methanogenic bacteria. Acetogenic bacteria are distinguished into two groups on the basis of their metabolism. The first group, the hydrogen-producing acetogens, produces acetate, H_2 and CO_2 from VFAs and alcohols. This group requires hydrogen pressure less than 10^{-4} atmosphere (Anderson *et al*., 2003). This is a syntrophic association between acetogenic bacteria and hydrogen consuming methanogens (hydrogenotrophic methanogen). So far, only a limited number of acetogenic bacteria have been isolated and identified namely *Syntrophomonas wolfei* and *Syntrophobacter woolinii* which oxidize butyrate and

propionate, respectively. The second group of acetogenic bacteria is homoacetogens which produce acetate from H_2 and CO_2. They compete with hydrogenetrophic methanogens for H_2. However, the numbers of the homoacetogens is rather lower than that of the methanogenic bacteria, about 10^5 CFU/ml, suggesting a relatively minor role. Homoacetogens are known in genera *Acetobacterium, Acetoanaerobium, Acetogenium, Butribacterium, Clostridium* and *Pelobacter* (Anderson *et al*., 2003; Kangle *et al*., 2012).

2.2.4 Methanogenic bacteria

Methanogenic bacteria (methanogens) are key organisms in the production of methane from acetate, H_2 and CO_2, and methyl groups in the last step of anaerobic digestion. They are classified to domain Archaea belonging to phylum *Euryarchaeota*. Archaea bacteria are differed from typical bacteria (Eubacteria) in properties such as cell walls of archaea lack muramic acid and cell membranes contain phytanyl ether lipids (Deublein and Steinhauser, 2011).

Methanogens have many coenzymes (F_{420}, F_{430}, methanopterin, methanofuran, HS-HTP and M) which are specific and unique for these microorganisms. For example, coenzyme M is used to reduce CO_2 to methane. The oxidized form of coenzyme F_{420} absorbs light at a wavelength of 420 nm and fluoresces blue-green. This fluorescence allows the rapid microscopic identification of the methanogens. The coenzyme F_{420} is important hydrogen carrier in methanogens. Methanogens are strictly anaerobe and grow slowly (doubling time 1-12 days) in wastewater in the rate limiting step of the anaerobic digestion process (Liu and Whitman, 2008). The methanogenic population in anaerobic reactors is around 10% of the total microorganisms (Garcia *et al*., 2000). The methanogens can divide into two groups: acetoclastic methanogen and hydrogenotrophic methanogen. Examples of methanogenic bacteria are listed in Table 2.2. Phylogenetic classification of methanogenic bacteria is presented in Figure 2.3.

1) Acetotrophic methanogen

Acetotrophic methanogen converts acetate into methane and CO_2. Acetate is the most important precursor for methane production and the accounted for up to 70% of the methane in reactors. This group comprises only two genera which are *Methanoseata (Methanothrix)* and *Methanosarcina*. These two genera have different

growth rate and differed in their ability to utilize acetate. *Methanosarcina* grows faster but cannot use acetate at low concentration. The lowest acetate concentration that *Methanosarcina* can utilize is ~20 mg/l compared to ~4 mg/l in *Methanoseata* (Schnürer and Jarvis, 2010). The presence of these methanogens is affected not only by the acetate concentration, but also by factors such a loading frequency and mixing (Liu and Whitman, 2008). In addition, *Methanosarcina* spp. can also use methanol, methylamine and sometimes H_2 and CO_2 as growth substrate (Anderson *et al*., 2003). The activity and efficiency of acetotrophic methanogens are important parameters in the process of anaerobic conversion of acetate (Ziemiński and Frąc, 2012).

2) Hydrogenotrophic methanogen

Hydrogenotrophic methanogens or hydrogen-utilizing methanogenic bacteria convert H_2 and CO_2 into methane, up to 30% of methane within anaerobic reactor produced by this group. However, many previous studies found that methane formation mostly resulted from conversion of H_2 and CO_2 rather than aceticlastic methanogenesis (Nettmann *et al*., 2008; Liu *et al*., 2009; Demirel, 2014). Order *Methanobacteriales*, *Methanomicrobiales* and *Methanococcales* belong to this group.

The predominance of acetotrophic or hydrogenotrophic methanogens seems to mainly relate to the levels of their substrates and their tolerance to a variety of inhibitors such as ammonium, H_2S or VFAs. However, these two types of methanogens must be coexisted to make the system stable (Lerm *et al*., 2012). Methanogenic communities in anaerobic reactors have been found to be more stable than bacterial communities (Akarsubasi *et al*., 2005; Wang *et al*., 2009). Their dynamic were related to main process parameters such as VFA, which is also used as indicators of process stability.

Table 2.2 Examples of methanogenic bacteria (Deublein and Steinhauser, 2011)

Methanogens	Morphology	Gram stain	Substartes
Order *Methanobacteriales*			
Genus *Methanobacterium*	long rod	positive	H_2+CO_2, formate
Methanobrevibacter	short rod	positive	H_2+CO_2, formate
Methanosphaera	cocci	positive	H_2, methanol
Methanothermobacter	rod	positive	H_2, formate
Order *Methanococcales*			
Genus *Methanococcus*	irregular cocci	negative	H_2+CO_2, formate
Order *Methanomicrobiales*			
Genus *Methanomicrobium*	short rod	negative	H_2+CO_2, formate
Methanolinea	cocci	negative	H_2+CO_2, formate
Methanoculleus	cocci	negative	H_2+CO_2, formate
Methanocorpusculum	irregular cocci	negative	H_2+CO_2, formate
Methanoregular	rod	negative	H_2+CO_2, formate
Methanospirillum	spirilla	negative	H_2+CO_2, formate
Order *Methanosarcinales*			
Genus *Methanosarcina*	irregular cocci in packets	positive	acetate, H_2+CO_2, methanol, methylamines
Methanoseata	long rod to filaments	negative	acetate
Methanococcoides	irregular cocci	negative	methanol, methylamines
Methanolobus	irregular cocci in aggregates	negative	methanol, methylamines

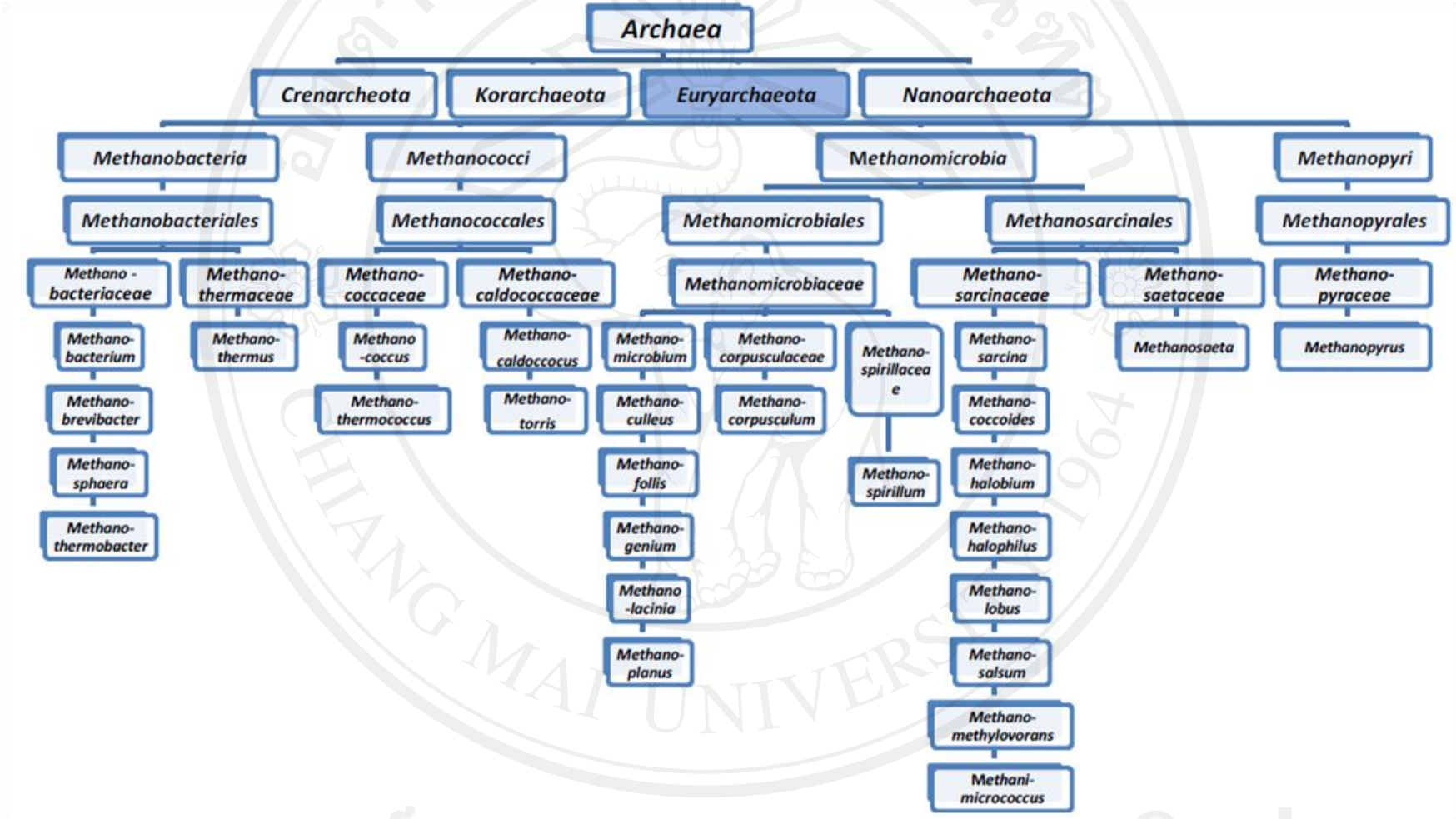

Figure 2.3 Phylogenetic classification of methanogenic bacteria (Ziemiński and Frąc, 2012).

2.3 Factors affecting anaerobic digestion

The operating parameters of the reactor must be controlled and monitored to enhance microbial activity and degradation efficiency of the system, and to avoid process failure. Factor affecting anaerobic digestion can be divided into two categories: environmental and operational factors.

2.3.1 Environmental factors

1) Temperature

Temperature is a major effect on the performance. It affects rate of biochemical and enzymatic reactions within cells. Therefore, a rising temperature leads to higher enzymatic activity, causing increased growth rate. Temperatures that exceed the optimal range can inhibit growth or even lethal, as protein and structural components of cell denatured and disfunctioned (Anderson *et al*., 2003). Anaerobic digestion can operate at various temperatures, including psycophilic (4-25 °C), mesophilic (25-40 °C) and thermophilic (50-60 °C). Normally, optimum temperature used in the anaerobic digestion are in mesophilic (35-37 °C) and thermophilic (50-55 °C) ranges (Gerardi, 2003; Schnürer and Jarvis, 2010). Research has shown that about 10% of the microorganisms in a mesophilic biogas process are actually thermophilic (Chen, 1983). Most commercial anaerobic reactors are operated at mesophilic or ambient temperatures (Chynoweth and Pullammanappallil, 1996). Methanogens are generally more sensitive to temperature fluctuation than other microorganisms in the process, particularly when change occurs rapidly (< 2 hours) (Anderson *et al*., 2003).

2) pH and alkilinity

pH is another important factor affecting growth of microorganisms. The best pH for anaerobic digestion is around neutral in a range of 7.0-7.2. Also, pH in a range of 6.8-7.2 is considered to be optimal. The pH values below 6 or above 8 may be harmful to bacteria, especially methanogens. Initially, pH in an anaerobic reactor will decrease with the production of VFAs. However, as methanogens consume the VFAs and alkalinity is produced, pH in reactor increases and then stable. Thus, the accumulation of VFAs due to high organic loading rate or presence of toxic material results in the decrease pH and methanogens growth inhibition (Gerardi, 2003). Generally, the concentration of VFAs, particularly acetate should be below 2,000 mg/l (Yadivika *et*

al., 2004). To avoid process failure and to get biogas yield, pH needs to be maintained within stable range by controlling the alkaline in the reactor. Most alkalinity is in form of bicarbonates which perform as a buffer in equilibrium with CO_2 to maintain neutral and stable pH. Decomposition of substrate with high protein and amino acid can increase alkalinity, because the ammonia released can react with CO_2 to form ammonium bicarbonate. Bicarbonate for stable processes usually varies in the range of 3,000-15,000 mg/l (Schnürer and Jarvis, 2010).

3) Nutrients and carbon to nitrogen ratio (C/N)

Nutrients can be classified into 2 types; macronutrients and micronutrients. Macronutrients are needed for all bacteria in large quantities such as carbon, nitrogen and phosphorus, while micronutrients refer to nutrients such as cobalt, iron, nickel and molybdenum that most bacteria need in small amounts (Gerardi, 2003). Some of the micronutrients such as tungsten, selenium and nickel are important in the enzyme systems of methanogenic bacteria (Stronach *et al*., 1986). Adequate balance of nutrients is needed for optimal growth of microorganisms and to achieve high biogas production. However, different nutrient source impacts on rate of anaerobic digestion and support different groups of microorganisms.

The carbon to nitrogen ratio (C/N ratio) is relative amount of carbon and nitrogen present in the substrate. Low C/N ratio substrates are human excreta, animal manure and sewage sludge. Agricultural residue, sawdust and wood have high C/N ratio. The ratio should not be too low or high. If the C/N ratio is low, high nitrogen leads to ammonia inhibition. If the C/N ratio is too high, bacteria in process may confront with nitrogen deficiency and result in lower gas production (Schnürer and Jarvis, 2010; Kangle *et al*., 2012). The combination of substrates with low and high C/N ratio is preferable to get the optimum gas production. The values of C/N ratio that work well in biogas process vary between 10-30, with optimal between 20-30 (Gerardi, 2003; Yadvika *et al*., 2004; Schnürer and Jarvis, 2010; Eaposito *et al*., 2012).

2.3.2 Operational factors

1) Mixing

Mixing is required to enhance contact between microorganisms and substrates, leading to a higher reactor performance. It is particularly important for hydrolytic

bacteria to make good contact with various molecules that they digest and their enzymes can distribute over a surface area of substrate. Moreover, mixing also minimizes toxicity caused by rapid digestion and stabilize temperature throughout the process (Schnürer and Jarvis, 2010; Kangle *et al*., 2012). Level and type of mixing affect growth rate and distribution of microorganisms, substrate availability and utilization rates, granule formation and gas production (Anderson *et al*., 2003). Excessive mixing can disrupt microorganisms so slow and gentle mixing are preferred (Schnürer and Jarvis, 2010).

2) Organic loading rate (OLR)

Organic loading rate (OLR) is the rate that organic matter flowing into the reactor per unit of time. According to slow growth and long doubling time of methanogens, if a large amount of substrate is suddenly added or overloading, this will lead to lower biogas yield and system failure due to build up VFAs that inhibited activity of methanogens. If OLR is too low, there will not be enough substrate for methane production. Typical values of OLR ranges between 0.5-3 kg VS/m^3/d (Kangle *et al*., 2012).

3) Retention time

Retention time refers to hydraulic retention time (HRT) or solid retention time (SRT). SRT is residence time of bacteria in the reactor. HRT is the time that substrate needs to be decomposed in the reactor. In many cases, HRT and SRT are equal. Retention time is usually referred to as HRT. It can be calculated by dividing operating volume by substrate flow rate (Arsova, 2010). HRT is usually between 10-25 days, but can also be longer depending on the composition of substrate, growth rate of microorganisms, type of reactor and temperature which must be enough for substrate digestion by anaerobic bacteria. The substrates that are easily broken down such as sugar and starch allows for short HRT while fiber and cellulose-rich plant matter may need longer time to break down (Schnürer and Jarvis, 2010). Although, a short retention time is desired for reducing reactor volume but too shot retention time can cause microbial wash out and lead to imbalance and deterioration of performance. So, balance must be made for stable operating conditions (Chynoweth and Pullammanappallil, 1996).

2.4 Co-digestion

Co-digestion is anaerobic digestion performed on a mixture of at least two different substrates (Mata-Alvarez *et al*., 2000; Yadvika *et al*., 2004; Esposito *et al*., 2012), for example, co-digestion of cattle slurry with fruit and vegetable wastes (Callaghan *et al*., 2002), crop and crop residue with cow manure (Lethomäki *et al*., 2007) or cassava pulp with pig manure (Panichnumsin *et al*., 2010). It is one of techniques for enhancing biogas production and also improving stability and performance of the process. It was introduced at the end of 1980s in Denmark for treating a mixture of different such as manure, food waste and organic household waste (Ahring, 2003).

2.4.1 Advantage and limitation of co-digestion

Co-digestion is preferably used for improving biogas yield of anaerobic digestion due to its numeral benefits which are (1) improve nutrient balance and increase biodegradable organic matter content, (2) widen range of bacterial strains taking part in the process and improve synergistic effect of microorganisms, (3) adjustment of moisture content and pH (4) supply necessary buffer capacity to the mixture (5) dilution of toxic compounds present in any of co-substrate and (6) produce a digested product of good quality (Khalid *et al*., 2011; Esposito *et al*., 2012). However, some limitations also exist such as increased effluent chemical oxygen demand (COD), require additional pre-treatment and mixing (Kangle *et al*., 2012).

2.4.2 Co-digestion of different substrates

The variety of organic compounds such as carbohydrates, proteins, lipids and cellulose can be digested by anaerobic bacteria. These compounds are mainly presented in different organic wastes that are suitable for using as substrate for anaerobic digestion. Some examples of organic waste which can be used as substrates are municipal solid waste, animal manure, meat and fish industrial wastes, dairy wastes, food waste, energy crops and harvest residues (Esposito *et al*., 2012). Gas yield varies with the content of carbohydrates, proteins and lipids. Lipids provide the highest biogas yield, but slow degradation, while proteins and carbohydrates show faster degradation but lower gas yield (Angelidaki and Ellegaard, 2003; Insam *et al*., 2010). In order to achieve a stable digestion process with a mixture of substrates, it is

important to know the composition of material to get a suitable mix of different components and provide a constant supply of substrate for the microorganisms.

2.4.3 Main substrates in anaerobic co-digestion for this study

Substrates such as pig manure, Napier grass and food waste were used in this study which has characteristics as follow.

1) Pig manure

Manure is a plentiful source of organic matter (Table 2.3) that can be used as feedstock in anaerobic digestion, and high buffering capacity which can protect digestion process against possible failures due to building up of VFAs and consequential dropping in the pH of the system. Pig manure contains various microorganisms and also a wide variety of nutrients necessary for optimal bacterial growth. However, pig manure is high in protein content, which can lead to problems with inhibition by ammonia when manure is digested individually (Schnürer and Jarvis, 2010; Cuetos *et al*., 2011). Moreover, it usually has a rather low total solid concentration and high water content. Therefore, pig manure is known as a substrate with low methane yield around 0.1-0.3 m^3/kg VS_{add} (Angelidaki and Ellegaard, 2003). However, the methane potential of manure is varied and influenced by factors such as growth stage of animals and feed (Liu, 2013). Many previous studies showed that when the manure is digested along with other types of materials, such as food waste, fruit and vegetable wastes or energy crop, the methane yield can be increased (Callaghan *et al*., 2002; Lethomäki *et al*., 2007; Li *et al*., 2010; Cuetos *et al*., 2011).

Table 2.3 Chemical compositions (in % of TS) of animal slurries (Steffen *et al*., 1998)

Chemical compositions	Cow slurry	Pig slurry
Fats	3.5-7.5	7.0-12.3
Proteins	13.7-15.6	16.0-28.9
Carbohydrates	59.9-62.1	53.8
Cellulose	14.5-25.0	10.3-22.9
Hemicellulose	2.0-19.3	17.1-20.8
Lignin	6.8	3.7
Inorganic residues	9.0 16.0	10.1 17.3

2) Napier grass

Napier grass is one of energy crops. It is a species of tropical grass native to the African grasslands. Napier grass has approximately more than 130 species, with 3 species are well-known to be grown in Thailand i.e. *Pennisetum purpureum*, King Grass and Mott Dwarf Elephant Grass. Napier grass is not weed. It has high nutritional value, which is used as animal feed (ruminants). The leaves of Napier grass are wide and the stalks are soft and thick (Department of Alternative Energy Development and Efficiency, 2014). Grass is interesting for biogas production because of its high yield, perennial nature, high VS content of around 92% and the associated relatively high methane yield (Nizami *et al*., 2009). However, the specific biogas yield of grass depends largely on the cutting time. If it is cut late, the content of raw fibers increases and the content of digestible compounds (carbohydrates, proteins and fats) decrease. Therefore, grass which is cut very late is not very productive for fermentation since only 15% of the energy can be transformed into biogas. Further, the methane yield is lower the greater the age of the plants, due to the increasing content of lignocellulose in the grass (Deublein and Steinhauser, 2011).

Grass is not commonly used as sole substrate in the anaerobic digestion process due to their high carbon and relatively low concentrations of trace elements. The unbalance in C/N ratio of grass may cause the failure of the anaerobic digestion process. Thus, mixing with nitrogen rich material such as animal manure can improve C/N ratio of feedstock and methane yield. Lethomäki *et al*. (2008) found that co-digestion of crop with manure generates 16-65% increase in methane production. However, it has high cellulose (25-40%), hemicelluloses (15-50%) and lignin (10-30%) content which are difficult to degrade in a biogas process due to their complex structure. In order to maximize digestion rate of cellulose-rich material, pre-treatment to break up complex structure of cellulose and make it more accessible for digestion is required (Nizami *et al*., 2009).

3) Food waste

Food waste is commonly used for biogas production. It has high VS content about 85-95%. The composition of food waste is varied depending on time of year, cultural habitat or region. Food waste usually contains proteins, fats, carbohydrates and various trace elements so it has potential to function very well in biogas production

process. However, potential for biogas production depends on concentration of these components. Lipids provide the highest biogas yield, but degrade slowing while proteins and carbohydrates show faster degradation. However, food waste with too much lipid or protein may have a problem due to accumulation of VFAs and ammonia, respectively (Arsova, 2010; Schnürer and Jarvis, 2010). Food waste is a desirable material to co-digest with manure because of its high biodegradability (El-Mashad and Zhang, 2010).

2.5 Process design

2.5.1 Continuous reactor

Continuous reactor (Figure 2.4B) has feed and discharge flows in continuous or semi-continuous manner (Arsova, 2010). In semi-continuous reactor, substrate is pumped in 1-8 times per day. The continuous digestion has advantage that it creates a very smooth inflow of substrate and a smooth production of gas. In addition, it also gets a more uniform supply of substrate for microorganisms. This helps interaction between various groups of microorganisms in breaking down chain and reducing risk that microorganisms will become overloaded due to the addition of a large volume of substrate at one time (batch reactor) (Figure 2.4A) (Schnürer and Jarvis, 2010). The continuous reactor is the most common form of full scale reactors (Bouallagui *et al*., 2005).

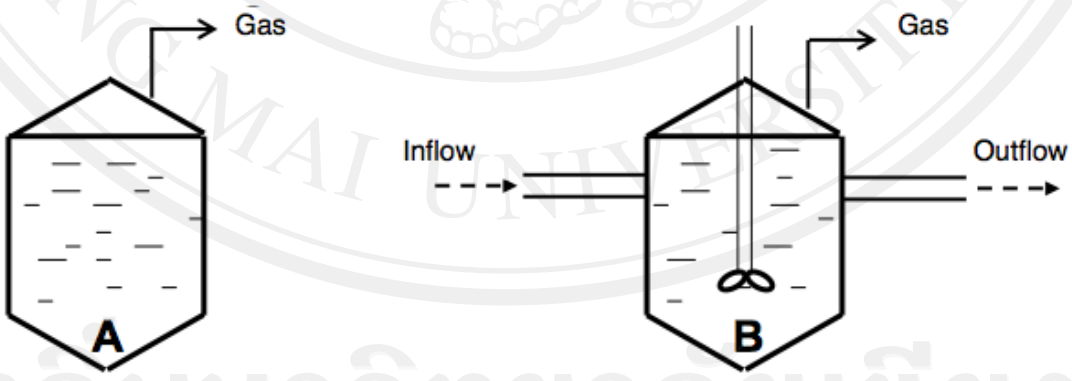

Figure 2.4 Schematic sketches of the batch (A) and continuous reactor (B) (Schnürer and Jarvis, 2010).

2.5.2 Single-stage system

The entire process occurs in one reactor and environment conditions are maintained at levels that optimal for all type of bacteria. This is the simplest model for biogas production. All stages in microbial breakdown i.e. hydrolysis, acidogenesis, acetogenesis and methanogenesis take place at the same time and in the same place. This system has advantage of low investment cost, simple operation and control, and increased process stability. However, it has some drawbacks that are longer start up period, sensitive to higher loading rate if the substrate that easily degradable are fed (Bouallagui *et al*., 2005) and non-optimized microbial condition.

2.5.3 Type of reactors

There are many different types of reactors that are used for biogas production such as anaerobic covered lagoon (ACL), anaerobic fixed film (AFF), completely stirred tank reactor (CSTR), anaerobic baffle reactor (ABR) and anaerobic fixed dome. The reactors used in this study are channel digester-up flow anaerobic sludge blanket (CD-UASB) (Rerkkriangkrai *et al*., 2009) and completely stirred tank reactor (CSTR) (Muenjee, 2010).

2.6. Denaturing Gradient Gel Electrophoresis (DGGE)

A large number of molecular methods have been developed for examination of microorganisms in environmental samples. Denaturing gradient gel electrophoresis (DGGE) is a widely used fingerprinting method for detection of the bacterial population and diversity in environmental samples. Fischer and Lerman (1983) were the first to describe the theoretical aspects of this method. The principle of DGGE is separation of DNA fragments that have the same size but different sequences. The mixture of DNA fragments are applied on a polyacrylamide gel with linearly increasing gradient of denaturant (urea and formamide). The electrophoresis with constant temperature about 60 °C is applied and the DNA fragments migrate through the gel. Differential migration occurs because more denaturant needed to separate sequence with higher guanine (G) and cytosine (C) content due to differences in the numbers of hydrogen bond between complementary nucleotide holding DNA strands together. There are 3 hydrogen bonds between guanine and cytosine whereas only 2 hydrogen bonds between adenine (A) and thymine (T). As DNA strands are separated, their migration becomes slow moving in

the gel (Figure 2.5). To prevent complete strand separation, the fragment migration is stabilized by adding a GC-rich sequence (GC clamp), 30-50 base pairs, to the 5'end of one primer (Sheffield *et al*., 1989) (Figure 2.5).

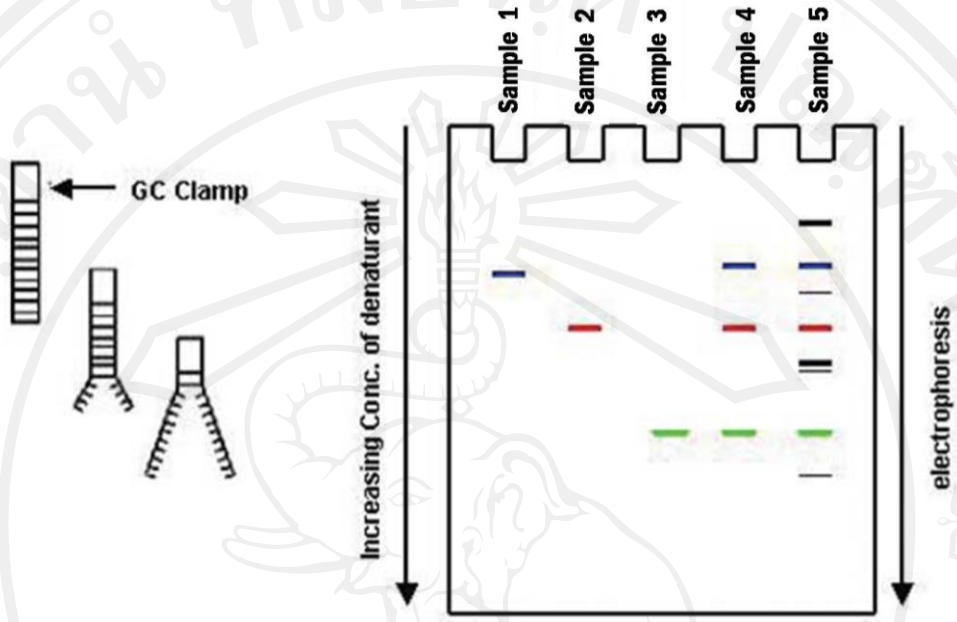

Figure 2.5 Migration of DNA bands by DGGE technique (Hovda, 2007).

Based on this principle, a sample containing many different bacteria will result in many bands on the gel. Comparing different samples will result in different profiles, reflecting bacterial structure and diversity of the sample. The number of bands in the gel was measure for richness, whereas the proportional abundance (evenness) of the bacterial community was calculated from the intensity of the bands (Muyzer, 1999)

There are similar techniques to DGGE like terminal restriction fragment length polymorphism (T-RFLP) or single strand conformation polymorphism (SSCP) which have been developed to estimate the level of diversity in environmental samples, to follow changes in community structure, and to compare microbial diversity and community in various samples (Zumstein *et al*., 2000; Peu *et al*., 2006; Wang *et al*., 2009; Ziganshin *et al*., 2013). However, DGGE is much more effective and easier method compared to other related techniques.

2.6.1 Advantage and limitation of DGGE techniques

DGGE technique is reliable, reproducible, and simple (Muyzer, 1999). Many samples can be analyzed at the same time, making it rapid to monitor change of microbial population in various samples. Information about bacterial profiles of the sample can be achieved within 24 hours (Temmerman *et al*., 2004). DGGE technique is relatively easy to obtain an overview of the dominant species in an ecosystem (Liu *et al*., 2009; Supaphol *et al*., 2011). Moreover, this technique is inexpensive compared to other molecular methods such as cloning. Therefore, this technique is suitable for analysis of different microbial communities from many environment samples.

The limitation of DGGE can be related to both the PCR amplification and the DGGE method. The drawbacks related to PCR biases such as DGGE can detect the chimeric sequence and heteroduplex formation which are formed in annealing step of PCR amplification (Muyzer and Smalla, 1998). Moreover, a single species with multiple rDNA copies can display a DGGE profile with multiple bands which may lead to overestimation in microbial diversity. Fragment to be resolved by DGGE technique should not be longer than 500 bp which limits the amount of sequence information used for phylogenetic analysis and design of primer and probe. DGGE can detect only dominant species in samples. Moreover, closely two bands can be difficult to excise. Co-migration of bands will also give a poor identification in the sequencing.

2.6.2 Procedure of DGGE technique

The DGGE technique consists of 6 major steps (Figure 2.6): (1) sample collection (2) DNA extraction from sample (3) PCR amplification of the target gene with primer to give a mixture of DNA fragments, all of the same length (4) PCR products are separated by DGGE technique on a polyacrylamide gel with increasing urea/formamide gradient. The DNA molecules migrate and stop on their corresponding force which depends on the DNA sequence. Every band on the gel corresponds to a different microorganism in the sample. (5) The band can be cut from the gel and sequenced to identify species (6) data analysis and phylogenetic tree can be made to display similarities graphically.

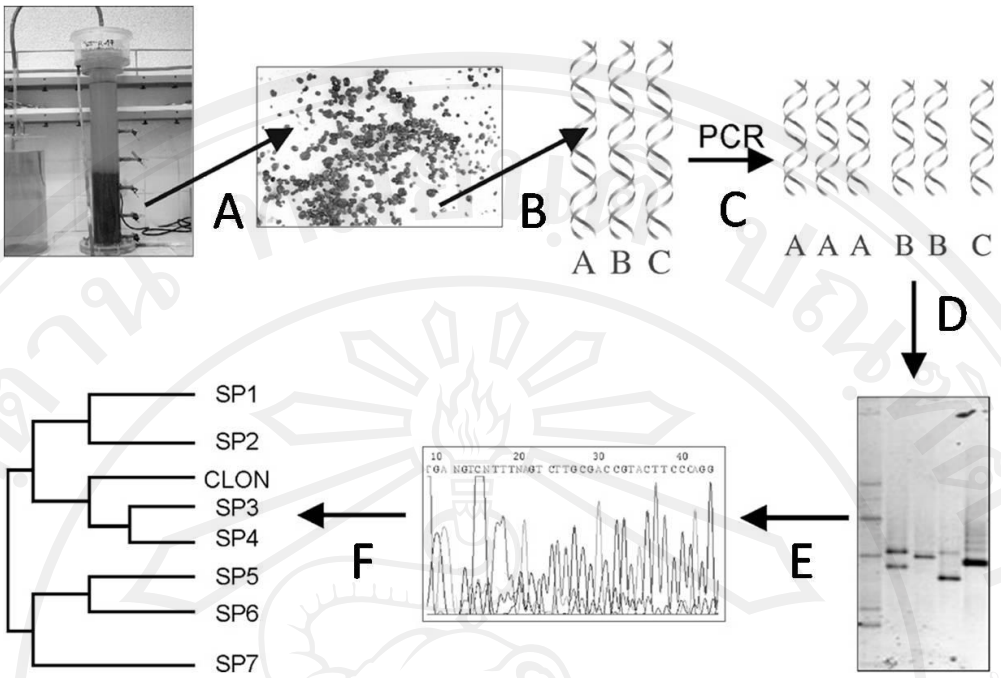

Figure 2.6 Diagram of steps in microbial community analysis by DGGE technique. Sample collection (A), Nucleic acid extraction (B), PCR amplification (C), Separated PCR product by DGGE technique (D), Cut DNA band from the gel, DNA extracted and sequencing (E) and data analysis (F). (adapted from Sanz and Köchling, 2007).

2.7 Application of DGGE technique for microbial community study in biogas reactors

Methanogens and some bacteria in anaerobic reactor are extremely difficult to study with culture-based method. Molecular technique such as DGGE is used to analyse microbial community structure in biogas reactor. There are many applications of DGGE related to anaerobic digestion process. It is usually used for comparative purpose, for example comparing microbial community under different operation conditions. One of the application of DGGE in anaerobic digestion is monitoring dynamic change in microbial community such as monitored change of microbial community during start-up of acidogenic anaerobic reactors under mesophilic (37°C) and thermophilic (55°C) condition (Liu *et al*., 2002). Studied found that the microbial community change was more significant and rapid in thermophilic than mesophilic reactor and a longer period up to 71 days was required to establish a stable microbial community. In addition, Lee *et al*. (2008) investigated bacterial and archaeal

community shift in anaerobic batch reactor treating dairy-processing wastewater. Lee found that bacterial community shift reconciled with change of performance and several bands were closely related to *Clostridium* species. Martín-González *et al*. (2011) monitored change of microbial structure in thermophilic co-digestion of municipal solid wastes with fat, oil and grease (FOG) waste. The result showed that archaeal community structure relatively unchanged along operation, while bacterial community structure had a dynamic change by FOG waste addition. Recently, Dinh *et al*. (2014) used DGGE to investigated change in the microbial community during acclimation stages of the reactor treating glycerol.

Another importance of DGGE is being used to determine microbial community structure and diversity in anaerobic reactor including study influence of some parameters on structure and diversity of microbial community. Curtis and Craine (1998) used DGGE to compare diversity of total microbial communities present in different activated plants. Wang *et al*. (2009) studied impact of feed component ratio and OLR on microbial community structure in co-digestion of grass silage with cow manure. Massive change in bacterial community was found when the ratio of grass increased, while archaea was slightly changed. Pholchan *et al*. (2010) found that an overall system performance and diversity of the microbial communities in the reactor were affected by change in operating parameter, and reactor configuration had effect on microbial diversity. Fliegerová *et al*. (2012) used DGGE to study effect of maize silage as co-substrate with swine manure on bacterial community structure. These authors detected a shift in the bacterial community associated with maize silage as co-substrate. Jianzheng *et al*. (2013) found six methanogenic groups in anaerobic baffled reactor (ABR) treating sugar refinery wastewater, indicating a high phylogenetic diversity of the methanogen in ABR. DGGE is also used to investigate the effect of HRT and temperature on bacterial community structure in CSTR treating swine wastewater. Results obtained from DGGE showed that bacterial community structure was more affected by temperature than HRT (Kim *et al*., 2013).